Copper is required for an array of physiological processes and is essential for proper fetal development. In ruminants, copper deficiency is a problem that manifests due to low dietary copper intake or when the diet provides high levels of copper antagonists, such as iron, molybdenum or sulfur. Genetics also contributes to the incidence of copper deficiency, as Simmental cattle have been shown to be more susceptible to copper deficiency than Angus. In pigs, copper deficiency is very rare and weanling pigs oftentimes are supplemented dietary copper as high as 50-fold above their requirement to obtain a pharmacological response. Furthermore, the use of ingredients such as phosphate supplements in nursery pig diets can provide superfluous amounts of dietary iron. Numerous transport and chaperone proteins have been identified in rodents that serve to regulate Cu homeostasis. However, regulation of these proteins has not been studied in cattle or pigs. The purpose of this research was to characterize mRNA of copper regulatory proteins in cattle and pigs, and to characterize the high affinity copper transporter 1 protein in bovine tissues. We examined how these molecular mechanisms are affected by level and source of dietary copper, breed, and high dietary iron in cattle and pigs. We found that severe copper deficiency in the bovine greatly reduced copper status and mRNA expression of cytochrome c oxidase assembly protein 17, an essential copper chaperone protein. In a subsequent study with pregnant Angus and Simmental cows, dietary copper did not affect expression of gene products involved in copper homeostasis in maternal duodenum or liver. However, expression of duodenal copper
transporter 1 mRNA in Simmentals was approximately 25% of that of Angus while mRNA expression of Atp7a was approximately 50% of that of Angus. Duodenal copper transporter 1 protein also tended to be lower in Simmental than in Angus. In the same experiment, breed did not affect expression of copper transporters and chaperones in fetal liver. Feeding a diet low in copper reduced copper in the bovine placentome and fetal liver, and consequently mRNA expression of antioxidant 1, cytochrome c oxidase assembly protein 17, and copper metabolism MURR1 domain 1 were up-regulated in liver of low copper fetuses. In a study with weanling pigs, we demonstrated that both level and source of dietary copper affects mRNA expression of copper regulatory proteins. Pigs supplemented 225 mg Cu/kg diet from Cu$_2$(OH)$_3$Cl (tribasic copper chloride) had approximately 50% less mRNA of duodenal copper transporter 1 than those supplemented 225 mg Cu/kg diet from CuSO$_4$ (copper sulfate) and controls. Duodenal metallothionein 1a mRNA was much higher in CuSO$_4$-supplemented pigs than in Cu$_2$(OH)$_3$Cl and control pigs. In the proximal jejunum, copper transporter 1 mRNA was down-regulated while antioxidant 1 mRNA was up-regulated in Cu-supplemented pigs. Interactions between iron and copper were examined in a subsequent study with pigs. Level of dietary iron also affected mRNA expression of copper transporters in liver of weanling pigs. Messenger RNA of the biliary copper exporter Atp7b was down-regulated in the pigs receiving low and high dietary iron, which agreed with the concomitant changes in liver copper concentrations. We also examined iron and copper interactions in the young bovine, utilizing Holstein calves. Data from this study demonstrated that high dietary iron increased copper efflux. Collectively these series of experiments herein address some of
the practical issues in regard to copper metabolism in cattle and pigs and provide a better understanding of the cellular regulation that occurs in these species.
Dietary and Genetic Effects on Cellular Copper Homeostasis in Bovine and Porcine Tissues

by
Robert Scott Fry

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Animal Science & Nutrition

Raleigh, North Carolina

2011

APPROVED BY:

Dr. Jerry W. Spears
Committee Co-Chair

Dr. Melissa S. Ashwell
Committee Co-Chair

Dr. Sung Woo Kim

Dr. Paul D. Siciliano

Dr. Terry E. Engle
BIOGRAPHY

R. Scott Fry, son of Eldon and Anne Fry, is a native of Arkansas. While growing up in his hometown of Quitman, Arkansas, he was actively involved in 4-H and Future Farmers of America. His passion for animal agriculture developed early in his childhood years as a result of working on the family owned and operated cattle operation. In August 2001, Scott began his undergraduate career at the University of Arkansas—Fayetteville, majoring in Animal Science with a minor in AgriBusiness. While at the U of A, he was a member of the collegiate Livestock Judging Team and was actively involved with undergraduate research. After completing his Bachelor’s degree in Animal Science in May 2005, Scott moved to North Carolina to attend graduate school at North Carolina State University. Under the supervision of Dr. Jerry Spears, in August 2007 he completed his degree requirements for his Master’s degree in Animal Science. Following the completion of his Master’s degree, Scott remained at NCSU to pursue his PhD under the direction of Drs. Spears and Ashwell. Scott has thoroughly enjoyed his time at NCSU and feels that his production background in combination with the training that he has received at NCSU has created a framework for him to delve into the core of the animal nutrition industry. Upon completion of his PhD degree requirements, he will begin his career as a swine nutritionist for Akey Nutrition in Ohio. In his spare time, Scott thoroughly enjoys spending time with his wife of 4 years, Kayte.
ACKNOWLEDGEMENTS

There are numerous people who have been instrumental in my educational endeavors and accomplishments. First to my parents, who from the very beginning of my decision to pursue a college education provided endless and unconditional support. Additionally, I am very grateful for the exceptional work ethic that they both instilled in me early in life. My entire family has been a great supporting cast, including Dr. Slaton E. Fry who has provided me encouragement over the years.

While at the University of Arkansas I was given the opportunity to work with Drs. Beth Kegley and Ellen Davis. Both served as mentors to me and I am extremely appreciative of their guidance in regard to animal nutrition research, as my undergraduate experience provided a solid foundation to my development as a scientist.

To Drs. Jerry Spears and Melissa Ashwell, I am forever grateful for the guidance you both have given me during my PhD research program. I feel very fortunate to have worked with two advisors who consistently have their students’ best interest in mind. It has truly been an enjoyable experience working with you both; I am not sure I will ever be able to express how appreciative I am of your mentorship. I would also like to thank Drs. Engle, Siciliano, and Kim for serving on my committee.

My technical development as a scientist during my graduate career is attributed to the exceptional training that I received from Karen “Missy” Murphy and Audrey O’Nan. It has been a blessing to have had such great technicians to work with over the past several years. I would also like to thank Dr. Stephanie Hansen for serving as a sounding board in the office while we were in graduate school together. The collaborative research we were able to work
on together was a unique and very developmental experience.

I am also very grateful to the farm crews at the outlining research facilities for their assistance with animal husbandry, specifically the employees at Butner Beef Cattle Field Labs. Dean, Greg, Jay, and Andrew, it was always a relief to know that the job was always done right. I would also like to thank Mebane Galloway, Marian Correll, Chris Brown, and Phil Williams for their camaraderie and assistance over the past several years; all that they have done and continue to do for graduate students and the department does not go unnoticed.

Last be not least, my wife and best friend, Kayte. I will never be able to clearly demonstrate to you how thankful I am to have you in my life. To say that you have been supportive and patient throughout this experience would definitely be an understatement. Thank you for always believing in me and making me a better man. I am truly blessed to have such a wonderful wife. I love you, and I am looking forward to the next chapter in our lives.
TABLE OF CONTENTS

LIST OF TABLES ........................................................................................................ ix
LIST OF FIGURES ....................................................................................................... xii
LITERATURE REVIEW .......................................................................................... 1
  Introduction ........................................................................................................ 1
  Overview of copper metabolism ........................................................................ 2
  Proteins involved in copper homeostasis .......................................................... 4
  Copper transporter 1 .......................................................................................... 4
  Antioxidant 1 ..................................................................................................... 9
  Copper-dependent ATPases .............................................................................. 10
  Copper alpha polypeptide ATPase (ATP7A) ................................................... 11
  Copper beta polypeptide ATPase (ATP7B) ..................................................... 14
  Copper metabolism MURR1 domain 1 ........................................................... 15
  Superoxide dismutase activating pathway ....................................................... 17
  Cytochrome c oxidase activating pathway ....................................................... 19
  Metallothionein ............................................................................................ 20
  Copper and iron interactions ........................................................................... 22
  Copper deficiency and genetic effects on copper metabolism in ruminants .... 26
  Responses to high dietary copper in weanling pigs ......................................... 28
  Conclusion ........................................................................................................ 30
  Literature Cited ............................................................................................... 30

EXPRESSON OF COPPER TRANSPORTERS AND CHAPERONES ARE AFFECTED BY LIFE-LONG, SEVERE COPPER DEFICIENCY AND GENDER IN BOVINE DUODENUM AND LIVER .................................................................................. 41
  Abstract ......................................................................................................... 42
  Introduction .................................................................................................... 42
  Experimental methods ................................................................................... 46
  Animals and experimental design .................................................................. 46
  Tissue collection ............................................................................................ 47
  RNA isolation and Real-Time PCR ............................................................... 47
  Protein extraction and western blot procedure ............................................. 48
  Statistical Analysis ......................................................................................... 50
  Results ............................................................................................................ 51
  Copper status ............................................................................................... 51
  Duodenal protein and mRNA expression ...................................................... 51
  Liver mRNA expression ................................................................................ 51
  Discussion ....................................................................................................... 53
  Conclusion ..................................................................................................... 61
  References ..................................................................................................... 62
LEVEL AND SOURCE OF DIETARY COPPER AFFECTS SMALL INTESTINE MORPHOLOGY, DUODENAL LIPID PEROXIDATION, AND HEPATIC OXIDATIVE STRESS IN WEANLING PIGS, AND ALSO AFFECTS mRNA EXPRESSION OF HEPATIC COPPER REGULATORY PROTEINS ..........................................................................................................141

Abstract ....................................................................................................142
Introduction ..............................................................................................143
Materials and methods .............................................................................144
Animals and experimental design ............................................................144
Performance measurements and blood sampling....................................145
Intestine and liver sampling procedures ..................................................145
RNA isolation and Real-Time PCR .........................................................147
Statistical Analysis ...................................................................................148
Results ......................................................................................................148
Performance and plasma measurements ..................................................148
Liver mineral, bile copper and total glutathione concentrations..............149
Mucosal histology and duodenal MDA concentrations ...........................149
Gene expression of hepatic copper regulatory proteins ...........................150
Discussion................................................................................................151
References................................................................................................156

DIETARY IRON MODULATES MECHANISMS INVOLVED IN COPPER ACQUISITION AND UTILIZATION IN PORCINE LIVER ..............................................................169

Abstract ....................................................................................................170
Introduction ..............................................................................................170
Materials and methods .............................................................................173
Animals and diets .....................................................................................173
Sample collection, RNA isolation, and quantitative RT-PCR .................173
Statistical analysis ....................................................................................174
Results ......................................................................................................174
Tissue mineral concentrations .................................................................174
Duodenal mRNA expression profiles ......................................................175
Hepatic mRNA expression profiles .........................................................175
Discussion................................................................................................175
References................................................................................................180

HIGH DIETARY IRON DOES NOT ANTAGONISTICALLY AFFECT EXPRESSION OF DUODENAL OR HEPATIC COPPER TRANSPORTER 1, BUT INCREASES COPPER EFFLUX AND HEPATIC Atp7b mRNA IN THE YOUNG RUMINANT.........................................................................................195

Abstract ....................................................................................................196
Introduction ..............................................................................................196
Materials and methods .............................................................................198
Animals and experimental design ............................................................198
Sample collection .....................................................................................198
LIST OF TABLES

CHAPTER 2

Table 1  Ingredient composition of the growing and finishing diets ........67
Table 2  Real-time polymerase chain reaction primers..........................68
Table 3  Effect of calf gender on copper status....................................69
Table 4  Gene expression profiles in duodenal mucosa of males ..........70
Table 5  Main effect of dietary copper on gene expression profiles in liver of beef cattle .................................................................71
Table 6  Main effect of gender on gene expression profiles in liver of beef cattle ........................................................................72
Table 7  Genes affected by a copper × gender interaction in liver of beef cattle ........................................................................73

CHAPTER 3

Table 1  Diet composition......................................................................96
Table 2  Real-time polymerase chain reaction primers.........................97
Table 3  Proposed function(s) of copper regulatory gene products ........98
Table 4  Effect of dietary copper and breed on plasma copper concentrations in pregnant Angus and Simmental cows ........99
Table 5  Effect of dietary copper and breed on plasma holo-Cp concentrations in pregnant Angus and Simmental cows ........100
Table 6  Effect of dietary copper and breed on duodenal mineral concentrations in pregnant Angus and Simmental cows ........101
Table 7  Effect of dietary copper and breed on liver copper concentrations in pregnant Angus and Simmental cows ............102
Table 8  Effect of dietary copper, breed of dam, and fetal gender on mineral concentrations in placentome and fetal liver ..........103
Table 9 Effect of dietary copper and breed on mRNA expression of copper regulatory proteins in duodenum and liver of pregnant Angus and Simmental cows ..........................................................104

Table 10 Effect of dietary copper and breed on mRNA expression of copper regulatory proteins in fetal liver collected from pregnant Angus and Simmental cows .........................................................105

Table 11 Effect of gender on mRNA expression of copper transporter 1 and metallothionein 1a in bovine fetal liver .........................................................106

CHAPTER 4

Table 1 Composition of nursery diets .................................................................129

Table 2 Level and source of dietary copper affects soluble copper concentrations in digesta and mucosa in the small intestine of weanling pigs ........................................................................130

Table 3 Level and source of dietary copper affects iron and zinc concentrations in the small intestine of weanling pigs ..........131

Supplemental
Table 1 Real-time polymerase chain reaction primers.................................132

CHAPTER 5

Table 1 Composition of diets ...........................................................................160

Table 2 Real-time polymerase chain reaction primers.................................161

Table 3 Proposed function(s) of copper regulatory gene products ..............162

Table 4 Effect of level and source of dietary copper on performance of weanling pigs ...........................................................................................................163

Table 5 Effect of level and source of dietary copper on plasma copper, holo-ceruloplasmin, and bile copper concentrations in weanling pigs .........................................................................................164

Table 6 Effect of level and source of dietary copper on liver mineral concentrations in weanling pigs .................................................................165
Table 7 Effect of level and source of dietary copper on duodenal malondialdehyde and hepatic total glutathione concentrations in weanling pigs ..........................................................166

Table 8 Effect of level and source of dietary copper on mRNA expression of copper regulatory proteins in liver of weanling pigs ..............167

CHAPTER 6

Supplemental Table 1 Real-Time polymerase chain reaction ........................................188

CHAPTER 7

Table 1 Ingredient composition of diets ............................................................211

Table 2 Real-time polymerase chain reaction primers.................................212

Table 3 Effect of adequate or high dietary iron on ceruloplasmin and plasma copper concentrations in young calves .....................213

Table 4 Effect of adequate or high dietary iron on duodenal, liver, and heart copper concentrations in young calves .......................214
LIST OF FIGURES

CHAPTER 1

Figure 1 Copper transport and chaperone proteins that are known to regulate mammalian copper homeostasis .................................................................3

Figure 2 Copper uptake in Hek293 cells transfected with human CTR1 is saturable and pH dependent .................................................................5

Figure 3 Intestinal Ctr1 deleted mice are characterized by reduced growth and survivability and display hypopigmentation .............................6

Figure 4 Deletion of Atox1 in mice reduces tyrosinase activity, hair pigmentation, and growth in mice .................................................................9

CHAPTER 2

Figure 1 Main effect of dietary copper and gender on duodenal copper transporter 1 (CTR1) protein expression ........................................74

CHAPTER 3

Figure 1 Effect of dietary copper and breed on expression of duodenal copper transporter 1 (CTR1) and copper chaperone for SOD1 (CCS) protein in Angus and Simmental cows .................................107

Figure 2 Effect of dietary copper and breed on expression of hepatic copper transporter 1 (CTR1) and copper chaperone for SOD1 (CCS) protein in Angus and Simmental cows .................................108

Figure 3 Effect of dietary copper, breed of dam, and fetal gender on expression of copper transporter 1 (CTR1) protein in fetal liver ..................................................................................................109

Figure 4 Effect of dietary copper, breed of dam, and fetal gender on expression of copper chaperone for SOD1 (CCS) protein in fetal liver ..................................................................................................110

CHAPTER 4

Figure 1 Relative expression of copper transporter 1 (Ctr1) in duodenum, proximal jejunum, and ileum of weanling pigs .................................133
Figure 2  Relative expression of antioxidant 1 (Atox1) in duodenum, proximal jejunum, and ileum of weanling pigs ............................134

Figure 3  Relative expression of the Cu-dependent ATPase, Atp7a, in duodenum, proximal jejunum, and ileum of weanling pigs ..........135

Figure 4  Relative expression of metallothionein 1a (Mtl1a) in duodenum, proximal jejunum, and ileum of weanling pigs ..................136

Figure 5  Relative expression of divalent metal transporter 1 (Dmt1) in duodenum, proximal jejunum, and ileum of weanling pigs ........137

Supplemental
Figure 1  Relative expression of cytochrome c oxidase assembly protein 17 (Cox17) in duodenum, proximal jejunum, and ileum of weanling pigs ........................................................................138

Figure 2  Relative expression of Atp7b in duodenum, proximal jejunum, and ileum of weanling pigs .........................................................139

Figure 3  Relative expression of hepheastin (Heph) in duodenum, proximal jejunum, and ileum of weanling pigs ..............................140

CHAPTER 5

Figure 1  Effect of level and source of dietary copper on villus height in duodenum, proximal jejunum, and ileum of weanling pigs ........168

CHAPTER 6

Figure 1  Relative expression of duodenal Ctr1 and Atox1 in weanling male pigs normalized to the geometric mean of Rpl4, Ywaz, and Tbp ......................................................................................184

Figure 2  Relative expression of duodenal Atp7a and Mtl1a in weanling male pigs normalized to the geometric mean of Rpl4, Ywaz, and Tbp ......................................................................................185

Figure 3  Relative expression of hepatic Ctr1 and Cp in weanling male pigs normalized to Rpl4, Hprt1, Ywaz, and Tbp .................186

Figure 4  Relative expression of hepatic Atp7b and Commd1 in weanling male pigs normalized to Rpl4, Hprt1, Ywaz, and Tbp ........187
Supplemental

Figure 1  Relative expression of duodenal Cox17 and Atp7b in weanling male pigs normalized to Rpl4, Ywaz, and Tbp ...............................189

Figure 2  Relative expression of duodenal Ccs and Sod1 in weanling male pigs normalized to Rpl4, Ywaz, and Tbp .................................190

Figure 3  Relative expression of hepatic Ccs and Sod1 in weanling male pigs normalized to Rpl4, Hprt1, Ywaz, and Tbp ..........................191

Figure 4  Relative expression of hepatic Cox17 in weanling male pigs normalized to Rpl4, Hprt1, Ywaz, and Tbp ..................................192

Figure 5  Relative expression of hepatic Mt1a in weanling male pigs normalized to Rpl4, Hprt1, Ywaz, and Tbp .................................193

Figure 6  Relative expression of hepatic Atox1 and Atp7a in weanling male pigs normalized to Rpl4, Hprt1, Ywaz, and Tbp .................194

CHAPTER 7

Figure 1  Effect of adequate or high dietary iron on duodenal and hepatic CTR1 protein expression in young calves ...............................215

Figure 2  Effect of adequate or high dietary iron on relative mRNA expression of duodenal copper transporters and chaperones in young calves..........................................................216

Figure 3  Effect of adequate or high dietary iron on relative mRNA expression of hepatic copper transporters and chaperones in young calves..........................................................217
CHAPTER 1

Literature Review

Introduction

It is well documented that copper (Cu) is required for a number of enzymes that are involved in an array of physiological processes (NRC, 2005). There are numerous practical dietary issues and uncertainties in regard to Cu homeostasis in cattle and young swine. In cattle, Cu deficiency is problematic in many areas of the world and genetics may contribute to this issue. Dietary Cu antagonist such as molybdenum (Mo), sulfur (S), and iron (Fe) can be found at high concentrations in soils, feedstuffs, and even water and can greatly reduce Cu status in ruminants (Suttle, 1991; Thornton, 2002; Kerr et al., 2008). One of the first reports to reveal differences in Cu metabolism between Simmental and Angus cattle reported that Simmental calves were less tolerant to dietary Mo than Angus (Gengelbach et al., 1994). Numerous studies have shown that Cu indices are lower in Simmental than in Angus when fed Cu-deficient diets (Ward et al., 1995; Mullis et al., 2003). From these data a strong case was made that Simmental cattle are more prone to Cu deficiency. The fetus has a high demand for Cu and several reports have also suggested that Simmental cattle may transfer less Cu to their fetus and that transfer may be further exacerbated by low dietary Cu (Ward et al., 1995; Mullis et al., 2003). Increasing levels of dietary Fe in monogastric species such as rodents has been shown to decrease apparent Cu absorption and biliary Cu excretion. While young, newborn pigs are quite susceptible to Fe deficiency, they can also be exposed to dietary Fe concentrations well above their requirement due to the use of ingredients such as phosphate supplements and blood meal in diet formulation. Moreover, nursery pig diets more
often than not contain phosphate supplements that provide very high concentrations of Fe even though these ingredients are at low inclusion levels in the diet. Furthermore, weanling pigs are often supplemented high concentration of Cu to stimulate growth. Definitive mechanisms responsible for enhanced performance are unknown and currently, we are unfamiliar with how the young pig tolerates such as high levels of dietary Cu. Within the past twenty years great strides have been made to better understand cellular control and regulation of Cu in mammals. A breadth of literature has involved utilizing model species and cell lines to elucidate regulation of Cu homeostasis at the cellular level. This literature review will highlight studies that are most relevant to the Cu homeostasis in domestic livestock.

Overview of Copper Metabolism

While Cu is an essential trace element, its ability to undergo redox chemistry can be detrimental to biological membranes and macromolecules such as DNA if not tightly regulated (Bremner, 1998). Thus, numerous transport and chaperone proteins exist that regulate Cu acquisition, intracellular distribution, and utilization and are essential in preventing both deficiency and toxicity (Kim et al., 2008; Figure 1). Dietary Cu from the intestinal lumen is taken up via the high affinity Cu transporter, copper transporter 1 (CTR1), but not before cupric Cu is reduced to cuprous Cu (Cu\(^{++}\) to Cu\(^{+}\)) by an intestinal reductase. In the liver, Cu\(^{+}\) is also taken up via CTR1 which is located in the plasma membrane. Once Cu\(^{+}\) is taken up by the enterocyte and hepatocyte, Cu is bound to one of several Cu chaperone or storage proteins. Metallothionein (MT) sequesters Cu in the intestine providing protection from excessive Cu efflux, while in the liver it serves as the primary Cu storage protein. Cytochrome c oxidase assembly protein 17 (COX17) is a cytosolic and mitochondrial
chaperone that delivers Cu to cytochrome c oxidase, the terminal enzyme in the electron transport chain. Another option for intracellular Cu is to be shuttled via the Cu chaperone for superoxide dismutase (CCS) that is responsible for delivering Cu to the antioxidant enzyme superoxide dismutase (SOD1) located in the cytosol.

Figure 1. Copper transport and chaperone proteins that are known to regulate mammalian copper homeostasis.

Lastly, Cu can be bound to antioxidant 1 (ATOX1), and thus be delivered to the
Cu-dependent ATPase, ATP7A (intestine) or ATP7B (liver). In the intestine, ATP7A is involved in basolateral Cu transport and also synthesis of cuproenzymes (i.e. lysyl oxidase, tyrosinase). In the liver, ATP7B is responsible for ceruloplasmin (Cp) synthesis, a Cu-dependent ferroxidase that is required for iron (Fe) oxidation and efflux from the liver. Bile is the major route for Cu excretion and ATP7B and Cu metabolism MURR1 domain 1 (COMMD1) are responsible for biliary Cu excretion.

**Proteins involved in copper acquisition, distribution, and utilization**

**Copper transporter 1**

Copper transporter 1 (CTR1) is a member of the CTR protein family, a family of transporters that are highly conserved in eukaryotes, providing Cu$^+$ to intracellular Cu chaperone proteins (van den Berghe et al., 2010). CTR1 is a three transmembrane protein with the unglycosylated and glycosylated forms having apparent molecular weights of 30 and 35 kDa, respectively (Gybina and Prohaska, 2006; Nose et al., 2010). This Cu transport protein exists as a homotrimer in the plasma membrane (Lee et al., 2002), and after much debate in the literature recent data has solidified that CTR1 is located on the apical membrane in the enterocyte (Nose et al., 2010). Functionality of CTR1 is dependent on conserved methionine residues located in the N-terminal extracellular domain and the second transmembrane domain as demonstrated in mutagenesis experiments (Puig et al., 2002). Lee and coworkers were some of the first researchers to characterize human CTR1. These authors reported that transfection of human *Ctr1* cDNA in human embryonic kidney (Hek293) cells stimulates $^{64}$Cu uptake. Furthermore, stimulated uptake by human CTR1 was saturable and pH dependent (Figure 2). In this study, authors also reported that CTR1 is specific for Cu$^+$ but
that zinc (Zn), iron (Fe), and cadmium (Cd) are possible reversible inhibitors or are low affinity substrates for CTR1 uptake when present at high concentrations (Lee et al., 2002).

CTR1 is ubiquitously expressed and over the past several years numerous studies have been conducted determining its essentiality in mammalian tissues. Several studies in rodents have demonstrated consequences of Ctr1 deletion and shown that CTR1 is the primary route of mammalian Cu uptake.

Lee et al. (2001) reported deletion of the Ctr1 gene resulted in compromised growth and development with mice dying in utero at mid-gestation. Nose et al. (2006) reported that intestinal epithelial cell-specific knock-out Ctr1 mice suffer greatly from Cu deficiency. These animals displayed neonatal defects, hepatic Fe overload, Cu accumulation in the periphery, cardiac hypertrophy, and had severe reductions in growth and survivability (Figure 3).
However, when Cu was injected, thereby by-passing intestinal absorption, clinical signs of Cu deficiency were reduced and survivability was increased demonstrating the essential role of CTR1 in intestinal Cu absorption.

Liver is the primary storage organ for Cu. However, hepatic Ctrl deletion is less disruptive to Cu metabolism than intestinal deletion of Ctrl. Hepatocyte cell-specific Ctrl knock-out mice were characterized by reductions in growth, liver Cu, biliary Cu excretion, and activity of the Cu-dependent enzymes, superoxide dismutase 1 (SOD1), CCO, and Cp (Kim et al., 2009). These authors suggested that other mechanisms involved in Cu homeostasis likely compensated for the 90% reduction in CTR1 protein expression because Cu deficiency was mild and not as severe as that observed with intestinal deletion of Ctrl (Nose et al., 2006).
Over the past several years numerous studies have been conducted in cell culture and in rodents examining regulation of CTR1. In a study by Lee et al. (2000), intestine and liver Cu in Cu deficient rats was approximately 90% lower than that of their Cu adequate counterparts. Although Cu concentrations were markedly reduced in the intestine and liver, Ctr1 mRNA was not affected by Cu deficiency, suggesting that Cu deprivation does not affect transcription of Ctr1. Utilizing immunohistochemical analysis Kuo et al. (2005) reported the presence of CTR1 in mouse retina, testes, mammary, heart, brain, intestine, kidney, and liver. Additionally, these authors showed that CTR1 protein, as demonstrated via western blot, was not affected by Cu deficiency in the liver, but was approximately 56% higher in the intestine and kidney as a result of Cu deficiency. The brain also has high Cu demand and work by Gybina and Prohaska (2006) has shown that Cu deficiency increases CTR1 protein in the choroid plexus of rats, a portion of the brain in which CTR1 protein is abundant (Kuo et al., 2005). Transcript levels of Ctr1 have been identified in the placenta of rats, but were not affected by Cu deficiency (Anderson et al., 2007). Recent research indicated that intestinal and cardiac expression of CTR1 protein was much higher in Cu-deficient rats than in-Cu adequate rats (Nose et al., 2010). Increases in CTR1 protein expression in the aforementioned studies likely occurred to increase Cu absorption via CTR1 in response to Cu deficiency.

Although results have been variable, research suggests that high Cu exposure affects Ctr1 at the level of transcription and translation. In the aforementioned study by Nose and coworkers, high Cu exposure, up to 100 µM concentrations, in Hek293 cells led to degradation of CTR1 protein (Nose et al., 2010), likely providing protection from excessive
Cu uptake. However, similar concentrations seem to not affect transcription of Ctr1. With the utilization of a polarized cell line that closely resembles the enterocyte, Tennant et al. (2002) reported that Ctr1 mRNA was not affected in Caco-2 cells exposed to 100 µM Cu. In vivo data by Bauerly et al. (2005) showed that Ctr1 mRNA and CTR1 protein was higher in the liver of rat pups supplemented with 10 and 25 µg Cu/d compared to controls receiving no supplemental Cu. In the small intestine only CTR1 protein was higher in pups supplemented with 25 µg Cu/d. Transcript levels of Ctr1 were recently identified and studied in liver of the bovine. This study showed that Ctr1 mRNA was negatively correlated with liver Cu concentrations in these animals. Liver Cu in these animals ranged from 86 to 801 mg Cu/kg DM, thus suggesting that high intracellular Cu concentrations may down-regulate expression of Ctr1 in the bovine liver (Han et al., 2009).

It was shown recently by Collins et al. (2009) that Fe deficiency affects expression of Ctr1 mRNA. In this experiment, these authors reported that duodenal Ctr1 mRNA expression was approximately 1.5 fold higher in Fe-deficient rats than in those receiving Fe-adequate diets. Iron deficiency can result in hypoxia as a result of compromised oxygen delivery to tissues. It is likely that the increase in Ctr1 mRNA reported in the study by Collins et al. (2009) occurred as a result of hypoxia as recent research in hypoxic macrophages has shown increases in CTR1 protein with concomitant Cu uptake (White et al., 2009). In contrast, in vitro data with Hek293 cells has shown that high Fe exposure does not affect CTR1 protein (Nose et al., 2010).
Antioxidant 1

One of the Cu chaperones to which CTR1 provides Cu\(^+\) is antioxidant 1 (ATOX1), the vital secretory Cu chaperone. Knock-out studies have demonstrated the biochemical significance of ATOX1 in mammals. Hamza et al. (2001) reported that consequences of  \( \text{Atox1} \) deletion were dramatic reductions in growth and survivability; in fact approximately half of the mouse pups died by mid-gestation. Those that survived were characterized by classic signs of clinical Cu deficiency such as hypopigmentation, skin laxity, and setbacks in growth and development, with these characteristics persisting well beyond weaning. In this study they also illustrated that \( \text{Atox1} \) deletion practically diminished the activity of tyrosinase, a cuproenzyme involved in hair pigmentation (Figure 4). Interestingly, growth and mortality of \( \text{Atox1}^{+/-} \) pups did not differ from \( \text{Atox1}^{+/-} \) pups, and in fact their phenotypes were indistinguishable from each other. Furthermore, deletion of \( \text{Atox1} \) in dams further exacerbated the consequences of \( \text{Atox1} \) deletion in pups. This can be explained by much greater Cu concentrations in maternal placenta than in the embryo, demonstrating that ATOX1 is essential for Cu efflux from the dam to embryo or fetus.

![Figure 4. Deletion of Atox1 in mice reduces tyrosinase activity, hair pigmentation, and growth in mice. Taken from Hamza et al., 2001; PNAS, 98, 6848-6852.](image)
Hamza and colleagues published subsequent data utilizing immortalized fibroblasts from Atox1+/- or Atox1+/- embryos to demonstrate the significance of Cu delivery by ATOX1 to ATP7A. Deletion of Atox1 greatly hindered the trafficking ability of ATP7A and thus Cu efflux (Hamza et al., 2003). Walker et al. (2002) reported similar data in regard to ATP7B, in which ATOX1 regulated the amount of Cu ions delivered to ATP7B as well as its catalytic activity. Other roles for ATOX1 have been revealed in the past decade. Kelner et al. (2000) reported that neuronal cell lines transfected to increase basal levels of ATOX1 were significantly protected from oxidative stress. More recently, Itoh et al. (2008) reported that ATOX1 contributes to cell proliferation by functioning as a novel transcription factor when activated by Cu.

Only limited research has examined the effects of Cu (or Fe) on Atox1 expression. Recent research has shown that Atox1 mRNA was not affected by Cu deficiency in the placenta of rats (Anderson et al., 2007). On the other hand, excesses of 275 fold do not seem to affect Atox1 expression in cultured HepG2 cells (Muller et al., 2007). The metabolism of Cu and Fe are closely related (Fox, 2003). A recent review suggested that Atox1 may be a link between Cu and Fe homeostasis (Collins et al., 2010). However, Atox1 mRNA was not affected in the liver of Fe-deficient rat dams and their respective fetal liver compared to their Fe-adequate counterparts (Gambling et al., 2004). It may be that Fe-deficiency affects translation rather than transcription of Atox1. Further research is warranted in examining Atox1 as a possible link.

**Copper-dependent ATPases**

Copper homeostasis is dependent on the Cu-dependent ATPases, ATP7A and ATP7B. These proteins are approximately 60% homologous and contain eight
transmembrane domains with their amino and carboxy termini located in the cytosol. Six metal binding domains reside in each of the proteins at the amino terminal end, and these domains are composed of methionine and cysteine residues (MXCXXC). Both proteins are dual-functioning in that they are involved in synthesis of cuproenzymes and Cu efflux. Unlike other Cu proteins that have been characterized, ATP hydrolysis is required for these proteins to be functional. Patients with Menkes disease and Wilson’s disease have mutations in the Atp7a and Atp7b gene, respectively. Menkes disease is often fatal and is an inherited X-linked disorder in which patients are characterized by perturbations in intestinal absorption of Cu and consequently become severely Cu deficient. Moreover, these individuals suffer from neurological disorders, compromised tissue maturation, and mental retardation (Velduis et al. 2009). On the other hand, Wilson’s disease patients suffer from inherent Cu toxicity in which biliary Cu excretion and Cp synthesis are both greatly reduced resulting in liver Cu accumulation to toxic levels. These individuals also experience high levels of Cu in the plasma and brain.

**Copper-dependent alpha polypeptide ATPase (ATP7A)**

An animal model that has been utilized to better understand biochemical characteristics of ATP7A is the brindled mottled mouse because Atp7a is mutated in these animals. Additionally, there have been numerous *in vitro* and *in vivo* studies conducted to better understand ATP7A biochemistry. Previous data has demonstrated that ATP7A localization is dependent on Cu concentrations. Lutsenko and Petris (2002) reported that when cells were exposed to high levels of Cu, movement of ATP7A from the trans golgi network to the plasma membrane occurred to promote Cu efflux. A more recent study by
Nyasae et al. (2007) examined ATP7A localization in polarized intestinal epithelia, rat enterocytes, and Caco-2 cells. In all cell types, the authors reported that under conditions of Cu deprivation an abundance of ATP7A was localized to the trans golgi network. However, when Cu levels were increased, ATP7A localized to the cell periphery. Because ATP7A was not primarily localized at the basolateral surface as reported in the study by Lutsenko and Petris (2002), the authors raised questions regarding the current models utilized to understand Cu efflux. In this same study, Nyasae et al. (2007) reported that ATP7A protein was most abundant in the upper jejunum and was the lowest in the ileum with duodenal expression being intermediate. From this work, authors suggested Cu absorption from the enterocyte for utilization may occur from the upper jejunum given the abundance of ATP7A protein in this section of the intestine.

Previous reports have been variable when examining the effects of Cu on \( Atp7a \) mRNA and protein. Bauerly et al. (2004) reported that the addition of 94 μM of Cu to media of Caco-2 cells for one week did not alter \( Atp7a \) mRNA. However, in a subsequent in vivo experiment from the same group, it was reported that 25 μg Cu/d for 20 days increased \( Atp7a \) mRNA in the intestine of rat pups relative to those supplemented with 0 and 10 μg Cu/d (Bauerly et al., 2005). More recent data by Anderson et al. (2007) showed that \( Atp7a \) mRNA in rat placenta is not affected by Cu deficiency. As mentioned previously ATP7A is present in the liver, but its function is unknown. Recently, Han et al. (2009) identified \( Atp7a \) mRNA in bovine liver, a species in which liver Cu can be quite high. Recent work showed that mice with cardiac specific deleted \( Ctr1 \) have higher expression of ATP7A protein in the intestine and liver. This occurred unexpectedly to authors, and an increase in serum Cu concentrations
led the authors to suggest that ATP7A in the liver may have a role in Cu mobilization (Kim et al. 2010).

The metabolism of Cu and Fe are closely related and previous studies have examined the effects of Fe deficiency on Atp7a mRNA and ATP7A protein. Gambling et al. (2004) reported that Fe deficiency had no affect on Atp7a mRNA in the placenta of Fe deficient rat dams. However, a series of experiments conducted by the same group of researchers has shown that Fe deprivation affects Atp7a and ATP7A in rats. Collins et al. (2005) reported that rats receiving Fe-deficient diets (3 mg Fe/kg diet) had on average 4 fold higher transcript levels of Atp7a in their duodenum than their Fe adequate (180 mg Fe/kg) counterparts. In a subsequent experiment from the same laboratory, it was reported that Fe deprivation also increased duodenal expression of ATP7A protein and altered ATP7A localization (Ravia et al., 2005). This experiment utilized similar dietary Fe treatments as the aforementioned study. By traditional measures, Fe-deprived rats were very anemic with severe reductions in red blood cells, hemoglobin, and hematocrit. Regardless of age or stage of development, duodenal ATP7A protein was markedly higher in Fe-deprived rats compared to those receiving-Fe adequate diets. ATP7A protein was localized to the brush border membrane and basolateral enterocytes in Fe-deprived rats, whereas ATP7A was primarily located on the apical domains of enterocytes in Fe-adequate rats. These authors suggested that this novel increase in ATP7A occurred to increase Cu efflux as liver Cu was markedly higher in Fe-deprived rats (Ravia et al., 2005).
**Copper-dependent beta polypeptide ATPase (ATP7B)**

The Long-Evans Cinnamon (LEC) rat is a useful animal model for Wilson’s disease as these animals exhibit many of the same characteristics as humans with Wilson’s disease (Wu et al., 1994). Interestingly, Terada et al. (1999) demonstrated that biliary Cu excretion in the LEC rat can be increased or rescued by introducing a full length \textit{Atp7b} cDNA, further illustrating the importance of ATP7B in biliary Cu excretion and the LEC rat as a useful model for Wilson’s Disease. As with ATP7A, trafficking of ATP7B also occurs in response to intracellular Cu concentrations. A recent study utilizing Can 10 cells reported that ATP7B translocates to the bile canalicular membrane and that an abundance of ATP7B is located in the tight junctions of the canalicular membrane under conditions of high Cu. Under conditions of low Cu exposure, ATP7B resided in the trans golgi network (Hernandez et al., 2008).

Recently \textit{Atp7b} mRNA was identified in bovine liver (Han et al., 2009), and experiments examining ATP7B trafficking has been conducted that are more applicable to ruminant nutrition. Lockhart and colleagues (2000; 2001) conducted a series of experiments examining ATP7B in the liver of sheep, a species, depending on breed, that is quite susceptible to both Cu deficiency and toxicity. Some researchers have compared Cu homeostasis in sheep liver to that of patients with Wilson’s Disease as biliary Cu excretion is quite low in sheep (Howell et al., 1984). In the initial studies by Lockhart and co-workers, an alternate form of \textit{Atp7b} cDNA that encoded a novel 79 amino acid N-terminus was discovered (Lockhart et al., 2000). To better understand the functionality of both forms, a subsequent study was conducted utilizing Chinese Hamster Ovary cells expressing both
forms of $Atp7b$ cDNA (Lockhart and Mercer, 2001). Results demonstrated that both forms of the protein underwent trafficking in response to Cu. In fact trafficking was specific to the presence of Cu as there were no effects of Fe or Zn. It was suggested that Cu toxicosis in sheep is likely not attributed to a defect in $Atp7b$. However, the breed of sheep used in these experiments may have affected their outcome, as Merino sheep are quite tolerant to Cu (Wiener et al., 1978).

Recently several studies have evaluated effects of Cu and Fe on $Atp7b$ mRNA in rodents. Bauerly et al. (2005) reported that $Atp7b$ mRNA expression was up-regulated in infant rats within 10 and 20 days postnatally when animals were fed 10 and 25 μg Cu/d Cu compared to no supplemental Cu. It may be that modulation of $Atp7b$ mRNA is dependent upon stage of development or tissue as a recent a report by Anderson et al. (2007) indicated that $Atp7b$ mRNA in rat placenta is not affected by Cu deficiency. Transcript levels of $Atp7b$ in liver of rat dams and their respective fetal liver was not affected by Fe deficiency (Gambling et al., 2004). Soon after, Ravia and colleagues (2005) reported data in agreement to Gambling’s work indicating that although liver Cu was on average 6 fold higher in Fe-deficient rats that hepatic $Atp7b$ mRNA was not differentiated. Rats do not have a gall bladder (Patterson et al., 2008), and biliary Cu and $Atp7b$ mRNA may be affected differently in the presence of a gallbladder.

**Copper metabolism MURR1 domain 1**

The Bedlington terrier is a breed of dog that is quite susceptible to Cu toxicosis (van de Sluis et al., 2002). Animals that are affected have very low biliary Cu excretion and consequently liver Cu accumulates to massive quantities leading to cirrhosis of the liver, very
similar to that of Wilson’s Disease patients. However, in contrast to patients with Wilson’s Disease, Cp concentrations are normal illustrating that ATP7B is functional in these animals. In a study utilizing liver samples from a population of Bedlington terriers, Klomp et al. (2003) revealed that the perturbation in liver Cu homeostasis in these animals was a result of COMMD1 protein being completely absent. Furthermore, these authors reported that COMMD1 is ubiquitously expressed with it being most abundant in kidney, spleen, liver, and colon. This protein is quite conserved throughout evolution but conservation seems to be restricted to bile containing organisms. In contrast to the Cu-dependent ATPases, there are no copper binding motifs in COMMD1, but a conserved domain does exist which is known as the copper metabolism MURR1 domain (De Bie et al., 2005). Previous data suggest that COMMD1 excretes Cu via the bile by interacting with ATP7B (Tao et al., 2003).

Recently there have numerous reports in literature illustrating that COMMD1 is involved in a plethora of cellular processes, such as NF-kB signaling, hypoxia inducible factor activity, as well as cell proliferation and cell death (De Bie et al., 2005; Maine and Burstein, 2007; van de Sluis et al., 2007). Recent reports have also identified COMMD1 in human placenta (Donadio et al. 2007). In this study, COMMD1 was localized to vascular endothelial cells and consequently the authors suggested that COMMD1 may have a role in regulating Cu transfer to the fetus which has a high demand for Cu. Few published reports exist examining the effects of Cu on COMMD1. Muller et al. (2007) reported that HepG2 cells exposed 100 µM of CuCl₂ resulted in down-regulation of Commd1 mRNA as well as reduced COMMD1 protein.
Superoxide dismutase activating pathway

In 1969, McCord and Fridovich were the first to purify and characterize superoxide dismutase (SOD1) from bovine erythrocytes. This enzyme is responsible for the reduction of the superoxide to hydrogen peroxide and oxygen in the cell. From these experiments, these authors found that Cu was required for the activity of SOD1 and that two Cu$^{+}$ ions are bound to the enzyme. More recently we have learned that Cu ions do not spontaneously become incorporated into SOD1, but that this relies on a Cu chaperone protein. Copper chaperone for SOD1 (CCS) was first identified by Culotta et al. (1997) in yeast. Soon after, Casareno et al. (1998) reported that CCS interacts with SOD1 in cultured COS-1 and HepG2 cells. Research utilizing knock-out mice revealed the consequences of $Ccs$ deletion and thus the importance of CCS in activating SOD1. Wong et al. (2000) reported that $Ccs^{-/-}$ mice have normal SOD1 protein levels, but SOD1 activity is severely reduced. Furthermore, challenging $Ccs^{-/-}$ mice with paraquat, an oxidative stressor, resulted in severe reductions in survivability.

Previous data has demonstrated that Cu deficiency results in higher expression of CCS protein but not its mRNA expression. Bertinato et al. (2003) reported that CCS protein in liver and erythrocytes increased in a linear fashion in rats as dietary Cu changed from normal to moderately deficient to deficient. Furthermore, these authors demonstrated that SOD1 activity in the liver and erythrocytes of Cu-deficient rats was reduced. In a subsequent study by Bertinato and L’Abbe (2003) it was revealed that CCS protein levels increase as a result of decreased protein degradation by the 26S proteosome rather than increased transcription. Since these reports, CCS has been deemed a useful experimental biomarker for Cu deficiency in rodents and also in the bovine. Severely Cu-deficient calves had higher
levels of CCS protein in their duodenum (Hansen et al., 2009), liver (Hepburn et al., 2009), and erythrocytes (Hepburn et al., 2009) than their Cu-adequate counterparts.

Some research has examined the effects of high Cu on Ccs mRNA. Muller et al. (2007) reported that HepG2 cells with 275 fold excesses of intracellular Cu did not have increased levels of Ccs mRNA. In vivo data by Suazo et al. (2008) demonstrated that peripheral mononuclear cells from humans with high Cp and receiving high Cu supplementation (10 mg Cu/d Cu; ~1.0 mg/d Cu human requirement) had lower levels of Ccs mRNA than cells from low Cp humans not supplemented with Cu. Authors suggested that this may have occurred in response to moderate Cu overload.

Some of the earlier work utilizing the pig to better understand Cu metabolism demonstrated that Cu-deficient pigs had lower SOD1 activity in erythrocytes and liver relative to their Cu-adequate counterparts (Williams et al., 1975). More recently Prohaska and colleagues have reported numerous studies illustrating that Cu deficiency lowers SOD1 protein but not mRNA levels, suggesting that regulation of SOD1 is posttranscriptionally controlled (Prohaska and Brokate, 2001; Prohaska et al., 2003; Broderius and Prohaska, 2009). A recent report in cattle is in agreement with the aforementioned rodent studies. Hansen et al. (2009) showed that hepatic Sod1 mRNA tended to be down-regulated in severely Cu-deficient cattle. On the other hand, Suazo et al. (2008) reported that Sod1 mRNA was down-regulated in peripheral mononuclear cells of individuals with a high Cu status and intake compared to their counterparts with low Cu status and intake. These authors speculated that this decrease in mRNA occurred as a result of oxidative stress due to high Cu intake and indices. Previous data in the bovine indicated that Sod1 mRNA was negatively
correlated with liver Cu concentrations (Han et al., 2009). Liver Cu concentrations were as high as 801 mg Cu/kg DM, which is high, but not toxic, levels in the bovine.

**Cytochrome c oxidase activating pathway**

Cytochrome c oxidase (CCO) is a Cu-dependent enzyme that is the terminal oxidase in the electron transport chain. There are numerous subunits (12-13) of this mitochondrial enzyme and several assembly proteins have been identified to be involved in mediating Cu transfer to and incorporation in this enzyme. Cytochrome c oxidase assembly protein 17 (COX17) is located in the cytosol and mitochondria and is responsible for delivering Cu$^+$ ions to one of several mitochondria Cu chaperones. Elegant knock-out studies have demonstrated that embryos from $Cox17^{-/-}$ mice had severely reduces CCO activity (Takahashi et al., 2002). It has been postulated that once COX17 delivers Cu to the mitochondria, the Cu chaperone proteins SCO1 and COX11 arbitrate Cu transfer into COX2 and COX1 subunits, respectively (Cobine et al., 2006). Zeng et al. (2007) reported that Cu deprivation hinders the assembly of and functionality of CCO in cardiac mitochondria, and recent research in Cu-deficient rats revealed that expression of SCO1 protein was higher, while COX1 and COX4 subunits were reduced in cardiac tissue of Cu-deficient rats (Getz et al., 2009). In cattle, whose growth was reduced, $Cox1$ mRNA was down-regulated in liver (Hansen et al., 2009). Characterizing and studying these proteins involved in activating and assembling CCO should provide us with a better understanding of how Cu deprivation decreases CCO activity, which has been demonstrated in several studies in cattle (Mills et al., 1963; Fell et al., 1975; Mills et al., 1976).
**Metallothionein**

Metallothionein (MT) is a low molecular weight, cysteine-rich metal binding protein that has been characterized in numerous tissues, particularly the intestine, liver, and kidney and provides protection from Cu, Zn, and cadmium toxicity (Cousins, 1985). Metallothionein can also be found in the blood, urine and bile (Bremner et al., 1986). In the intestine MT sequesters Cu when Cu accumulates in the enterocytes as demonstrated in humans with Menkes’ disease in which entrapment of Cu occurs due to comprised Cu efflux (Hunt and Port, 1979). On the other hand, MT in the liver serves as the primarily storage protein for Cu as indicated in humans with Wilson’s disease (Cox and Moore, 2002). Additionally some reports in the literature have suggested that MT may also act as a Cu chaperone providing Cu to metalloproteins (Palmiter, 1998). The majority of mammalian tissues have two isoforms of MT, MT1 and MT2. Amino acid composition is quite similar between the isoforms, as the differences lie in affinity in which MT2 has a higher Zn affinity than MT1. In mammals Zn is a strong inducer of MT, but Cu has a much higher affinity for MT (Cousins, 1985). Consequently, Zn therapy has been used to treat patients with Wilson’s disease to induce MT synthesis in the small intestine so intracellular Cu concentrations can be sequestered by MT providing protection from unbound Cu ions (Hoogenraad, 2006). In these cases, Cu bound to MT in the intestine can be lost via endogenous losses and thus fecal Cu concentrations are elevated (Cox and Moore, 2002).

Recent work in cattle indicated that hepatic MT concentration was not correlated to liver Cu but was correlated to liver Zn (Lopez-Alonso et al., 2005). However, when authors considered the amount of Cu and Zn bound to MT, both minerals were correlated to MT
content. Some of the earlier work characterizing MT was conducted using fetal bovine liver. Munder et al. (1985) showed that both isoforms contained approximately equal amounts of Cu and Zn. Concentrations of Cu and Zn are quite high in fetal liver (Graham et al., 1994; Gambling and McArdle, 2004), and development is a factor that affects MT expression (Cousins, 1985). In the report by Munder and coworkers, the authors indicated that adult bovine liver had much lower MT content than that of fetal liver. Some breeds of sheep are quite susceptible to Cu toxicity, and work by Saylor et al. (1980) showed that sheep have a limited ability to synthesize MT in the liver and intestine when high levels of dietary Cu are fed.

In addition to providing protection against metal toxicity, MT can be induced by physiological stressors, chemical stressors, and compounds that promote oxidative stress (Sato and Bremner, 1993). Previous data has indicated that Fe does not bind to MT in vivo (Bremner, 1991), however there have been reports that Fe deficiency and excess affects MT. Iron deficiency in rodents induces the expression of duodenal Mt1 mRNA in the duodenum (Collins et al., 2009) and MT1 protein in the blood (Robertson et al., 1989). Fleet et al. (1990) reported that Fe administration to chicks increased mRNA and protein of MT. Andrews et al. (2000) reported that H2O2 induces Mt1 mRNA in NIH 3T3 and L929 cells. It has been suggested that the thiolate groups in the MT protein react with hydroxyl radicals to provide protection against oxidative damage. Oxidative stress, as a result of Fe deficiency and excess may be a way in which induction of Mt1a and MT occurs.

Chen et al. (1995) reported that liver Mt1 mRNA was up-regulated 75 fold in Cu-deficient (0.4 mg Cu/kg diet) rats relative to those fed a Cu-adequate (5.7 mg Cu/kg diet)
diet. Although such a robust up-regulation of Mt1 mRNA occurred, MT1 protein levels were not affected by diet. Induction of Mt1 mRNA may have occurred in response to Cu deficiency induced oxidative stress. Some of the earlier work by Bremner et al. (1986) demonstrated that MT1 protein in the liver of rats does not increase until liver Cu concentration reach concentrations of 600 µg of Cu/g fresh tissue. Animals in this study were provided 1000 mg Cu/kg for several weeks. This is in stark contrast to data by Bauerly et al. (2005) reporting that hepatic Mt1 mRNA was robustly up-regulated in infant rats receiving 25 µg Cu/d even though liver Cu did not change when infant rat diets were supplemented 0, 10, or 25 µg Cu/d. In this study, Mt1 mRNA in the intestine was up-regulated in both Cu treatment groups, but intestinal Cu was only higher in rats given 25 µg Cu/d.

**Copper and iron interactions**

* Copper-dependent ferroxidases

To date we are only aware of two mechanisms that provide explanations for the close metabolic link between Cu and Fe (Fox, 2003). The Cu-dependent ferroxidases, hephaestin and ceruloplasmin (Cp), are required for the oxidation of Fe²⁺ to Fe³⁺. It has been shown in Cu-deficient mice that hephaestin activity is markedly reduced and consequently mice become anemic (Chen et al., 2006). The essentiality of Cp in Fe metabolism was first reported by Roeser et al. (1970), who found that Cu deprived pigs had low levels of plasma Fe and became anemic even though tissue Fe concentrations were normal. These authors showed that when pigs were administered Cp, plasma Fe levels began to rise. Furthermore, injections of inorganic Cu resulted in increased Cp in circulation resulting in a subsequent increase in plasma Fe concentrations. These data demonstrated the essentiality of Cp in the
oxidation of Fe$^{2+}$ to Fe$^{3+}$ for proper Fe mobilization from the liver.

Previous data demonstrated that Cu does not affect transcription of Cp (McArdle et al., 1990). However, in recent years there have been several reports examining the effects of Fe on Cp mRNA. Mukhopadhyay et al. (1998) reported that Fe deficient media increases Cp mRNA and protein in HepG2 cells. In this same report the authors showed that high Fe media decreased Cp protein by 50% compared to control media with no additional Fe. Subsequent data by Mukhopadhyay et al. (2000) illustrated that increased Cp occurred as a result of increased transcription and found that Cp contains hypoxia-responsive elements. Iron deficiency increases hypoxia inducible factor 1 and data by Martin et al. (2005) reported that Cp mRNA was up-regulated approximately 2 fold in hypoxic mice. However, data more relevant to animal nutrition indicated that Cp mRNA was not affected in rats that were exposed to deficient, normal, or high levels of Fe (Tran et al., 2002).

**Antagonisms between Cu and Fe**

The young pig is susceptible to Fe deficiency as a result of low body Fe stores at birth and low Fe content in milk; thus, in practice, Fe injections are given to prevent deficiency. On the other hand, weanling pig diets generally contain phosphorus and calcium supplements and other ingredients (i.e. blood meal) that can be high in Fe, and consequently dietary Fe concentrations can be well above their 100 mg Fe/kg requirement (NRC, 1998). Several years ago, a study conducted by Yu et al. (1994) in rats demonstrated changes in Cu homeostasis that are likely relevant to swine nutrition. These authors fed diets there were either low (7 mg Fe/kg), normal (40 mg Fe/kg), or high (389 mg Fe/kg) in dietary Fe to examine Cu and Fe interactions in rats. From this study, it was reported that increasing
dietary Fe resulted in linear decreases in apparent Cu absorption and biliary Cu excretion and a slight increase in biliary Fe excretion. Although the aforementioned decreases occurred, liver Cu was only higher in low Fe rats compared to the other treatment groups. Increased liver Cu in low Fe rats is in agreement with increased liver Cu in anemic dogs (Hahn and Fairman, 1936). To date it is unclear why the previously mentioned changes in Cu status occurred and additional studies are warranted to further examine these interactions.

It is well documented that dietary Fe can greatly reduce Cu indices in ruminants when present in superfluous amounts (Standish et al., 1969; Humphries et al., 1983; Gengelbach et al., 1994). Previous data in cattle has shown that dietary Fe concentrations can reduce apparent Cu absorption (Standish et al., 1971) which may explain why liver Cu is often reduced when dietary Fe is high. The Fe requirement for beef cattle is 50 mg Fe/kg DM (NRC, 1996), but many feedstuffs such as alfalfa, soyhulls, and corn silage, can provide high levels of dietary Fe (NRC, 1996; DePeters et al., 2000). Soil contamination during harvest is likely the reason for such high levels in corn silage. Hansen and Spears (2009) examined the effects of soil contamination on solubility of Fe. These authors used three soil types of varying Fe concentrations to contaminate greenchop silage before or after ensiling at 0%, 1%, and 5% on a wet basis. Soil types were Cecil clay loam (3.4% Fe), Georgeville silt loam (4.3%) and Dyke clay loam (6.9%). Silage was ensiled in small experimental silos for 90 days. Water solubility of Fe in soil contaminated silage was much higher than that of silage contaminated with soil after the ensiling, illustrating that the ensiling process affects Fe solubility. As determined via an *in vitro* simulated rumen, abomasum, and intestine, soluble Fe concentrations were much higher in each of these sections of the ruminant gastrointestinal
as a result of the ensiling process (Hansen and Spears, 2009).

Humphries et al. (1983) demonstrated that high dietary Fe cannot only affect liver Cu, but can also affect activity of the Cu-dependent antioxidant enzyme, superoxide dismutase. In this study, the authors utilized 3 week old Hereford-Friesian and Aberdeen Angus heifers. These calves were fed a diet containing 4 mg Cu/kg DM for approximately 3 months to reduce liver Cu concentrations to approximately 100 mg/kg DM. Subsequently, calves were allocated to dietary treatments that provided supplemental Fe in the form of saccharated ferrous carbonate and dietary treatment were fed for 32 weeks. Dietary treatments consisted of 0 supplemental molybdenum (Mo) and Fe, 0 supplemental Mo and 800 mg supplemental Fe/kg DM, 5 mg supplemental Mo/kg DM and 0 supplemental Fe, or 5 mg supplemental Mo/kg DM and 800 mg supplemental Fe/kg DM. Supplemental Fe reduced liver Cu and plasma Cu by week 8, and in fact, levels at this point and beyond were below levels indicative of Cu deficiency in cattle (liver Cu, ≤ 20 mg Cu/kg DM; plasma Cu, ≤ 0.5 mg/L; Underwood and Suttle, 1999). By the end of the study, 800 mg supplemental Fe/kg DM had the most dramatic effect in which liver Cu was reduced by approximately 95% and liver SOD activity was reduced by approximately 66% when compared to the controls.

Gengelbach et al. (1994) reported that ferrous carbonate supplemented at 600 mg Fe/kg DM reduces plasma Cu and Cp in bred heifers and their offspring. In this study, 40 two-year old heifers were fed a diet containing 20 mg of supplemental Mo/kg DM for 17 days to deplete their Cu stores. Subsequently, heifers were allocated to diets containing 10 mg supplemental Cu/kg DM or a high Fe diet containing no supplemental Cu and 600 mg supplemental Fe/kg DM. Dietary treatments were fed for 280 days and heifers gave birth to
offspring between day 70 and 125. By day 56, plasma Cu was reduced to concentrations below (< 0.5 mg/L) levels indicative of Cu deficiency (Underwood and Suttle, 1999). Copper status of offspring was greatly affected by high dietary Fe in which plasma Cu decreased markedly from day 168 (0.35 mg/L) to 280 (0.12 mg/L) of the study.

**Copper deficiency and genetic effects on copper metabolism in ruminants**

Copper deficiency is problematic in many areas of the world. Dietary Cu antagonist such as Fe, sulfur (S), and molybdenum (Mo) further exacerbates the problem, particularly when dietary Cu is low. Molybdenum and S can form strong Cu chelating complexes known as thiomolybdates, and the reducing environment in the rumen potentiates the formation of these insoluble complexes. Thiomolybdates greatly affect Cu homeostasis in ruminants by decreasing Cu absorption, increasing biliary excretion of Cu, and also removing Cu from vital cuproenzymes (NRC, 2005). Numerous studies in Cu-deficient cattle have reported decreased growth (Mills et al., 1963; Hansen et al., 2009). Hansen et al. (2009) reported severe reductions in plasma Cu and liver Cu as a result of calves being exposed to Cu deficiency *in utero* and for 493 days of life. Some of the earlier work examining Cu deficiency in cattle reported decreased activity of cytochrome c oxidase in liver and intestine (Mills et al., 1963; Fell et al., 1975; Mills et al., 1976).

Breed can also affect Cu homeostasis in ruminants, and numerous studies have reported that Simmental and Angus cattle differ in their Cu indices (Gooneratne et al., 1994; Ward et al., 1995; Mullis et al., 2003). Some of the first data demonstrating these differences reported that Simmental calves were less tolerant to dietary Mo than Angus calves, in which two Simmentsals died from systemic infection and one had to receive Cu therapy to regain
health and thriftiness (Gengelbach et al., 1994). Subsequent studies showed that Cu status of Simmentals is lower than Angus when dietary Cu is low and clear Cu by breed interactions were revealed. These interactions occurred as a result of plasma Cu, liver Cu, and plasma Cp being lower in Cu-deficient Simmental than in Cu-deficient Angus, but not when Cu adequate diets were fed (Ward et al., 1995; Mullis et al., 2003). Studies have been conducted to examine possible differences in absorption and excretion of Cu between breeds. Gooneratne and colleagues reported that bile Cu concentrations and biliary Cu excretion was two-fold higher in Simmental heifers than in their Angus counterparts. These authors indicated that biliary Zn concentration and excretion were not affected by breed, suggesting breed specificity to Cu metabolism (Gooneratne et al., 1994). Ward et al. (1995) reported that apparent Cu absorption tended to be lower in Simmental steers than in Angus steers, while retention tended to be lower in Simmentals. Definitive mechanisms responsible for these genetic differences have yet to be elucidated.

The importance of Cu in fetal development became evident in early studies where lambs born to Cu deficient dams suffered from the nervous disorder known as neonatal ataxia or “sway back” that is characterized by severe incoordination and high mortality rates (Underwood and Suttle, 1999). In cattle it has been reported that the Cu status of the dam dictates the amount of Cu transported to the fetus (Graham et al., 1994; Gooneratne and Christensen, 1989) and that the amount of maternal liver Cu stores is critical for fetal development (Gooneratne and Christensen, 1989). The fetus has a high Cu demand for growth and development and Cu accretion is even higher during the last trimester, a period in which growth is the highest (Gooneratne and Christensen, 1989). Furthermore, the
accumulated liver Cu is vital for postnatal utilization because milk is quite low in Cu (Underwood and Suttle, 1999) and synthesis of Cp does not occur for several days post-conception (Chang et al., 1975; Chang et al., 1976). Ward et al. (1995) reported that while both Angus and Simmental calves born to deficient dams had low plasma Cu concentrations, plasma Cu was much lower in Simmental offspring. Plasma Cu was also lower in Cu-adequate Simmental compared to Angus, and from these data the authors suggested Angus dams may have transported more Cu to their fetus. Thus genetics may also dictate the amount of Cu accrued by the bovine fetus.

**Responses to high dietary copper in weanling pigs**

In 1948, Braude reported the first observations that Cu stimulated growth when pigs licking Cu pipes grew faster than their counterparts without access to Cu pipes. This report initiated the inclusion of dietary Cu in the range of 125 to 250 mg/kg Cu, which is well above the growing pig’s NRC requirement of 5 mg/kg Cu (NRC, 1998). Since this initial observation many studies have reported increases in average daily gain, feed intake, and enhanced feed efficiency when Cu is fed at pharmacological levels (Braude, 1967; Edmonds et al., 1985; Cromwell 1991; Cromwell, 1997). Research indicates no additive effects on performance when Cu is supplemented above 250 mg/kg Cu (Cromwell et al., 1989), and to date, definitive mechanisms responsible for the pharmacological effects of Cu are unknown.

Ammerman et al. (1995) reported that bioavailability of Cu from tribasic copper chloride (Cu$_2$(OH)$_3$Cl) was equal to that of Cu from Cu sulfate (CuSO$_4$). In a later report, Cromwell et al. (1998) reported that the growth promoting efficacy of Cu from Cu$_2$(OH)$_3$Cl was similar to Cu from CuSO$_4$. Liver is the primary storage organ for Cu, and the authors
also reported that 100 mg Cu/kg diet from both Cu$_2$(OH)$_3$Cl and CuSO$_4$ did not significantly increase liver Cu concentrations. However, supplementing 200 mg Cu/kg from either source increased liver Cu, but liver Cu in CuSO$_4$ supplemented pigs increased to a greater magnitude relative to control pigs. Liver Cu in Cu$_2$(OH)$_3$Cl supplemented pigs moderately increased, and as a result, these pigs tended to have lower liver Cu concentrations than those supplemented with CuSO$_4$ at 200 mg Cu/kg. Previous data in weanling pigs have shown that supplementing CuSO$_4$ at 225 mg Cu/kg increases bile Cu concentrations (Armstrong et al., 2000). However, these authors did not provide explanations as to why liver Cu increased to a greater magnitude in CuSO$_4$ pigs than in Cu$_2$(OH)$_3$Cl-supplemented pigs when compared to controls. Marked chemical differences between CuSO$_4$ and Cu$_2$(OH)$_3$Cl may affect Cu uptake and absorption. Miles et al. (1998) reported that CuSO$_4$ is very soluble (99%) in water, while very little (< 1%) of Cu$_2$(OH)$_3$Cl is soluble. *In vitro* acid solubility is quite different between these Cu sources as well. Pang and Applegate (2007) reported that *in vitro* solubility of CuSO$_4$ at pH 2.5 was approximately 100% while the solubility of Cu$_2$(OH)$_3$Cl was approximately 80%. Furthermore, solubility of CuSO$_4$ was 99.8% and 75.5% at pH of 5.5 and 6.5, respectively, while solubility of Cu$_2$(OH)$_3$Cl was 29.2% and 9.1% at pH of 5.5 and 6.5, respectively.

Copper absorption primarily occurs in the duodenum and upper jejunum (Linder and Hazegh-Azam, 1996), and, as expected, supplementing high levels of dietary Cu increases Cu concentrations in the digesta and intestinal mucosa. Drouliscos et al. (1970) reported that supplementing Cu at 250 mg Cu/kg diet from CuSO$_4$ increased Cu concentrations in the digesta contents and intestinal mucosa of weanling pigs, but did not affect Cu concentrations
in the portal blood plasma. The portal blood plasma represents the portion of Cu entering the liver from the gastrointestinal tract, and lack of an effect suggests robust regulation of Cu absorption by the small intestine when dietary Cu is high. Minimal studies have examined Cu homeostasis throughout the small intestine when dietary Cu is supplemented at pharmacological levels and it is unknown how the young pig regulates these high levels.

**Conclusion**

Rodents and cell culture systems have provided much needed information in regard to cellular Cu homeostasis. Examination of these mechanisms in livestock will likely advance our knowledge of practical dietary issues in ruminant and nonruminant nutrition. In cattle, Cu deficiency is of practical concern and factors such as high dietary Fe, Mo, S, and genetics can greatly affect Cu homeostasis. The young pig is quite susceptible to Fe deficiency and can also be exposed to high concentrations of dietary Fe. Furthermore, high levels of Cu enhance pig performance, and are often supplemented during the weanling or nursery period. Currently we are unfamiliar with how such high levels of dietary Cu are controlled by the young pig.

**Literature Cited**


Nose, Y., B.E. Kim, and D.J. Thiele. 2006. Ctr1 drives intestinal copper absorption and is essential for growth, iron metabolism, and neonatal cardiac function. Cell Metab. 4: 235-244.


CHAPTER 2

Expression of copper transporters and chaperones are affected by life-long, severe copper deficiency and gender in bovine duodenum and liver

R. Scott Fry*, Melissa S. Ashwell*, Terry E. Engle†, Hyungchul Han†, Stephanie L. Hansen*, and Jerry W. Spears*,§

*Department of Animal Science, North Carolina State University, Raleigh, NC 27695, USA
†Department of Animal Science, Colorado State University, Fort Collins, CO 80523, USA
*Current address: Department of Animal Science, Iowa State University, Ames, IA 50011
†Corresponding author: Dr. Jerry W. Spears, fax +1 919 515 4463, email: jerry_spears@ncsu.edu
Abstract

A study was conducted to determine the effects of life-long, severe copper (Cu) deficiency and gender on cellular Cu metabolism in the bovine. Duodenal scrapings and liver were obtained for Cu analysis and analysis of gene products involved in cellular Cu metabolism from Angus calves (n = 14) after receiving dietary treatments for 493 days. Treatments consisted of 1) 10 mg supplemental Cu/kg DM (copper adequate, CuA; n = 6) or 2) no supplemental Cu and 2 mg supplemental molybdenum (Mo)/kg DM (copper deficient, CuD; n = 8). Dietary Mo was used to induce deficiency. Treatments contained equal numbers of males and females. Cu status in CuD calves was much lower than in CuA calves, but gender did not affect ceruloplasmin activity or Cu concentrations in liver, plasma, and duodenum. Duodenal CTR1 protein was higher ($P = 0.03$) in males than in females, but hepatic $ctr1$ mRNA was higher ($P = 0.03$) in females compared to males. Hepatic $cox17$ was markedly lower ($P = 0.002$) in CuD than in CuA calves, but was higher ($P = 0.03$) in males than in females. Furthermore, $cox17$ and $atp7b$ were lower ($P = 0.001; P = 0.04$), while $atp7a$ tended ($P = 0.08$) to be lower in CuD males vs. CuA males, but these genes were not down-regulated in CuD females vs. CuA females. These data indicate that life-long, severe Cu deficiency affects important Cu regulatory mechanisms in the bovine and that these mechanisms are affected differently by gender.

Introduction

It has been well documented that dietary copper (Cu) is essential for an array of Cu-dependent enzymes that are involved in a number of physiological processes (NRC, 2005). Cu deficiency is a problem in ruminants in many areas of the world. As expected low dietary
Cu intake contributes to Cu deficiency, but potent Cu antagonists such as molybdenum (Mo) and sulphur (S) exacerbate the problem. Throughout the British Isles Mo can be found at elevated levels (Suttle, 1991; Thornton, 2002) and many of the by-product feedstuffs that are currently being fed to cattle in the United States often times contain relatively high concentrations of dietary S (Kerr et al., 2008). Thiomolybdates, comprised of Mo and S can form in the developed rumen, and bind with high affinity to dietary Cu (Suttle, 1991). Cu metabolism is antagonized by thiomolybdates by decreasing absorption, increasing biliary excretion of Cu, and chelating Cu from metalloenzymes (NRC, 2005).

Absorption, excretion, and Cu incorporation into metalloenzymes does not occur via passive diffusion or at any degree of spontaneity, as free or unbound Cu is toxic to the cell. Thus, specific Cu transporters and chaperones are in place to regulate Cu metabolism at the cellular level. The essentiality and function of these mechanisms have been evaluated in a breadth of studies using rodents and cell lines. Cu is taken up via a high affinity Cu transporter, called copper transporter 1 (CTR1) that specifically transports Cu+ (Prohaska and Gybina, 2004; Kim et al., 2008). Upon entering the cell, Cu is bound to one of several Cu chaperones. Cu can be delivered to cytochrome c oxidase (CCO) in the mitochondria via COX17 where Cu is delivered to a series of other CCO assembly proteins and subsequently CCO subunits (i.e. SCO1, SCO2, COX11, and COX4). Cu can also be sequestered via metallothionein 1 (MT1), which can regulate Cu absorption in the enterocyte and serves as the primary Cu storage protein in the liver. Antioxidant 1 (ATOX1) chaperones Cu down the secretory pathway in the trans golgi network where Cu is delivered to one of two Cu dependent Cu-ATPases, ATP7A in the intestine and ATP7B in the liver (Hamza et al., 2003;
Hamza et al., 1999). These ATPases are dual function Cu transporters. ATP7A is responsible for basolateral Cu export from the enterocyte and incorporation of Cu into cuproenzymes (i.e. lysyl oxidase, tyrosinase). A mutation in this Cu exporter results in Menkes’ disease in humans, a state of Cu deficiency that is due to a lack of Cu utilization from the enterocyte and often results in death (Cox and Moore, 2002). In the liver, ATP7B interacts with COMMD1 to excrete Cu via bile, the major route of Cu excretion in mammals. ATP7B is also responsible for incorporating Cu into ceruloplasmin (Cp), a protein that contains approximately 95% of the plasma Cu in circulation. Individuals with mutations in \textit{atp7b} or \textit{commd1} suffer from Cu toxicity. Wilson disease patients have a mutated \textit{atp7b} that results in hyperaccumulation of liver Cu, resulting in lipid peroxidation and decreased apo-Cp synthesis (Prohaska and Gybina, 2004; Kim et al., 2008). Bedlington terriers have an inherit defect in \textit{commd1}, thus biliary Cu excretion is markedly reduced (Klomp et al., 2003).

While the majority of the studies in model species have evaluated transporter and chaperone essentiality using knock-out models and evaluated these mechanisms with immunohistochemical analysis, some studies have evaluated the effect of Cu deficiency on mRNA and protein expression of various transporters and chaperones \textit{in vivo}. Lee et al. (2000) reported that hepatic \textit{ctr1} mRNA was not affected in Cu deficient rats although liver Cu concentrations were reduced by approximately 90% compared to Cu adequate rats. In another rat study, SCO1 and CCS proteins were significantly higher, and the CCO subunit, COX4, was significantly reduced in cardiac tissue of Cu deficient rats compared to rats receiving CuA diets (Getz et al., 2009). These authors also reported that cardiac COX17 did not differ between dietary treatments.
Several studies have reported that gender affects Cu status. Liver Cu was higher in male than in female calves (Miranda et al., 2006). Haywood (1979) reported similar results in rats, with males having more liver Cu than females. However, in humans, females generally have higher plasma Cu and Cp than males (Mason, 1979). This difference may be attributed to differences in circulating hormone profiles between males and females (Mason, 1979; Solomons, 1979). Hormones seem to affect Cu transporters that are involved in Cu homeostasis. Research in cell culture demonstrated that \textit{atp7a} contains estrogen response elements and that CTR1, ATP7A, and ATP7B respond to hormonal treatment as well (Hardman et al., 2007; Kelleher and Lonnerdal, 2006; Michalczyk et al., 2008; Hardman et al., 2006). Although the aforementioned studies were conducted in placental cell lines and mammary glands, it is very plausible that hormones, particularly estrogen, could have a similar effect on these transporters \textit{in vivo} in intestine and liver tissue.

Although some of the key Cu transporters and chaperones have been previously identified at the mRNA level in the bovine (Han et al., 2009), the effect of Cu deficiency on these mechanisms in the bovine have not been evaluated nor has bovine CTR1 protein been characterized. A recent study with cattle from our laboratory reported that life-long exposure to diets deficient in Cu resulted in marked reductions in Cu status and decreased expression of hepatic \textit{cox1} and \textit{sod1} (Hansen et al., 2009). Therefore animals from the aforementioned study were utilized to determine the effects of life-long, severe copper deficiency and gender on end products associated with cellular Cu homeostasis in bovine duodenum and liver tissue, and to characterize CTR1 protein in the duodenum of the bovine.
Experimental methods

*Animals and Experimental Design*

Experimental procedures were approved by the North Carolina State University Animal Care and Use Committee prior to initiation of the experiment. Fourteen Angus steers (castrated males; n = 7) and heifers (females; n = 7) from a previous study (Hansen et al., 2009) with an average body weight of 560 (SE 19·6) kg at harvest were utilized in the present study. Calves in the present study were the second set of offspring from cows utilized in an experiment evaluating the effects of long-term, severe Cu deficiency on brain prion protein biology (Legleiter, 2006). These dams had been consuming dietary treatments for approximately 410 d prior to conception. As a result, animals in the present study were exposed to dietary treatments *in utero* and subsequently for 493 d of life. Average calf birth date was considered d 0 of the study, thus d 493 is the average calf age at harvest. Other details in regard to animal vaccinations, handling, and management have been previously reported in detail (Hansen et al., 2009).

Dietary treatments consisted of 1) 10 supplemental Cu/kg DM (Cu adequate, CuA; n = 6) or 2) no supplemental Cu and 2 mg supplemental Mo/kg DM (Cu deficient, CuD; n = 8). Both dietary treatments contained an equal number of steers and heifers. Treatments were provided to calves in a corn-silage-based growing diet and a corn-based finishing diet. Cu content in the growing and finishing diet was 7 and 4 mg/kg DM, respectively. Growing and finishing diets were fed for 136 and 139 d, respectively, and were individually fed via electronic Calan gate feeders (American Calan, Northwood, NH; Hansen et al., 2009). Diets were formulated to meet or exceed NRC recommended requirements (NRC, 1996) with the
exception of Cu. Cu was supplemented as Cu$_2$(OH)$_3$Cl to CuA diets, while Mo was supplemented as NaMoO$_4$ to CuD diets. Mo has been used in numerous studies to induce Cu deficiency in cattle (Genegelbach et al., 1994; Legleiter et al., 2007; Suttle and Angus, 1976).

**Tissue Collection**

Calves were harvested on d 493 of the study at a commercial abattoir. Animals were humanely euthanized as they were stunned with captive bolt and exsanguinated, and a segment of liver and duodenal mucosa were obtained for mineral as well as protein and mRNA analysis as described by Hansen et al. (2009). Samples obtained for mineral analysis were placed on ice while samples for protein and mRNA analysis were snap frozen in liquid nitrogen and subsequently stored at -80°C until isolation procedures. Liver and duodenal mucosa mineral content was determined as described by Gengelbach et al. (1994).

**RNA Isolation and Real Time Polymerase Chain Reaction**

Total RNA was isolated from duodenal mucosa and liver using the RNeasy kit as per manufacturer’s instructions with an on-column DNase digestion (Qiagen Inc., Valencia, CA, and USA). Duodenal RNA was isolated from males only, because RNA integrity of female samples was lost during storage. Quantity and integrity of RNA was determined via Nanodrop-100 Spectrophotometer and agarose gel (Hansen et al., 2009). Briefly, real-time primers were designed using Beacon Designer Software to be compatible with SYBR Green I as indicated by Hansen et al. (2009). Target genes and the tissue in which they were evaluated are noted in Table 2. Primers were designed to amplify across a predicted intron-exon boundary when possible to allow for detection of any genomic DNA contamination. Ribosomal protein 9 (*rps9*) was utilized as an internal housekeeping gene as it has been
previously reported by Janovick-Guretzky et al. (2007) to be stable under various nutritional conditions in cattle and has been previously used under conditions of severe Cu deficiency (Hansen et al., 2009). Product specificity was accessed by generating melting curves for each PCR reaction and one amplicon from each primer pair was sequenced to confirm product identity.

Briefly, 1 µg of total RNA was used to synthesize cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc., Foster City, CA, USA) as per manufacturer’s instructions. Real-time PCR was performed as specified by Hansen et al. (2009). Standard curves for each gene was generated using a five point, 1:3 series dilution of pooled cDNA and ran in triplicate within each sample tissue. These curves were used to generate PCR amplification efficiencies for each primer pair as described by Hansen et al. (2009).

**Protein Extraction and Western Blot Procedure**

Protein from liver samples was not isolated as protein integrity was lost during storage. Isolation procedure of the duodenal protein has been described previously (Hansen et al., 2009). A pooled sample was generated by obtaining equal molar amounts of total protein from each animal for normalization of unknown samples. Samples were separated with polyacrylamide gel electrophoresis using the Novex X-Cell Surelock Minicell system (Invitrogen Corp, Carlsbad, CA, USA). All supplies such as buffers, gels, and membranes were purchased from Invitrogen Corp. Approximately 69 µg of protein was heated at 70°C for 10 min with loading buffer and 2-mercaptoethanol. Samples, pooled samples, and a Novex XP low molecular weight protein standard were then loaded into precast 10 well, 10%
Bis-Tris gels. Proteins were separated under denaturing conditions and were subsequently transferred onto a polyvinylidene difluoride membrane for immunoblotting. At the completion of the transfer, membranes were stained with Ponceau S stain (Sigma, St. Louis, Mo, USA) to determine equal loading and then washed with 0.02 M tris buffered saline (TBS; pH 7.4) to rid the membrane of stain. Membranes were then blocked at room temperature in TBS containing 3% (w/v) bovine serum albumin (BSA-TBS). After blocking, membranes were probed for 1 h with a polyclonal rabbit anti-CTR1 at a dilution of 1:1,000 (vol/vol) in TBS containing 0.05% (vol/vol) tween-20 and 3% (wt/vol) BSA (BSA-TTBS; anti-CTR1 was kindly provided by Dr. Dennis J. Thiele). Members of the CTR protein family contain three predicted membrane-spanning domains and methionine- and histidine-rich amino terminals that are important in copper binding (Sharp, 2003). The anti-CTR1 used in the present study targets the sequence H2N-VSIRYNSMPVPGPNGTILC-COOH that resides in the cytosolic loop between the first and second transmembrane domain of mouse and human CTR1 (Nose et al., 2006). Bovine CTR1 contains the identical peptide sequence in the same region as mouse and human. Subsequently, an anti-rabbit alkaline phosphatase-linked secondary antibody was diluted at 1:10,000 (vol/vol) in BSA-TTBS and incubated for 30 min. Membranes were briefly washed and incubated with Lumi-Phos™ WB substrate as per manufacturer’s instructions (Thermo Scientific) prior to autoradiography film exposure. Band densities on the captured film were detected using Image Quant TL software (Amersham Biosciences, Piscataway, NJ, USA). Pooled samples were run in duplicate on each gel, one at each end of the gel. Since all unknown samples could not be analyzed within one gel (10 well gels), half (n = 7) of the experimental samples were ran across two gels with
one replicate per gel, balancing for dietary treatment and gender. This was repeated for the other half of the samples. Band densities of experimental replicates were averaged and expressed as a ratio to the mean density of the pooled samples within the sample gels. Thus results are expressed as a ratio to the experimental pool.

**Statistical Analysis**

Duodenal CTR1 protein data and Cu status data were analyzed by ANOVA using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC, USA). To further investigate gender effects Cu, gender, and Cu × gender were all included in the model. Liver Cu and liver gene expression were correlated using PROC CORR in SAS (SAS Institute). Significance was declared at $P \leq 0.05$ and tendencies are discussed at $P < 0.15$ due to relatively low animal numbers.

Briefly, as described by Hansen et al. (2009), CT values from target genes were normalized to the CT value of their respective housekeeping gene as indicated below:

$$CT_N = CT_T / CT_{rps9},$$

where $CT_T$ is the target gene CT value and $CT_N$ is the normalized CT value that was tested for significance. Samples containing greater amounts of the target cDNA will have a lower or smaller $CT_N$. $CT_N$ values for duodenal and liver gene expression were analyzed by ANOVA using the MIXED procedure of SAS (SAS Institute Inc.). The model for duodenal gene data included Cu, whereas the model for liver gene data included Cu, gender, and Cu × gender. Significance was declared at $P \leq 0.05$, and tendencies will be discussed at $P < 0.15$ due to low animal numbers. Fold changes for each target gene between dietary treatments and gender were determined via the Pfaffl method (Pfaffl et al., 2002) and PCR amplification.
efficiencies were accounted for in the calculation (Hansen et al., 2009). Amplification efficiencies for *rps9* and all target genes ranged from 75 to 125 percent.

**Results**

**Copper Status**

After receiving experimental diets for 493 d of life, calves receiving CuD diets had markedly lower Cu concentrations in plasma, liver, and duodenal mucosa than CuA fed calves and these data are reported elsewhere (Hansen et al., 2009; Hansen et al., 2010). Briefly, CuD calves had 72.6%, 96.7%, 86.7%, and 79.4% percent reductions in duodenal Cu, liver Cu, Cp, and plasma Cu, respectively compared to CuA calves. Gender effects in each of the aforementioned variables were tested. Duodenal Cu, liver Cu, and plasma Cu was not affected by gender (Table 3).

**Duodenal Protein and mRNA expression**

Duodenal CTR1 protein was detected at an apparent molecular weight of approximately 30 kDa and was not affected (*P* = 0.72) by diet but was affected by gender. Males had higher (*P* = 0.03) expression of this high affinity Cu importer than females regardless of dietary treatment (Figure 1). Duodenal expression of *ctr1* was not affected by diet (*P* = 0.24), but the major enterocyte Cu-exporter, *atp7a* tended to be higher (*P* = 0.12) in CuD vs. CuA males. Gene expression profiles of duodenal *cox17*, *atox1*, and *atp7b* mRNA were not affected by diet (Table 4).

**Liver mRNA expression**

Hepatic *ctr1* mRNA was not affected by diet (*P* = 0.35; Table 5) but was affected by gender (*P* = 0.03; Table 6). In contrast to duodenal CTR1 protein, hepatic *ctr1* mRNA was
higher in females than in males regardless of diet. Cox17, the chaperone responsible for delivering Cu to the mitochondria for Cu incorporation into CCO, was affected at the transcript level by Cu (Table 5), gender (Table 6), and a Cu × gender interaction (Table 7). CuD calves had markedly ($P = 0.002$) lower cox17 expression vs. CuA calves. Furthermore, males had greater ($P = 0.03$) cox17 expression compared to females regardless of diet. A Cu × gender interaction ($P = 0.01$) occurred as a result of CuD males, but not CuD females ($P = 0.49$), having lower ($P = 0.001$) cox17 expression than their counterparts fed CuA diets. The function of atp7a in the liver is unknown, but in the present study atp7a was affected by a Cu × gender interaction ($P = 0.03$). CuD males tended ($P = 0.08$) to have less atp7a than CuA males, but atp7a was numerically higher ($P = 0.14$) in CuD females than in CuA fed females. The dual function hepatic Cu exporter, atp7b, was lower ($P = 0.04$) in CuD vs. CuA males, but was not affected ($P = 0.38$) by diet in females resulting in a Cu × gender interaction ($P = 0.04$). Interactions in these transporters and chaperones can be further explained with liver Cu and mRNA correlations. In males, cox17 and liver Cu was significantly correlated ($R^2 = 0.90; P = 0.01$) while atp7b ($R^2 = 0.70; P = 0.08$) tended to be correlated with liver Cu. Liver Cu and atp7a were numerically ($R^2 = 0.65; P = 0.11$) correlated in males. However, in females liver Cu was not correlated to any degree with these genes. Transcript levels of atox1, mt1, commd1 and cp were not affected by diet or gender. Expression of the mitochondrial Cu chaperones sco1, sco2, and cox11 along with cox4 subunit were not affected by diet or gender (Table 5; Table 6).
Discussion

The current study is unique in that animals were exposed to dietary treatments *in utero* and post parturition. Not only was the Cu status of these animals severely depressed (Hansen et al., 2009; Hansen et al., 2010), but CCS protein levels were higher in duodenum (Hansen et al., 2009), liver (Hepburn et al., 2009), and erythrocytes (Hepburn et al., 2009) when compared to CuA calves, further indicating the severity and degree of Cu deficiency. CCS protein levels have been shown to increase in response to Cu deficiency in rodents and has been proven useful as an experimental biomarker for Cu deficiency (Bertinato et al., 2003). Thus, considering the extremely low Cu status of the calves in the present study (Hansen et al., 2009; Hansen et al., 2010), in combination with their respective dam’s Cu status (Legleiter, 2006) a unique opportunity was available to evaluate the effects of life-long Cu deficiency on cellular Cu metabolism in the bovine. Additionally, gender of calves was fortunately equal across both dietary treatments, thus an additional opportunity was available in the present study to evaluate the effect of gender on gene products involved in cellular Cu metabolism. Numerous studies have reported differences in Cu status due to gender and results have been variable within and across species. Males accumulated more Cu in the liver than females in cattle (Miranda et al., 2006) and rodents (Haywood, 1979), with contrasting results reported in humans (Mason, 1979). However, in the present study Cu status was not affected by gender. Males in the present study were castrated which may explain why no differences occurred between gender. Differences in Cu metabolism between intact and castrated males have been reported, indicating that testosterone can affect Cu metabolism (Fields et al., 1987). It is unclear if animals in the study by Miranda et al. (2006) were intact
or castrated.

Intracellular Cu is tightly regulated by specific Cu transporters and chaperones that are involved in Cu acquisition, distribution, and utilization (Prohaska and Gybina, 2004; Kim et al., 2008). To our knowledge the present study is the first to show the presence of the high-affinity Cu import protein, CTR1 in the duodenum of the bovine. Following immunoblotting, bovine CTR1 was detected at approximately 30 kDa, which is consistent with previous reports in rodents (Lee et al., 2000; Nose et al., 2006). CTR1 has been shown to be present in many murine tissues (Kuo et al., 2006) and has been shown to be essential for Cu uptake in the intestine. The generation of intestinal epithelial cell-specific $ctr1$ knock-out mice resulted in neonatal defects, iron accumulation in peripheral tissues, and severe growth and viability defects (Nose et al., 2006). In the present study, although duodenal Cu concentrations were decreased by approximately 72%, duodenal CTR1 protein in both males and females was not affected by severe Cu deficiency, nor was $ctr1$ mRNA in males. Lack of an effect of dietary Cu on transcript levels of $ctr1$ in the present study agrees with data from CuD male rats. Lee et al. (2000) reported that despite the fact that rats were severely CuD, $ctr1$ was unaffected in duodenal mucosa, indicating that Cu does not affect transcription of $ctr1$. Few studies have shown increased CTR1 protein expression in response to Cu deficiency, but very recent work in CuD rat duodenum demonstrated higher expression of CTR1 than CuA counterparts after receiving diets for just five days (Nose et al., 2010). Furthermore, immunohistochemical analysis in mice has demonstrated that CTR1 expression increases at the apical membrane in response to Cu deficiency in duodenum and in the newborn mouse (Kuo et al., 2006; Nose et al., 2010). Localization of CTR1 in the adult mouse is in the intracellular vesicles, suggesting
that CTR1 can undergo trafficking in response to dietary Cu (Kuo et al., 2006). It is plausible that cellular localization differed between treatment groups in our study even though expression of CTR1 was similar between these animals.

In patients with Menkes disease, \textit{atp7a} is mutated and as a result basolateral utilization of Cu from the enterocyte is hindered, resulting in a state of Cu deficiency that is oftentimes fatal (Cox and Moore, 2002). The tendency for \textit{atp7a} mRNA to be up-regulated in CuD males vs. CuA males suggests the enterocytes from these animals were responding to Cu deprivation and attempting to increase Cu utilization. Analysis of \textit{atp7a} mRNA in females would allow a more definitive conclusion to be drawn, but at any rate these data suggest that Cu deficiency may affect transporters involved in efflux of Cu rather than uptake in the intestine. To our knowledge, the effect of severe Cu deficiency on \textit{atp7a} has not been evaluated, as the majority of studies in model species has evaluated the effect of high Cu intake and low dietary Fe on \textit{atp7a} expression (Bauerly et al., 2005; Collins et al., 2005). Transcript levels of duodenal \textit{cox17}, \textit{atox1}, and \textit{atp7b} were not affected by Cu deficiency and little to no published data exists evaluating these mechanisms \textit{in vivo} under conditions of severe Cu deficiency.

Although liver Cu concentrations in CuD animals were reduced by approximately 96%, \textit{ctr1} was not affected by severe Cu deficiency. These data agree with previous research in severely CuD rats. Lee et al. (2000) reported that despite liver Cu being reduced by approximately 90%, \textit{ctr1} mRNA was similar between CuD and CuA rats. In the present study, mean liver and plasma Cu in CuD animals was 0.11 µmol/g DM and 3.6 µmol/l, respectively (Hansen et al., 2009). In the bovine, levels below 0.31 µmol/g DM and 7.9
μmol/l for liver and plasma Cu respectively, are considered clinically deficient (Underwood & Suttle, 1999). Lack of an effect of dietary Cu on 

\textit{ctr1} mRNA agrees with our duodenal results and further indicates that transcription does not appear to change in response to Cu status.

COX17 is the Cu chaperone protein located both in the cytosol and intermembrane space of the mitochondria responsible for Cu delivery to the mitochondria for Cu incorporation into CCO, the terminal enzyme in the electron transport chain (Prohaska and Gybina, 2004). Animals fed CuD diets had markedly less \textit{cox17} than their CuA fed counterparts. Furthermore, in a separate report from our laboratory (Hansen et al., 2009), CuD animals in this study had lower \textit{cox1} mRNA as well. The effect of severe Cu deprivation on cytochrome c oxidase activity in these animals is unknown as it was not measured. However, studies have been reported that CCO activity is lower in the liver (Mills et al., 1963; Mills et al., 1976) and even the enterocyte (Fell et al., 1975) of CuD vs. CuA calves. Some recent work in cardiac tissue from rats indicates that Cu deficiency affects the assembly of a fully functional CCO enzyme (Zeng et al., 2007). The assembly of CCO is carried out by many different mitochondrial Cu chaperones that are responsible for delivering Cu to the various subunits of CCO. Thus to further assess the reductions in \textit{cox17} and \textit{cox1}, \textit{cox4} and the mitochondrial Cu chaperones \textit{sco1}, \textit{sco2}, and \textit{cox11} were measured at the mRNA level. Once Cu is chaperoned via COX17, it has been postulated and observed that SCO1 and COX11 mediate Cu transfer into COX2 and COX1 subunits, respectively (Cobine et al., 2006). SCO2 is another chaperone that is involved in Cu delivery to the COX2 subunit and its function does not overlap that of SCO1 (Leary et al., 2004). In the present study,
severe Cu deficiency did not affect mRNA levels of sco1, sco2, cox11, or the cox4 subunit. Getz et al. (2009) reported that cardiac COX17 protein was not affected by Cu deficiency, but that SCO1 protein was higher while COX1 and COX4 were lower in Cu deficient rats. Although, we only examined mRNA expression, it appears that Cu deficiency does not significantly affect the key players in CCO assembly in the bovine. Studies in rodents evaluating the effect of Cu deficiency on CCO activity and assembly have shown concomitant reductions.

No differences in mRNA expression of atox1, mt1, cp, or commd1 were observed in the present study. Although plasma Cp was reduced by approximately 86% in CuD animals, cp mRNA was not affected by diet and these data agree with previous reports that Cu deficiency does not affect cp at the mRNA level (McArdle et al., 1990). Additionally, cp mRNA was not affected by gender. Data from Cu deficient rats indicated that hepatic mt1 mRNA was increased approximately 75 fold relative to Cu adequate counterparts (Chen et al., 1995). In contrast we found mt1 mRNA levels to be similar between dietary treatment groups. Because Cu status was severely reduced over an extended period of time, it is plausible that CuD animals adapted to low dietary Cu intake and as a result mt1 was not up-regulated by the end of the study. COMMD1 is involved in biliary Cu excretion and is mutated in the Bedlington terriers, who suffer from Cu toxicity as a result of reduced Cu excretion via the bile (Klomp et al., 2003). ATOX1 is the essential Cu chaperone that delivers Cu to the Cu-ATPases, ATP7A and ATP7B (Hamza et al., 2003; Hamza et al., 1999). To our knowledge no data exist evaluating the effect of Cu deficiency and gender on these mechanisms and our data suggests that neither Cu, nor gender affects mRNA
expression of either gene.

In the liver, ATP7B is the Cu efflux pump involved in biliary Cu excretion and incorporation of Cu into Cp. Human subjects with Wilson’s disease and the Long Evans Cinnamon rat have a mutated \textit{atp7b} (Wu et al., 1994), thus these individuals experience Cu toxicosis as biliary Cu excretion and synthesis of holo-Cp are markedly reduced. Until recently, the dogma has existed that little to no ATP7A is present in the adult liver (Prohaska and Gybina, 2004), but recent work by Kim et al. (2010) demonstrated the presence and differential expression of this transporter in the liver of cardiac cell-specific \textit{Ctr1} knock-out mice. We, as well as others (Han et al., 2009), have detected \textit{atp7a} in bovine liver. Additionally, we observed that \textit{atp7a} is present in the liver in abundances similar to \textit{atp7b} as indicated via quantitative RT-PCR analysis (data not shown). In the present study, a Cu × gender interaction occurred with \textit{atp7a}, \textit{atp7b} and even \textit{cox17}. Expression of \textit{atp7b} and \textit{cox17} was lower in males fed CuD vs. CuA diets but significant changes did not occur in females fed CuD vs. CuA diets. Additionally, \textit{cox17} mRNA was 24% higher in males than females regardless of dietary Cu. Although only tendencies were present, expression of \textit{atp7a} was 35% lower \((P = 0.08)\) in CuD vs. CuA males, but in CuD females \textit{atp7a} was numerically higher \((P = 0.14; 46\%)\) than in CuA fed females resulting in a significant \((P = 0.03)\) Cu × gender interaction. These novel results occurred without gender differences in liver Cu or other indicators of Cu status, suggesting that a signal independent of Cu can alter the expression of these mechanisms. Elegant work by Hardman et al. (2007) demonstrated that \textit{atp7a} contains an estrogen response element and that \textit{atp7a} expression increased following estrogen treatment. In the present study, liver Cu was correlated with \textit{cox17}, \textit{atp7a}, and \textit{atp7b}
expression in males but not in females. Because females in the present study were cycling, it seems plausible that their estrogen profiles affected *atp7a* differently than in males because expression of the previously mentioned genes and liver Cu were not correlated. Why reductions in *atp7b* and *cox17* occurred in CuD males but not CuD females is unclear. Recent work in cell models reported that ATP7A and ATP7B are regulated by lactation hormones (Michalczyk et al., 2008). Although these females were not lactating it is possible that unknown signals independent of Cu are having an effect on certain Cu transporters. Other signaling phenomena have been eluded to by Kim et al. (2010) as a result of increased expression of ATP7A protein in human primary umbilical vein endothelial cells and human intestinal epithelial-like cell line (i.e. Caco-2 cells) following treatment with serum from cardiac *ctr1* knockout mice.

Hepatic *ctr1* mRNA was approximately 40% higher while duodenal CTR1 protein was approximately 34% lower in females versus males regardless of dietary Cu. These differences were not expected and to our knowledge previous research has not reported gender differences in Ctr1 expression. Because duodenal and liver Cu was not affected by gender, it seems as though cellular signals independent of Cu may be regulating the expression of this high affinity Cu importer. Work in placenta and choriocarcinoma Jeg-3 cells (Hardman et al., 2006), as well as cultured mammary epithelial cells (Kelleher and Lonnerdal, 2006), suggests that CTR1 responds to hormone treatment. Hardman et al., (2006) reported that western blot analysis of CTR1 in Jeg-3 cells treated with insulin, estrogen, and progesterone showed an approximately two fold increase. Prolactin treatment of cultured mammary epithelial cells altered the cellular localization of CTR1 and greater
band intensity at approximately 70 kDa rather than 30 and 35 kDa (Kelleher and Lonnerdal, 2006). No such differences or observations were discovered in the present study. Even though these hormonal effects are in placental and mammary gland cell lines and not in intestine and liver, it is possible that the menstrual cycle and associated changes in hormone profiles in females may have affected CTR1 expression differently. As previously mentioned, the integrity of duodenal RNA samples from females and liver protein samples from all animals were lost during a storage malfunction. Limited reports in this area and the fact that mRNA and protein were not able to be evaluated in both tissues from all animals does not allow us to provide further explanations as to why gender differences were tissue dependent. The abundance of \textit{ctr1} in the duodenum of females and hepatic CTR1 data in the present study would provide more information into this novel finding.

It has been well documented that dietary antagonist such as Mo and S decrease Cu utilization in the bovine (Suttle, 1991; Gengelbach et al., 1994). While the number of experimental animals in the present study could ideally be higher, the opportunity to evaluate cellular Cu metabolism during such an extreme case of Cu deficiency is rare. In the present study indices of Cu status were first measured in calves at 114 d of age. By this time plasma and liver Cu concentration in CuD calves were below the thresholds for clinical Cu deficiency (Hansen et al., 2009). It is likely that liver and plasma Cu were reduced prior to that first measurement, but nonetheless by d 493, animals had experienced a continuum of Cu deficiency for 379 days. Marked differences in hair pigmentation occurred, as tyrosinase is a Cu-dependent enzyme required for melanin synthesis (Petris et al., 2000). Changes in hair pigmentation are often associated with some degree of Cu deprivation in cattle. Over the
length of the animals’ life in the present study, we observed the most dramatic changes in hair pigmentation during the early, more developmental stages of life. By the end of the finishing phase, changes in pigmentation were not as dramatic suggesting a possible adaptation or changes in cellular distribution of Cu. Some work with Cu deprivation in zebrafish embryos indicates that during the early stages of Cu deficiency, tyrosinase activity is the first to be eliminated as a means to increase Cu utilization for more vital processes within the body (Turski and Thiele, 2009). As the state of Cu deficiency becomes more robust, cellular distribution then repartitions to prioritize intracellular Cu utilization. If repartitioning of Cu does indeed occur in the bovine, then it is possible that the end point measurement in the present study likely missed other transcriptional and translational changes in these Cu transporters and chaperones. Nonetheless the present study provides information at the cellular level in the bovine, demonstrating that cellular mechanisms involved in Cu acquisition, distribution, and utilization are affected by Cu deficiency and gender in the bovine.

**Conclusion**

The present study demonstrates the presence of duodenal CTR1 protein in the bovine along with characterization of many of the known Cu transporters and chaperones that are involved in acquisition, distribution, and utilization of intracellular Cu. Transcription and translation of Ctr1 does not appear to be affected by severe Cu deficiency in duodenum or liver. Although no effects of gender on Cu status were found in the present study, Ctr1 expression was affected by gender in the duodenum and liver. *Cox17* was the only gene affected by Cu regardless of gender. Liver Cu and *cox17* were positively correlated in males
but not in females. Furthermore, it appears that the extremely low liver Cu in males had a
more dramatic effect on liver \textit{atp7b and atp7a} expression than in females, even though liver
Cu did not differ between CuD males and females.

\textbf{References}

copper absorption, tissue distribution, and copper transporter expression in an infant rat

Bertinato, J., M. Iskandar, and M.R. L’Abbe. 2003. Copper deficiency induces the
upregulation of the copper chaperone for Cu/Zn superoxide dismutase in weanling male rats.


Cobine, P.A., F. Pierrel, and D.R. Winge. 2006. Copper trafficking to the mitochondrion and

differentially expressed genes in response to dietary iron deprivation in rat duodenum. Am. J.
Physiol. Gastrointest. Liver Physiol. 288: 964-971.


Gengelbach, G.P., J.D. Ward, and J.W. Spears. 1994. Effect of dietary copper, iron, and
molybdenum on growth and copper status of beef cows and calves. J. Anim. Sci. 72: 2722-
2727.

Getz, J., D. Lin, and D.M. Medeiros. 2009. Dietary copper deficiency up-regulates selected


Nose, Y., B.E. Kim, and D.J. Thiele. 2006. Ctr1 drives intestinal copper absorption and is essential for growth, iron metabolism, and neonatal cardiac function. Cell Meta. 4: 235-244.


Table 1. Ingredient composition of the growing and finishing diets

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Growing phase (%DM)*</th>
<th>Finishing phase (%DM)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize silage</td>
<td>86·88</td>
<td>-</td>
</tr>
<tr>
<td>Ground maize</td>
<td>-</td>
<td>83·80</td>
</tr>
<tr>
<td>Soyabean meal (48%)</td>
<td>9·00</td>
<td>7·00</td>
</tr>
<tr>
<td>Cottonseed hulls</td>
<td>-</td>
<td>5·00</td>
</tr>
<tr>
<td>Urea</td>
<td>1·00</td>
<td>0·80</td>
</tr>
<tr>
<td>CaSO₄</td>
<td>0·80</td>
<td>0·80</td>
</tr>
<tr>
<td>Limestone</td>
<td>0·10</td>
<td>0·40</td>
</tr>
<tr>
<td>NaCl</td>
<td>0·20</td>
<td>0·20</td>
</tr>
<tr>
<td>Vitamin premix‡</td>
<td>0·01</td>
<td>0·01</td>
</tr>
<tr>
<td>Trace mineral premix</td>
<td>0·01</td>
<td>0·01</td>
</tr>
<tr>
<td>Monensin</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Treatment supplement¶</td>
<td>2·00</td>
<td>2·00</td>
</tr>
</tbody>
</table>

*Analyzed 7 mg Cu/kg DM and 59 mg Mn/kg DM
†Analyzed 4 mg Cu/kg DM and 32 mg Mn/kg DM
‡Provided (per kg premix): 1·98 g all-trans retinol; 38 mg cholecalciferol; 4·2 g DL-α-tocopheryl acetate.
§Provided (per kg diet): 30 mg Zn as ZnSO₄; 0·5 mg I as Ca(IO₃)₂(H₂O); 0·2 mg Se as Na₂SeO₃; 0·1 mg Co as CoCO₃.
¶Provided 33 mg monensin/kg DM.
¶A ground maize supplement provided the following treatments: +Cu (10 mg Cu/kg DM, 20 mg Mn/kg DM); -Cu (20 mg Mn/kg DM, 2 mg Mo/kg DM).
Table 2. Real-time polymerase chain reaction primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Accession number</th>
<th>Tissue Analyzed</th>
<th>Primer sequence (5’- 3’)‡</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rps9§</td>
<td>DT860044</td>
<td>†, *</td>
<td>F: CCTCGACCAAGAGCTGAAG</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: CCTCCAGACCTACGTTTTGTTTC</td>
<td></td>
</tr>
<tr>
<td>ctrl</td>
<td>NM00110038</td>
<td>†, *</td>
<td>F: AAGAGTCTCTGGAGGTGTG</td>
<td>200</td>
</tr>
<tr>
<td>1.1</td>
<td></td>
<td></td>
<td>R: GGTCAGATGAAGTGGTTG</td>
<td></td>
</tr>
<tr>
<td>atox1</td>
<td>XM877831.2</td>
<td>†, *</td>
<td>F: CGGAAGCAGAGTTCTCC</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: TGTGGGCGAGGTCAATGTC</td>
<td></td>
</tr>
<tr>
<td>cox17</td>
<td>AF031558.1</td>
<td>†, *</td>
<td>F: GAGCAGACCACCATTCATAC</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: GCGAAGCCGACAAAATGG</td>
<td></td>
</tr>
<tr>
<td>atp7a</td>
<td>XM615430.3</td>
<td>†, *</td>
<td>F: GAAGATTATAGTATGGCTCAACC</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: CCTCTGCTGTGATAAGATGG</td>
<td></td>
</tr>
<tr>
<td>atp7b</td>
<td>XM596258.3</td>
<td>†, *</td>
<td>F: AAGTCTTGGTCCAGTCC</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: GAGATGGATCGTGAAGAACC</td>
<td></td>
</tr>
<tr>
<td>Cp</td>
<td>XM592003.4</td>
<td>†</td>
<td>F: GGTATGGGTAATGAAATGGTGT</td>
<td>275</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>R: TCACAGGCTGTAGTAGTG</td>
<td></td>
</tr>
<tr>
<td>commd1</td>
<td>NM00104638</td>
<td>†</td>
<td>F: CTGGAGGCAATTTTGACTGCT</td>
<td>112</td>
</tr>
<tr>
<td>4.1</td>
<td></td>
<td></td>
<td>R: GACTCTTCGAGATTTTTGTCTTATG</td>
<td></td>
</tr>
<tr>
<td>mt1</td>
<td>NM00104049</td>
<td>†</td>
<td>F: ATGGACCCGAACTGCTCCTGC</td>
<td>182</td>
</tr>
<tr>
<td>2.1</td>
<td></td>
<td></td>
<td>R: GCCGACGCTGACTTGTCCG</td>
<td></td>
</tr>
<tr>
<td>cox11</td>
<td>NM00108340</td>
<td>†</td>
<td>R: GTTGTGCTAGTAGATTCTAGC</td>
<td>182</td>
</tr>
<tr>
<td>3.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cox4</td>
<td>NM00100143</td>
<td>†</td>
<td>F: CTCTCTCTGCTGCCTCC</td>
<td>111</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td></td>
<td>R: ATCTCACTTCAACACTTCA</td>
<td></td>
</tr>
<tr>
<td>sco1</td>
<td>NM00108024</td>
<td>†</td>
<td>F: AGGAGGAAGTAGGACTACATA</td>
<td>196</td>
</tr>
<tr>
<td>3.1</td>
<td></td>
<td></td>
<td>R: TGGCAGGAAATAGAGCATGG</td>
<td></td>
</tr>
<tr>
<td>sco2</td>
<td>NM00110549</td>
<td>†</td>
<td>F: TGTAAGTGGAGAGTGTTG</td>
<td>169</td>
</tr>
<tr>
<td>3.1</td>
<td></td>
<td></td>
<td>R: GAAGTTGCCAGGAGG</td>
<td></td>
</tr>
</tbody>
</table>

*Analyzed in duodenum of males
†Analyzed in liver of both males and females
‡F, Forward; R, Reverse
§Reported by Janovick-Guretzky et al. 2007.
Table 3. Effect of calf gender on Cu status

<table>
<thead>
<tr>
<th>Item</th>
<th>Males</th>
<th>Females</th>
<th>SE</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenal Cu, µmol/g DM</td>
<td>0.16</td>
<td>0.28</td>
<td>0.070</td>
<td>0.26</td>
</tr>
<tr>
<td>Liver Cu, µmol/g DM</td>
<td>1.80</td>
<td>1.59</td>
<td>0.152</td>
<td>0.36</td>
</tr>
<tr>
<td>Cp, µmol/l</td>
<td>0.04</td>
<td>0.03</td>
<td>0.004</td>
<td>0.13</td>
</tr>
<tr>
<td>Plasma Cu, µmol/l</td>
<td>11.67</td>
<td>11.86</td>
<td>1.188</td>
<td>0.91</td>
</tr>
</tbody>
</table>

Males, n = 7; Females, n = 7
Table 4. Gene expression profiles in duodenal mucosa of males

<table>
<thead>
<tr>
<th>Target</th>
<th>CuA Mean</th>
<th>CuA SE</th>
<th>CuA Fold</th>
<th>CuD Mean</th>
<th>CuD SE</th>
<th>CuD Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>ctr1</td>
<td>1·21</td>
<td>0·026</td>
<td>1·00</td>
<td>1·17</td>
<td>0·023</td>
<td>1·77</td>
</tr>
<tr>
<td>atox1</td>
<td>1·22</td>
<td>0·021</td>
<td>1·00</td>
<td>1·21</td>
<td>0·018</td>
<td>1·14</td>
</tr>
<tr>
<td>cox17</td>
<td>1·63</td>
<td>0·018</td>
<td>1·00</td>
<td>1·61</td>
<td>0·016</td>
<td>1·04</td>
</tr>
<tr>
<td>atp7a</td>
<td>1·45</td>
<td>0·024</td>
<td>1·00</td>
<td>1·39</td>
<td>0·021</td>
<td>1·96</td>
</tr>
<tr>
<td>atp7b</td>
<td>1·29</td>
<td>0·017</td>
<td>1·00</td>
<td>1·27</td>
<td>0·015</td>
<td>1·32</td>
</tr>
</tbody>
</table>

ctr1, copper transporter 1; atox1, antioxidant 1; cox17, cytochrome c oxidase assembly protein 17; atp7a, copper transporting, alpha polypeptide; atp7b, copper transporting, beta polypeptide.

CuA, Cu adequate (n = 3); CuD, Cu deficient (n = 4).

*CT ratios are shown as the target gene:rps9 ratio; lower CT ratio = higher expression.
†The mRNA fold change was calculated relative to CuA steers using the Relative Expression Software Tool (REST©) adjusting for PCR amplification efficiencies for the target genes and rps9. Δ, change.
Table 5. Main effect of dietary Cu on gene expression profiles in liver of beef cattle

<table>
<thead>
<tr>
<th>Target</th>
<th>CT ratio*</th>
<th></th>
<th></th>
<th></th>
<th>CT ratio*</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CuA</td>
<td></td>
<td></td>
<td></td>
<td>CuD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
<td>Fold Δ†</td>
<td>Mean</td>
<td>SE</td>
<td>Fold Δ†</td>
<td>Mean</td>
<td>SE</td>
</tr>
<tr>
<td>ctr1</td>
<td>1.02</td>
<td>0.008</td>
<td>1.00</td>
<td>1.01</td>
<td>0.007</td>
<td>1.10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>atox1</td>
<td>0.91</td>
<td>0.011</td>
<td>1.00</td>
<td>0.91</td>
<td>0.011</td>
<td>1.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cox17</td>
<td>1.12</td>
<td>0.006</td>
<td>1.00</td>
<td>1.15b</td>
<td>0.005</td>
<td>0.60</td>
<td></td>
<td></td>
</tr>
<tr>
<td>atp7a</td>
<td>1.11</td>
<td>0.008</td>
<td>1.00</td>
<td>1.12</td>
<td>0.007</td>
<td>0.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>atp7b</td>
<td>1.09</td>
<td>0.011</td>
<td>1.00</td>
<td>1.12</td>
<td>0.009</td>
<td>0.77</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cp</td>
<td>0.84</td>
<td>0.017</td>
<td>1.00</td>
<td>0.84</td>
<td>0.015</td>
<td>0.91</td>
<td></td>
<td></td>
</tr>
<tr>
<td>commd1</td>
<td>1.23</td>
<td>0.011</td>
<td>1.00</td>
<td>1.24</td>
<td>0.010</td>
<td>0.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mt1</td>
<td>0.79</td>
<td>0.016</td>
<td>1.00</td>
<td>0.80</td>
<td>0.014</td>
<td>0.81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cox11</td>
<td>1.20</td>
<td>0.014</td>
<td>1.00</td>
<td>1.22</td>
<td>0.012</td>
<td>0.83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cox4</td>
<td>1.32</td>
<td>0.021</td>
<td>1.00</td>
<td>1.36</td>
<td>0.016</td>
<td>0.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sco1</td>
<td>1.23</td>
<td>0.013</td>
<td>1.00</td>
<td>1.22</td>
<td>0.012</td>
<td>0.81</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sco2</td>
<td>1.26</td>
<td>0.014</td>
<td>1.00</td>
<td>1.27</td>
<td>0.012</td>
<td>0.98</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ctr1, copper transporter 1; atox1, antioxidant 1; cox17, cytochrome c oxidase assembly protein 17; atp7a, copper transporting, alpha polypeptide; atp7b, copper transporting, beta polypeptide; cp, ceruloplasmin; commd1, copper metabolism domain containing 1; mt1, metallothionein 1; cox11, cytochrome c oxidase assembly protein 11; cox4, cytochrome c oxidase subunit 4; sco1, SCO cytochrome oxidase deficient homolog 1; sco2, SCO cytochrome oxidase deficient homolog 2.

CuA, copper adequate (n = 6); CuD, copper deficient (n = 8)

*Mean values within a row with unlike superscript letters were significantly different (P < 0.01)

†The mRNA fold change was calculated relative to CuA steers using the Relative Expression Software Tool (REST©) adjusting for PCR amplification efficiencies for the target genes and rps9. Δ, change.
Table 6. Main effect of gender on gene expression profiles in liver of beef cattle

<table>
<thead>
<tr>
<th>Target</th>
<th>CT ratio*</th>
<th>SE</th>
<th>Fold Δ†</th>
<th>CT ratio*</th>
<th>SE</th>
<th>Fold Δ†</th>
</tr>
</thead>
<tbody>
<tr>
<td>ctr1</td>
<td>1.02a</td>
<td>0.007</td>
<td>1.00</td>
<td>0.99b</td>
<td>0.007</td>
<td>1.40</td>
</tr>
<tr>
<td>atox1</td>
<td>0.91</td>
<td>0.012</td>
<td>1.00</td>
<td>0.91</td>
<td>0.012</td>
<td>1.05</td>
</tr>
<tr>
<td>cox17</td>
<td>1.12a</td>
<td>0.005</td>
<td>1.00</td>
<td>1.14b</td>
<td>0.005</td>
<td>0.76</td>
</tr>
<tr>
<td>atp7a</td>
<td>1.11</td>
<td>0.008</td>
<td>1.00</td>
<td>1.12</td>
<td>0.008</td>
<td>0.81</td>
</tr>
<tr>
<td>atp7b</td>
<td>1.10</td>
<td>0.010</td>
<td>1.00</td>
<td>1.12</td>
<td>0.010</td>
<td>0.73</td>
</tr>
<tr>
<td>cp</td>
<td>0.84</td>
<td>0.016</td>
<td>1.00</td>
<td>0.85</td>
<td>0.016</td>
<td>0.84</td>
</tr>
<tr>
<td>commd1</td>
<td>1.23</td>
<td>0.011</td>
<td>1.00</td>
<td>1.24</td>
<td>0.011</td>
<td>0.91</td>
</tr>
<tr>
<td>mtl</td>
<td>0.79</td>
<td>0.015</td>
<td>1.00</td>
<td>0.79</td>
<td>0.015</td>
<td>1.07</td>
</tr>
<tr>
<td>cox11</td>
<td>1.22</td>
<td>0.013</td>
<td>1.00</td>
<td>1.20</td>
<td>0.013</td>
<td>1.19</td>
</tr>
<tr>
<td>cox4</td>
<td>1.34</td>
<td>0.017</td>
<td>1.00</td>
<td>1.34</td>
<td>0.019</td>
<td>1.02</td>
</tr>
<tr>
<td>sco1</td>
<td>1.23</td>
<td>0.013</td>
<td>1.00</td>
<td>1.23</td>
<td>0.012</td>
<td>1.51</td>
</tr>
<tr>
<td>sco2</td>
<td>1.28</td>
<td>0.013</td>
<td>1.00</td>
<td>1.26</td>
<td>0.013</td>
<td>1.26</td>
</tr>
</tbody>
</table>

 ctr1, copper transporter 1; atox1, antioxidant 1; cox17, cytochrome c oxidase assembly protein 17; atp7a, copper transporting, alpha polypeptide; atp7b, copper transporting, beta polypeptide; cp, ceruloplasmin; commd1, copper metabolism domain containing 1; mtl, metallothionein 1; cox11, cytochrome c oxidase assembly protein 11; cox4, cytochrome c oxidase subunit 4; sco1, SCO cytochrome oxidase deficient homolog 1; sco2, SCO cytochrome oxidase deficient homolog 2.

Males (n = 7), Females (n = 7)

a,b Mean values within a row with unlike superscript letter were significantly different (P < 0.05)

*CT ratios are shown as the target gene: rps9 ratio; lower CT ratio = higher expression

†The mRNA fold change was calculated relative to males using the Relative Expression Software Tool (REST©) adjusting for PCR amplification efficiencies for the target genes and rps9. Δ, change.
Table 7. Genes affected by a Cu × gender interaction in liver of beef cattle

<table>
<thead>
<tr>
<th>Target</th>
<th>Males CT ratio*</th>
<th>Males Fold ∆†</th>
<th>Females CT ratio*</th>
<th>Females Fold ∆†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>cox17</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CuA</td>
<td>1.09(^a)</td>
<td>0.007</td>
<td>1.00</td>
<td>1.14(^{ab})</td>
</tr>
<tr>
<td>CuD</td>
<td>1.15(^b)</td>
<td>0.007</td>
<td>0.44</td>
<td>1.15(^{ab})</td>
</tr>
<tr>
<td>atp7a</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CuA</td>
<td>1.09(^c)</td>
<td>0.012</td>
<td>1.00</td>
<td>1.14(^{cd})</td>
</tr>
<tr>
<td>CuD</td>
<td>1.12(^d)</td>
<td>0.010</td>
<td>0.65</td>
<td>1.11(^{cd})</td>
</tr>
<tr>
<td>atp7b</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CuA</td>
<td>1.07(^e)</td>
<td>0.016</td>
<td>1.00</td>
<td>1.13(^{ef})</td>
</tr>
<tr>
<td>CuD</td>
<td>1.12(^f)</td>
<td>0.014</td>
<td>0.45</td>
<td>1.11(^{ef})</td>
</tr>
</tbody>
</table>

*CT ratios are shown as the target gene:rps9 ratio; lower CT ratio = higher expression
†The mRNA fold change was calculated relative to CuA steers using the Relative Expression Software Tool (REST\(©\)) adjusting for PCR amplification efficiencies for the target genes and rps9. ∆, change.

cox17, cytochrome c oxidase assembly protein 17; atp7a, copper transporting, alpha polypeptide; atp7b, copper transporting, beta polypeptide.

Males; CuA, copper adequate (n = 3) and CuD, copper deficient (n = 4).
Females; CuA, copper adequate (n = 3) and CuD, copper deficient (n = 4).

\(^{ab}\) Mean value within a column with unlike superscript letters were significantly different (\(P < 0.01\))
\(^{cd}\) Mean value within a column with unlike superscript letters were different (\(P < 0.10\))
\(^{ef}\) Mean value within a column with unlike superscript letters were significantly different (\(P < 0.05\))
Figure 1. Main effects of dietary copper and gender on duodenal copper transporter 1 (CTR1) protein expression. CuA, copper adequate; CuD, copper deficient. ROI, relative optical intensities. Values are means, expressed as a ratio to the experimental pool, with treatment standard errors represented by vertical bars. Duodenal CTR1 was not affected by dietary Cu, $P = 0.72$, but was affected by gender, $P = 0.03$. 
CHAPTER 3

Effect of dietary copper and breed on gene products involved in copper acquisition, distribution, and utilization in Angus and Simmental cows and their fetuses

R.S. Fry, J.W. Spears, K.E. Lloyd, A.T. O’Nan, and M.S. Ashwell

North Carolina State University, Raleigh, NC, USA, 27695

3Corresponding author: melissa_ashwell@ncsu.edu
Abstract

Copper (Cu) deficiency is a problem in ruminants and breed differences in Cu metabolism may contribute to this issue. Intracellular Cu is tightly regulated by transport and chaperone proteins, and to date these mechanisms have not been elucidated to address breed differences in Cu metabolism, nor have they been characterized in bovine fetal liver. Mature, pregnant Angus (n = 8) and Simmental (n = 8) cows (~4 mo into gestation) were used in a 2 × 2 factorial design. Cows were randomly assigned to corn-silage based diets that were either adequate (+Cu) or deficient (-Cu) in Cu. Diets were fed for 112 d via electronic Calan gates. Jugular blood samples were taken at 28 d intervals while liver biopsies were taken on d 0 and 56 for mineral analysis. At the end of the study, cows were harvested to collect duodenal mucosa scrapes, liver samples, and fetal liver samples for mineral analysis and also for mRNA and protein analysis of Cu transport and chaperone proteins. Placentome samples were also obtained for mineral analysis. Plasma Cu and liver Cu were affected by Cu, breed, and Cu × breed. Both of these Cu indices were lower (P ≤ 0.05) in –Cu Simmentals (–CuS) than in –Cu Angus (–CuA), but were similar among +Cu Simmental (+CuS) and +Cu Angus (+CuA). Duodenal Cu was lower (P = 0.01) in –Cu vs. +Cu cows. Placentome Cu was lower (P = 0.003) in –Cu vs. +Cu cows, and was also lower (P = 0.03) in Simmentals vs. Angus. Fetal liver Cu was lower (P = 0.002) in –Cu vs. +Cu fetuses, and was also lower (P = 0.05) in Simmentals vs. Angus. Levels of Cu transporter1 (CTR1) protein and transcripts for Cu transporters and chaperones were not affected by Cu or breed in liver and were not affected by Cu in the intestine. Duodenal Ctr1 was lower (P = 0.04) and CTR1 tended (P = 0.10) to be lower in Simmentals vs. Angus. Furthermore, expression of Atp7a tended (P = 0.08) to be
lower in Simmentals than in Angus. In fetal liver, relative expression of antioxidant 1 \((Atox1)\), cytochrome c oxidase assembly protein 17 \((Cox17)\), and Cu metabolism MURR1 domain 1 \((Commd1)\) were up-regulated \((P \leq 0.05)\) in –Cu fetuses vs. +Cu fetuses. In conclusion, lower expression of duodenal \(Ctr1\) and slightly lower \(CTR1\) and \(Atp7a\) suggest that Simmentals have a lesser ability to absorb and utilize dietary Cu, and may explain why they are more prone to Cu deficiency than Angus. Furthermore, up-regulation of fetal liver \(Atox1\), \(Cox17\), and \(Commd1\) in –Cu fetuses reflects the high Cu demand by the fetus.

**Introduction**

Copper (Cu) deficiency is a problem in ruminants and can manifest when the diet supplies less than their requirement. This problem is further enhanced when superfluous amounts of Cu antagonists, such as molybdenum (Mo), sulfur (S), or iron (Fe), are present in the diet \((NRC, 2005)\). Breed can also affect Cu metabolism as several studies in cattle have reported that Simmentals have lower Cu indices than Angus when fed Cu-deficient diets \((Ward et al., 1995; Mullis et al., 2003)\). Clinical signs of deficiency have also been more pronounced in Simmentals fed Cu-deficient diets than in Angus \((Gengelbach et al., 1994)\). The bovine fetus has a high demand for Cu \((Underwood and Suttle, 1999)\), and previous research has indicated that maternal Cu status, and possibly breed of dam, may affect Cu transport to the fetus \((Gooneratne and Christensen, 1989; Ward et al., 1995)\).

Numerous transport and chaperone proteins tightly regulate intracellular Cu ions to prevent both deficiency and toxicity \((Prohaska and Gybina, 2004)\), and a breadth of data exists in model species evaluating these mechanisms. We have previously characterized and identified mRNA for many of the Cu transporters and chaperones known to regulate Cu
homeostasis in the bovine, as well as copper transporter 1 (CTR1) protein (Fry et al., 2009). Copper transporter 1 is a high affinity Cu transporter that is essential for proper intestinal Cu uptake (Nose et al., 2006) and the primary transport for hepatic Cu uptake (Kim et al., 2009). Mechanistic data have not been elucidated to explain why Cu status among Angus and Simmental cattle differs, nor have gene products involved in Cu metabolism been characterized in bovine fetal liver. The present study was conducted to determine the effect of dietary Cu and breed (Angus vs. Simmental) on gene products associated with Cu acquisition, distribution, and utilization in the duodenal mucosa and liver of pregnant cows and liver of their fetuses.

Materials and Methods

Animal care and experimental design

Prior to the initiation of the study, animal care, use, and sampling procedures were approved by the North Carolina State University Animal Care and Use committee. Sixteen mature, pregnant multiparous Angus (n = 8) and Simmental (n = 8) cows bred to purebred Angus bulls were utilized in a 2 × 2 factorial design. Within breed cows were stratified by initial Cu status, age, and estimated days pregnant and then randomly assigned to diets that were either deficient (-Cu) or adequate (+Cu) in Cu (Table 1). Mean cow age across all dietary treatments was approximately 6 y and mean stage of pregnancy at the beginning of the study was approximately 4 m. Copper-adequate diets contained 10 mg supplemental Cu/kg DM in the form of Cu2(OH)3Cl. The –Cu diet analyzed 6.6 mg Cu/kg DM and was supplemented with 5 mg Mo/kg DM as NaMoO4 and 0.15% S from NH4SO4 to further induce Cu deficiency. Urea was added to the +Cu diet to make diets isonitrogenous. Diets
were fed once daily at 1.75% of BW on a DM basis, and were formulated to meet or exceed all NRC requirements with the exception of Cu (NRC, 1996). Cows were individually fed via electronic Calan gates for 112 d (American Calan, Northwood, NH).

**Sampling procedures**

Jugular blood samples were obtained on d 0, 28, 56, 84, and 112 for determination of plasma mineral concentrations and holo(active)-ceruloplasmin (holo-Cp). Liver biopsies were obtained for liver mineral analysis on d 0 and 56 as described previously (Tiffany et al., 2003). On d 113 and 114 of the study an equal number of cows from each breed and dietary Cu treatment were humanely euthanized via captive bolt at a commercial abattoir to collect duodenal mucosal scrapes, liver samples, and fetal liver samples for mineral analysis and mRNA and protein analysis of Cu transporters and chaperones. Placentome samples were also collected for mineral analysis. Mucosal scrapes were obtained as described by Hansen et al. (2009). Tissues obtained for mineral analysis were placed in whirl-pak bags and stored on ice. Samples taken from each tissue for mRNA and protein analysis were placed in a 50 mL tube containing RNAlater preservative as per manufacturer’s instructions (Ambion, Austin, TX). In tables and text, end sampling (d 113 and 114) will be referred to as d 112 for simplicity.

**Analytical procedures**

Determination of holo-Cp was conducted as described by Houchin (1958), and plasma Cu was prepared and analyzed as described by Hansen et al. (2008). Tissues and feed samples were dried in a forced-air oven and were ashed for mineral analysis via the microwave procedures described by Gengelbach et al. (1994). Plasma and tissue mineral
concentrations were determined using atomic absorption spectrometry (AA-6701F, Shimadzu, Japan). A bovine NIST certified liver standard was used to validate instrumental accuracy.

**Quantitative Real Time PCR**

Total RNA was isolated from duodenal mucosal scrapes, liver samples, and fetal liver samples using the RNeasy kit (Qiagen, Valencia, CA) with an on-column DNase digestion. Subsequently, quantity and integrity of RNA was determined via a Nanodrop-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Integrity was assessed further by running the isolated RNA on 1.2% agarose gel to determine the integrity of the 28S and 16S ribosomal subunits. Real-time primers (Table 2) were designed using Beacon Designer Software (Premier Biosoft Intl., Palo Alto, CA) to be compatible with SYBR Green I (Ambion, Austin, TX), as described by Hansen et al. (2009). The proposed function(s) of each of the gene products examined are outlined in Table 3. Multiple housekeeping genes obtained from a report by Lisowski et al. (2008) were screened to determine the most stable for normalization in duodenum. Of those tested (β-actin, glyceraldehyde 3-phosphate dehydrogenase, succinate dehydrogenase, TATA-box binding protein), TATA-box binding protein (Tbp) was the most stable between breed and dietary treatment. In the liver, ribosomal protein subunit 9 (Rps9) was used as the housekeeping gene and was stable between dietary treatment and breed and has been reported by others to be a good housekeeping gene in bovine liver (Janovick-Guretzky et al. 2007; Hansen et al., 2009, Hansen et al., 2010). Specificity of each reaction was accessed via melt curve analysis and
one amplicon from each primer reaction was sequenced to confirm identity. One µg of total RNA from each tissue was used to synthesize cDNA utilizing the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc., Foster City, CA, USA) as per manufacturer’s instructions. Quantitative RT-PCR reactions were conducted as previously reported (Hansen et al., 2009), and relative expression of target genes was determined using the $2^{-\Delta\Delta CT}$ method (Lavak and Schmittgen, 2001).

**Protein isolation and immunoblotting**

Protein from duodenum, maternal liver, and fetal liver were isolated as described by Hansen et al. (2009). Within each tissue, an experimental pool sample was generated by obtaining equal molar concentrations of protein from each animal for normalization of unknown protein bands. Samples were separated using polyacrylamide gel electrophoresis using the Novex X-Cell Surelock Minicell system (Invitrogen Corp, Carlsbad, CA, USA). All supplies and buffers for immunoblotting procedures were purchased from Invitrogen. Approximately 40 µg of duodenal protein, 65 µg of liver protein, and 78 µg of fetal liver protein were heated at 70°C for 10 min with loading buffer and 2-mercaptoethanol. Samples, low molecular weight protein standard (Novex XP, Invitrogen), and experimental pool samples were then loaded into precast 10% Bis-Tris, 10 well gels. Proteins were separated under denaturing conditions and then transferred to a polyvinylidene difluoride membrane for immunoblotting. Subsequently membranes were stained with Ponseau S stain (Sigma, St. Louis, Mo, USA) to confirm equal loading of protein and then washed several times with 0.02 M tris buffered saline (pH 7.4) to rinse the membrane of the stain. After membranes were blocked for 1 h at room temperature, 1 of the following antibodies were probed for 1 h
at room temperature: 1) a polyclonal rabbit anti-CTR1 antibody diluted 1:1000 (kindly provided by Dr. Dennis Thiele) or 2) a polyclonal rabbit anti-CCS antibody diluted 1:500 (kindly provided by Dr. Joseph Prohaska). Bovine CTR1 contains the identical peptide sequence in the same region as mouse and human (Nose et al., 2006). The anti-CCS antibody has been used in a previous report from our laboratory and reacts with high specificity in bovine tissues (Hansen et al., 2009). At the completion of the primary antibody incubation, membranes were washed and then incubated with a secondary antibody at 1:10,000. Band densities were detected using Image Quant TL software (Amersham Biosciences, Piscataway, NJ, USA). Experimental pool samples were ran in duplicate on each gel, one at each end of the gel. Band densities of experimental replicates were averaged and expressed as a ratio to the mean density of the experimental pool samples within the sample gels.

**Statistical Analysis**

Data were analyzed using the mixed procedure of SAS (Cary, NC), and animal was the experimental unit. Plasma minerals, holo-Cp, and maternal liver minerals were analyzed as repeated measures and model included the fixed effects Cu and breed, and cow was considered a random effect. Duodenal mineral, placentome mineral, fetal liver mineral, maternal protein and gene data were analyzed using ANOVA. For maternal data the model included the fixed effects Cu and breed. The model for fetal liver data included the fixed effects of Cu, breed, and fetal gender. All possible interactions were considered in the model, and were removed if $P > 0.15$. Data are expressed as least square means ± SEM. Significance was declared at $P \leq 0.05$ and tendencies are discussed at $P \leq 0.10$.  

82
Results

On d 70 of the study ultrasound measurements were obtained to confirm pregnancy and stage of pregnancy. During this time a +CuA cow was found to be open. Data from this animal was not considered in any of the analysis.

Maternal Plasma Measurements

Repeated measures analysis of plasma Cu revealed it was affected by Cu ($P = 0.01$), breed ($P = 0.003$), and a Cu × breed interaction ($P = 0.01$; Table 4). Overall plasma Cu was lower ($P = 0.0004$) in –CuS vs. –CuA, but did not differ among +CuS vs. +CuA cows. Plasma Cu was also affected by day ($P = 0.002$) and tended ($P = 0.10$) to be affected by a Cu × day interaction. On d 28 plasma Cu was lower ($P = 0.04$) in –Cu cows than in their +Cu counterparts. Plasma Cu was not affected by Cu on d 56. However, d 84 plasma Cu tended ($P = 0.07$) to be lower while d 112 plasma Cu was lower ($P = 0.004$) in –Cu vs. +Cu cows (Table 4). Overall plasma holo-Cp tended ($P = 0.06$) to be affected by Cu and breed and was affected ($P = 0.05$) by a Cu × breed interaction and day ($P < 0.001$; Table 5). Like that of plasma Cu, holo-Cp was lower ($P = 0.01$) in –CuS vs. –CuA, but did not differ ($P = 0.95$) among breeds fed +Cu diets. Overall plasma Fe tended ($P = 0.06$) to be lower in –Cu vs. +Cu cows (1.9 ± 0.09 vs. 2.2 ± 0.10 mg/L), but was not affected by breed or Cu × breed. Plasma Zn was not affected by Cu or breed, but tended ($P = 0.08$) to be affected by a Cu × breed interaction. Plasma Zn was higher ($P = 0.03$) in –CuS vs. –CuA (1.19 ± 0.03 vs. 1.07 ± 0.03 mg/L), but was similar ($P = 0.82$) among +CuS and +CuA (1.09 ± 0.03 vs. 1.10 ± 0.04 mg/L).
**Maternal Tissue Mineral Concentrations**

At harvest, duodenal Cu concentrations were lower \((P = 0.01)\) in –Cu cows compared to those fed +Cu diets (Table 6). Duodenal Cu concentrations were not affected by breed or a Cu × breed interaction. Duodenal Zn concentrations tended \((P = 0.09)\) to be lower in –Cu cows compared to +Cu cows, but were not affected by breed or a Cu × breed interaction. Concentrations of Fe in the duodenum were not affected by Cu, breed, or Cu × breed. Overall liver Cu concentrations were affected by Cu \((P = 0.001)\), breed \((P = 0.03)\), and Cu × breed \((P = 0.04; \text{Table 7})\).

Liver Cu concentrations were lower \((P = 0.005)\) in –CuS vs. –CuA, but did not differ \((P = 0.90)\) among +CuS vs. +CuA treatment groups. Liver Cu concentrations were also affected by day \((P = 0.006)\) and a Cu × day interaction \((P = 0.002)\). On d 56 \((P = 0.001)\) and 112 \((P < 0.0001)\) liver Cu concentrations were lower in –Cu cows than in +Cu cows. Liver Fe concentrations were not affected by Cu, breed, or Cu × breed (data not shown). Liver Zn concentrations were also not affected by Cu or a Cu × breed interaction (data not shown), but tended \((P = 0.06)\) to be affected by breed. Simmentals tended \((P = 0.06; 122.9 \pm 5.1 \text{ vs. } 138.4 \pm 5.4 \text{ mg Zn/kg DM})\) to have lower liver Zn concentrations than Angus.

**Placentome and Fetal Liver Mineral Concentrations**

Placentome Cu concentrations were not affected by fetal gender, but were lower \((P = 0.003)\) in –Cu cows compared to their +Cu counterparts, and were also lower \((P = 0.03)\) in Simmentals than in Angus (Table 8). Concentrations of Zn and Fe in the placentome were not affected by Cu, breed, or fetal gender. Fetal liver Cu concentrations were affected by Cu \((P = 0.002)\), breed \((P = 0.05)\), gender \((P = 0.03)\) and tended \((P = 0.07)\) to be affected by a Cu
breed interaction (Table 8). Copper concentrations in liver of +CuS fetuses were lower ($P = 0.02$) than that of +CuA fetuses ($508 \pm 66$ vs. $831 \pm 90$ mg Cu/kg DM), but did not differ ($P = 0.84$) among –CuS and –CuA fetuses ($317 \pm 66$ vs. $296 \pm 81$ mg Cu/kg DM), which is the opposite of what occurred in their dams. Liver Cu concentrations were higher ($P = 0.03$) in male fetuses than in females. Fetal liver Zn concentrations were not affected by breed or gender, but tended ($P = 0.08$) to be lower in –Cu fetuses than in +Cu fetuses. Iron concentrations in fetal liver were not affected by Cu, breed, or gender.

**Maternal Tissue Gene and Protein Expression**

In the duodenum, dietary Cu did not affect mRNA expression of Cu transporters ($Ctr1$, $Atp7a$, $Atp7b$) and chaperones ($Atox1$, $Cox17$), nor did Cu affect metallothionein 1a ($Mt1a$) and superoxide dismutase 1 ($Sod1$) mRNA (Table 9). Breed did not affect mRNA expression of $Atp7b$, Cu chaperones, $Mt1a$, or $Sod1$. However, duodenal $Ctr1$ mRNA was approximately 4 fold lower ($P = 0.04$) and $Atp7a$ mRNA tended ($P = 0.08$) to be lower ($\sim 2$ fold) in Simmentals than in Angus. Furthermore, duodenal CTR1 protein tended ($P = 0.10$) to be lower in Simmentals than in Angus, but was not affected by Cu or Cu $\times$ breed (Figure 1). Abundance of duodenal CCS protein was not affected by Cu, breed, or Cu $\times$ breed (Figure 1). In the liver, mRNA expression of Cu regulatory proteins was not affected by Cu, breed, or Cu $\times$ breed (Table 9), nor was CTR1 (Figure 2) or CCS protein (Figure 2).

**Fetal Liver Gene and Protein Expression**

Breed did not affect mRNA expression of fetal liver Cu transporters and chaperones, $Mt1a$, $Sod1$, and $Cp$ (Table 10). Dietary Cu did not affect transcript levels of $Ctr1$, $Atp7a$, $Atp7b$, $Cp$, or $Mt1a$. However, relative expression of fetal liver $Atox1$ in –Cu fetuses was 4.6
fold higher \((P = 0.05)\) than that of +Cu fetuses (Table 10). Abundance of Cox17 was 1.3 fold higher \((P = 0.04)\) in –Cu fetuses compared to their +Cu counterparts. Relative expression of Comm11 was 2 fold higher \((P = 0.002)\) in –Cu fetuses compared +Cu fetuses. There was a tendency for Sod1 to be higher \((P = 0.10)\) in –Cu fetuses compared their +Cu counterparts. Abundance of fetal liver Ctr1 mRNA \((P = 0.06)\) and CTR1 protein \((P = 0.08)\) tended to be higher, while Mt1a was higher \((P = 0.03)\) in bulls than in heifers (Table 11). Expression of CTR1 (Figure 3) and CCS (Figure 4) was not affected by diet or breed in fetal liver.

**Discussion**

A series of experiments from our laboratory has shown that Cu indices in Simmentals are lower than in Angus when –Cu diets are fed (Ward et al., 1995; Mullis et al., 2003). These experiments have also shown that plasma Cu concentrations are much lower in Simmental offspring than Angus offspring, particularly when dietary Cu was deficient (Ward et al., 1995; Mullis et al., 2003). More recently, research from our laboratory has examined impacts of long-term exposure to Cu-deficient diets, in which animals were exposed to diets in utero and for 493 d of life. Copper deficiency reduced growth in these animals and also reduced mRNA expression of hepatic Cu regulatory proteins such as Sod1 (Hansen et al., 2009), Cox17, and Atp7b (Fry et al., 2009). Cattle in this study were severely Cu-deficient as indicated by plasma and liver Cu concentrations and increased CCS protein levels in their duodenum (Hansen et al., 2009), liver (Hepburn et al., 2009), and erythrocytes (Hepburn et al., 2009). Examination of CCS protein has been deemed a useful experimental biomarker to examine Cu deficiency as data from rodents has shown that it increases during Cu deficiency (Bertinato et al., 2003; Gybina and Prohaska, 2006). Expression of CCS protein in cows in
the present study was not affected by Cu or breed and this likely occurred as a result of cows not being Cu deficient based on traditional measures of liver Cu and plasma Cu. Only CuS dams had liver Cu and plasma Cu that approached concentrations indicative of Cu deficiency by d 112 in the present study. The fact that cows were not Cu-deficient likely explains why gene products involved in Cu homeostasis were not affected by dietary Cu in maternal duodenum and liver.

Earlier research in Angus and Simmental cattle demonstrated that biliary Cu excretion was 2 fold higher in Simmental than in Angus (Gooneratne et al., 1994). In our study, breed did not affect CTR1 protein or mRNA expression of other Cu regulatory proteins examined in the liver. However, we did find that breed affected expression of two key duodenal Cu transporters that are essential for Cu uptake and export as illustrated in mice and humans. Nose et al. (2006) demonstrated that mice with cell specific knock-out Ctr1 in the intestinal epithelium have less Cu accumulation in peripheral tissue, suffer greatly from Cu deficiency, and survivability of these animals is greatly reduced. When Cu was injected to bypass intestinal absorption, Cu status and survivability were rescued, thus further demonstrating the critical role of Ctr1 in intestinal Cu absorption. The Cu dependent ATPase, Atp7a, is mutated in humans with Menkes’ disease and these subjects are severely Cu deficient. In fact, this disease is often times fatal as Cu export to peripheral tissues, such as the liver, is markedly hindered (Cox and Moore, 2002). In the present study, lower Ctr1 mRNA and slightly lower CTR1 protein in the duodenum of Simmentals suggest that they have a lesser ability to absorb Cu. Slightly lower duodenal Atp7a mRNA in Simmentals suggests that export from the intestinal epithelium may also be reduced. Our data does not by
any means suggest that Ctr1 or Atp7a are mutated in Simmentals, but that their basal level of expression is lower. These data agree with previous data by Ward et al. (1995), who reported that apparent Cu absorption was lower in Simmentals than in Angus. The rate at which Cu was absorbed in the present study may have been affected by lower expression of duodenal Cu transporters in Simmentals, which may explain why duodenal Cu was not affected by breed. Higher expression of duodenal Cu transporters and greater Cu concentrations in the periphery suggests that Cu is being utilized from the intestine to a greater extent in Angus than in Simmentals. Our data suggest that when dietary Cu is adequate (≥ 10 mg Cu/kg DM), lower expression of the aforementioned duodenal Cu transporters in Simmentals is sufficient to maintain proper Cu homeostasis as liver Cu and plasma Cu were equal among breeds. However, when Simmentals receive diets low in available Cu, lower expression of Cu transporters may adversely affect Cu homeostasis resulting in lower liver Cu and plasma Cu concentrations than Angus. This may explain why Simmental cattle are more susceptible to Cu deficiency and why they are less tolerant to Cu antagonist, such as Mo, as demonstrated by Gengelbach et al. (1994).

Breed and dietary Cu affected Cu concentrations in placentome and fetal liver. Lower Cu concentrations in placentome and fetal liver of Simmentals would suggest that their lower expression of duodenal Cu transporters is also affecting the amount of Cu being delivered to the fetus. Effects of Cu on placentome and fetal liver Cu is in agreement with data from rats indicating that both placenta Cu and fetal liver Cu concentrations were lower in –Cu dams than in those fed +Cu diets (Wapnir et al., 1996). The developing fetus has a high demand for Cu, particularly during the last trimester, as Cu is essential for proper growth and
development (Gooneratne and Christensen, 1989). Furthermore, the bovine neonate depends heavily on accumulated liver Cu for postnatal utilization because of delayed synthesis of Cp (Chang et al., 1975) and their dam’s milk is quite low in Cu (Underwood and Suttle, 1999). Previous data has indicated that fetal liver Cu concentrations at birth should be at least 300 mg Cu/kg DM to prevent Cu deficiency within the early months of life (Gooneratne and Christensen, 1989). Liver Cu concentrations were reduced to 307 mg Cu/kg DM in –Cu fetuses. Expression of CCS protein in fetal liver was not affected by Cu, but mRNA expression of several Cu regulatory proteins were up-regulated due to Cu deficiency.

A small increase in Commd1 mRNA occurred in liver of –Cu fetuses compared to +Cu fetuses. In Bedlington terriers, Commd1 is mutated and consequently biliary Cu excretion is hindered, resulting in accumulation of toxic liver Cu concentrations (Klomp et al., 2003). Biliary Cu excretion in the fetus is quite rudimentary (Schaefer et al., 1999), thus it seems as though COMMD1 might have a different role in fetal liver. COMMD1 is ubiquitously expressed in mammalian tissues (de Bie et al., 2006), and was recently characterized in human placenta (Donadio et al., 2007). From these data authors suggested that COMMD1 is involved in regulating Cu transfer into fetomaternal circulation. Additionally it has been demonstrated that deletion of Commd1 in mice results in embryonic death (van de Sluis et al., 2007). If COMMD1 in fetal liver has a similar role to the putative role described in human placental tissue, then our data suggests that Commd1 mRNA up-regulated in response to an increased demand for Cu transport from the dam to the fetus. Previous research in cattle has indicated that the fetus has the ability to accrue Cu from its dam even though the dam may be Cu deficient (Gooneratne and Christensen, 1989). This
may explain why fetal liver Cu concentrations were similar among –CuS and –CuA fetuses, but not in +CuS and +CuA, which was in contrast to maternal Cu indices.

Numerous studies in the mature bovine have demonstrated that Cu deficiency decreases the activity of the Cu-dependent enzyme cytochrome c oxidase (CCO) in bovine hepatocytes and enterocytes (Mills et al., 1963; Fell et al., 1975; Mills et al., 1976). Although activity of CCO is unknown in the present study, up-regulation of Cox17 mRNA in –Cu fetuses likely occurred to assure adequate Cu delivery to CCO. Growth of the fetus is the highest during the last trimester which supports the aforementioned theory for higher Cox17 mRNA in –Cu fetuses.

Relative expression of Atox1 was up-regulated 4.6 fold in liver of –Cu fetuses compared to their +Cu counterparts. Antioxidant 1 is a vital component to the secretory pathway of Cu metabolism (Walker et al., 2002; Hamza et al., 2003). Hamza et al. (2001) demonstrated that Atox1 is essential in perinatal Cu homeostasis, in which Atox1 deleted mice exhibit similar phenotypes seen in severely Cu depleted animals. These animals are characterized by increased mortality, hypopigmentation, and growth retardation. Additionally, these authors demonstrated that there were marked reductions in liver Cu, brain Cu, and brain CCO in Atox1<sup>-/-</sup> mice (Hamza et al., 2001). Copper is required for plethora of physiological processes including angiogenesis (Harris, 2004). Therefore, up-regulation of Atox1 mRNA may have occurred to increase Cu efflux for fetal organ growth and development and synthesis of cuproenzymes (Walker et al., 2002; Hamza et al., 2003).

Fetal liver Cu was affected by gender, in which liver Cu concentrations were higher in males than in females, and this is in agreement with data reported in adult cattle (Miranda
et al., 2006) and in adult rodents (Haywood, 1979). However, these data are in contrast to earlier work in the bovine fetus by Gooneratne and Christensen (1989) reporting that female fetuses had higher liver Cu concentrations than males. These authors attributed the gender difference to higher liver Cu concentrations in dams of females than those that conceived males, suggesting a systemic effect by the dam. Gender differences in fetal liver Cu concentrations in our study occurred independent of maternal liver Cu. Gender differences in our study likely occurred as a result of slightly higher $Ctr1$ and $CTR1$ in males than in females. Previous data in rodents with $Ctr1$ deleted in the liver has shown that $CTR1$ is the primary route for hepatic Cu uptake (Kim et al., 2009). Greater Cu uptake via $CTR1$ in males likely explains the 8.5 fold increase in $Mt1a$ mRNA in males compared to females. Metallothionein serves as a vital Cu storage protein in the liver and provides cellular protection from free Cu ions. This Cu storage protein is abundant in neonatal animals due to large concentrations of Cu that accumulates in their liver during gestation (Cousins et al., 1985). To our knowledge this is the first demonstration that gender affects $Ctr1$, $CTR1$, and $Mt1a$, and definitive explanations for changes are unknown.

In conclusion, feeding a low Cu diet did not affect mRNA or protein levels of Cu regulatory proteins in maternal duodenum or liver. However, we did demonstrate that two essential Cu transporters are lower in the duodenum of Simmentals than in Angus. Lower expression of $Ctr1$, and slightly lower $CTR1$ and $Atp7a$, may explain why Cu indices were lower in Simmentals than in Angus when fed –Cu diets. Although placentome Cu and fetal liver Cu concentrations were affected by breed, molecular mechanisms involved in Cu homeostasis were not affected by breed in fetal liver. Copper deficiency reduced Cu
concentrations in placentome and fetal liver and increased mRNA expression of several Cu proteins. Up-regulation of Commd1 suggests it may have a putative role in fetomaternal circulation. Increased expression of Cox17 and Atox1 suggests an increased demand from synthesis of vital cuproenzymes such as CCO. Furthermore, Cu effects in the fetal liver suggest that the fetus is impacted to a greater extent by Cu deficiency than its dam. Further research is warranted in examining impacts of breed on mRNA and protein levels of Cu transporters and chaperones, and also future work is needed in examining Cu metabolism in the bovine fetus.

References


Nose, Y., B.E. Kim, and D.J. Thiele. 2006. Ctr1 drives intestinal copper absorption and is essential for growth, iron metabolism, and neonatal cardiac function. Cell Meta. 4: 235-244.


Table 1. Diet composition

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>% of diet, DM basis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn silage</td>
<td>93.0</td>
</tr>
<tr>
<td>Corn</td>
<td>3.51</td>
</tr>
<tr>
<td>Corn supplement</td>
<td>2.0</td>
</tr>
<tr>
<td>Urea</td>
<td>0.75</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>0.28</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>0.21</td>
</tr>
<tr>
<td>White salt</td>
<td>0.02</td>
</tr>
<tr>
<td>Vitamin premix</td>
<td>0.03</td>
</tr>
<tr>
<td>Rumensin 80</td>
<td>0.02</td>
</tr>
<tr>
<td>Trace mineral mix</td>
<td>0.01</td>
</tr>
</tbody>
</table>

1 Analyzed 6.6 mg Cu, 128.9 mg Fe, and 40.9 mg Zn/kg DM.
2 A ground corn supplement that provided dietary treatments: +Cu (10 mg supplemental Cu/kg DM from Cu₂(OH)₃Cl) and –Cu (5 mg supplemental Mo/kg DM from NaMoO₄; 0.15% S from NH₄SO₄).
3 Provided (per kg of premix): 2,045,455 IU of vitamin A, 454,545.5 IU of vitamin D, and 909.1 IU of vitamin E.
4 Ionophore, provided 33 mg monensin/kg DM.
5 Provided (per kg of DM): 30 mg Zn as ZnSO₄, 20 mg Mn as MnSO₄, 0.2 mg Se as Na₂SeO₃, 0.5 I as Ca(IO₃)₂, 0.1 Co as CoCO₃.
Table 2. Real-time RT-PCR primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Accession number</th>
<th>Primer sequence (5'– 3')</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rps9²</td>
<td>DT860044</td>
<td>F:CCTCGACCAAGAGCTGAAG</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:CCTCCAGACCTCAGTTTGTC</td>
<td></td>
</tr>
<tr>
<td>Tbp³</td>
<td>NW001503202</td>
<td>F:ACAACAGCTCCCCACCTATGC</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:GTGGAGTCAGTCTGCGTGAAG</td>
<td></td>
</tr>
<tr>
<td>Ctr1</td>
<td>NM001100381.1</td>
<td>F:AAAGATTCCTGGAGGTGTG</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:GGTCAGATGAAATGTTGG</td>
<td></td>
</tr>
<tr>
<td>Atox1</td>
<td>XM877831.2</td>
<td>F:CCGAGACTGGTCTCCTCC</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:TGTGGGGCAGGTCAATGTC</td>
<td></td>
</tr>
<tr>
<td>Cox17</td>
<td>AF031558.1</td>
<td>F:GAGCAGACCCACATTTCTATC</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:GCGAAGCGGCGATAATTG</td>
<td></td>
</tr>
<tr>
<td>Atp7a</td>
<td>XM615430.3</td>
<td>F:GAAGATTATAGTATGGCTCAACC</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:CCTCTGCTGTGATAAGATGG</td>
<td></td>
</tr>
<tr>
<td>Atp7b</td>
<td>XM596258.3</td>
<td>F:AAGTCGTCGTCCTAGTCC</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:GAGATGGATCGTAGAGAACC</td>
<td></td>
</tr>
<tr>
<td>Cp</td>
<td>XM592003.4</td>
<td>F:GGTAGGGTAATGAAATGGTG</td>
<td>275</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:TCAGCAGGATGGTAATGG</td>
<td></td>
</tr>
<tr>
<td>Commd1</td>
<td>NM001046384.1</td>
<td>F:CTGGAGGCATTGTGGCTGCT</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:GACTCTCTCGGATTTTTCTATG</td>
<td></td>
</tr>
<tr>
<td>Mt1a</td>
<td>NM001040492.1</td>
<td>F:ATGGACCCGAACTGCTTGCG</td>
<td>182</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:GCCAGACGCTGACCTTGCC</td>
<td></td>
</tr>
<tr>
<td>Sod1</td>
<td>NM174615.2</td>
<td>F:AGATACAGCTCGTGGTAAAC</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:ACAGGAGGATTAAATGG</td>
<td></td>
</tr>
</tbody>
</table>

¹F: Forward; R: Reverse
²Primers for Rps9 were previously reported by Janovick-Guretzky et al. (2007).
³Primers for Tbp were previously reported by Lisowski et al. (2008).
<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Acronym</th>
<th>Proposed function(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper transporter 1</td>
<td>Ctr1</td>
<td>Primary route of Cu absorption in mammals</td>
</tr>
<tr>
<td>Antioxidant 1</td>
<td>Atox1</td>
<td>Delivers Cu to Atp7a in the intestine and Atp7b in the liver</td>
</tr>
<tr>
<td>Cytochrome c oxidase assembly protein 17</td>
<td>Cox17</td>
<td>Delivers Cu to mitochondria for incorporation into cytochrome c oxidase</td>
</tr>
<tr>
<td>Cu chaperone for Cu/Zn superoxide dismutase</td>
<td>CCS</td>
<td>Delivers Cu to Sod1</td>
</tr>
<tr>
<td>Cu/Zn superoxide dismutase</td>
<td>Sod1</td>
<td>Scavenges superoxide ion</td>
</tr>
<tr>
<td>Cu transporting alpha-polypeptide Atpase</td>
<td>Atp7a</td>
<td>Basolateral Cu transport and synthesis of cuproenzymes in the intestine; function in liver is unknown</td>
</tr>
<tr>
<td>Cu transporting beta-polypeptide Atpase</td>
<td>Atp7b</td>
<td>Incorporation of Cu into Cp liver and Cu biliary excretion; function in intestine unknown</td>
</tr>
<tr>
<td>Metallothionein 1a</td>
<td>Mt1a</td>
<td>Copper storage protein and may also function as a chaperone protein</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>Cp</td>
<td>Copper dependent ferroxidase secreted from liver</td>
</tr>
<tr>
<td>Copper metabolism MURR domain 1</td>
<td>Commd1</td>
<td>Interacts with Atp7b to excrete Cu via bile</td>
</tr>
</tbody>
</table>
Table 4. Effect of dietary Cu and breed on plasma Cu concentrations in pregnant Angus and Simmental cows\(^1,2,3\)

<table>
<thead>
<tr>
<th>Treatment(^4)</th>
<th>Cu</th>
<th>Breed</th>
<th>Cu × Breed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>1.21</td>
<td>1.17</td>
<td>0.88</td>
</tr>
<tr>
<td>Day 0</td>
<td>1.36</td>
<td>1.24</td>
<td>1.57</td>
</tr>
<tr>
<td>28</td>
<td>1.13</td>
<td>1.21</td>
<td>0.99</td>
</tr>
<tr>
<td>56</td>
<td>1.23</td>
<td>1.17</td>
<td>1.25</td>
</tr>
<tr>
<td>84</td>
<td>1.19</td>
<td>1.11</td>
<td>1.10</td>
</tr>
<tr>
<td>112</td>
<td>1.31</td>
<td>1.15</td>
<td>1.14</td>
</tr>
</tbody>
</table>

\(^1\)Values expressed as milligrams per liter.
\(^2\)Cu × day (\(P = 0.10\))
\(^3\)Day (\(P = 0.002\))
\(^4\)+CuA, Cu-adequate Angus (n = 3); +CuS, Cu-adequate Simmental (n = 4); -CuA, Cu-deficient Angus (n = 4); -CuS, Cu-deficient Simmental (n = 4).
Table 5. Effect of dietary Cu and breed on plasma holo-Cp concentrations in pregnant Angus and Simmental cows$^{1,2}$

<table>
<thead>
<tr>
<th>Treatment$^3$</th>
<th>Overall</th>
<th>Day 0</th>
<th>28</th>
<th>56</th>
<th>84</th>
<th>112</th>
</tr>
</thead>
<tbody>
<tr>
<td>+CuA</td>
<td>25.6</td>
<td>22.4</td>
<td>18.3</td>
<td>28.5</td>
<td>34.2</td>
<td>26.2</td>
</tr>
<tr>
<td>+CuS</td>
<td>25.7</td>
<td>20.7</td>
<td>20.5</td>
<td>28.4</td>
<td>30.9</td>
<td>26.0</td>
</tr>
<tr>
<td>-CuA</td>
<td>25.7</td>
<td>25.3</td>
<td>15.6</td>
<td>30.4</td>
<td>31.4</td>
<td>27.9</td>
</tr>
<tr>
<td>-CuS</td>
<td>19.9</td>
<td>18.7</td>
<td>13.4</td>
<td>23.6</td>
<td>25.8</td>
<td>18.2</td>
</tr>
<tr>
<td>SEM</td>
<td>1.40</td>
<td>2.33</td>
<td>2.05</td>
<td>3.41</td>
<td>2.21</td>
<td>3.66</td>
</tr>
<tr>
<td>$^P$-value</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cu</td>
<td>0.06</td>
<td>0.85</td>
<td>0.03</td>
<td>0.68</td>
<td>0.10</td>
<td>0.20</td>
</tr>
<tr>
<td>Breed</td>
<td>0.06</td>
<td>0.10</td>
<td>0.99</td>
<td>0.33</td>
<td>0.07</td>
<td>0.43</td>
</tr>
<tr>
<td>Cu × Breed</td>
<td>0.05</td>
<td>0.31</td>
<td>0.31</td>
<td>0.34</td>
<td>0.60</td>
<td>0.21</td>
</tr>
</tbody>
</table>

$^1$Values expressed as milligrams per 100 milliliters.
$^2$Day ($P < 0.0001$)$^3$+CuA, Cu-adequate Angus (n = 3); +CuS, Cu-adequate Simmental (n = 4); -CuA, Cu-deficient Angus (n = 4); -CuS, Cu-deficient Simmental (n = 4).
Table 6. Effect of dietary copper and breed on duodenal mineral concentrations in pregnant Angus and Simmental cows\textsuperscript{1,2}

<table>
<thead>
<tr>
<th></th>
<th>Dietary Cu\textsuperscript{3}</th>
<th>Breed\textsuperscript{4}</th>
<th>(P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+Cu (n = 7)</td>
<td>-Cu (n = 8)</td>
<td>SEM</td>
</tr>
<tr>
<td>Cu</td>
<td>10.3</td>
<td>6.5</td>
<td>0.84</td>
</tr>
<tr>
<td>Zn</td>
<td>103.5</td>
<td>78.2</td>
<td>9.04</td>
</tr>
<tr>
<td>Fe</td>
<td>168.7</td>
<td>115.8</td>
<td>30.16</td>
</tr>
</tbody>
</table>

\(1\) Values expressed as milligram per kilogram of DM.

\(2\) Cu × breed (\(P > 0.15\)).

\(3\) +Cu, Cu-adequate (n = 7); -Cu, Cu-deficient (n = 8).

\(4\) Angus (n = 7); Simmental (n = 8).
Table 7. Effect of dietary Cu and breed on liver Cu concentrations in pregnant Angus and Simmental cows1,2,3

<table>
<thead>
<tr>
<th>Treatment</th>
<th>+CuA</th>
<th>+CuS</th>
<th>-CuA</th>
<th>-CuS</th>
<th>SEM</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall</td>
<td>200.5</td>
<td>197.1</td>
<td>160.5</td>
<td>77.3</td>
<td>16.61</td>
<td>0.001 0.02 0.04</td>
</tr>
<tr>
<td>Day 0</td>
<td>191.0</td>
<td>186.9</td>
<td>259.7</td>
<td>158.9</td>
<td>40.56</td>
<td>0.63 0.22 0.26</td>
</tr>
<tr>
<td></td>
<td>216.5</td>
<td>206.4</td>
<td>114.5</td>
<td>55.2</td>
<td>24.34</td>
<td>0.001 0.06 0.12</td>
</tr>
<tr>
<td></td>
<td>193.9</td>
<td>197.9</td>
<td>77.2</td>
<td>17.7</td>
<td>24.07</td>
<td>&lt;0.001 0.26 0.20</td>
</tr>
</tbody>
</table>

1Values expressed as milligrams per kilogram of DM.
2Cu × day ($P = 0.002$)
3Day ($P = 0.006$)
4+CuA, Cu-adequate Angus (n = 3); +CuS, Cu-adequate Simmental (n = 4); -CuA, Cu-deficient Angus (n = 4); -CuS, Cu-deficient Simmental (n = 4).
Table 8. Effect of dietary Cu, breed of dam, and fetal gender on mineral concentrations in placentome and fetal liver

<table>
<thead>
<tr>
<th></th>
<th>Dietary Cu¹</th>
<th>Breed of Dam²</th>
<th>Fetal Gender³</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>¹+Cu ¹</td>
<td>¹-Cu ¹</td>
<td>Angus ²</td>
<td>Simm ²</td>
</tr>
<tr>
<td>Placentome⁴</td>
<td></td>
<td></td>
<td>Males ³</td>
<td>Females⁵</td>
</tr>
<tr>
<td>Cu⁵</td>
<td>12.1</td>
<td>8.6</td>
<td>0.58</td>
<td>11.3</td>
</tr>
<tr>
<td>Zn</td>
<td>80.8</td>
<td>78.3</td>
<td>3.20</td>
<td>76.2</td>
</tr>
<tr>
<td>Fe</td>
<td>110.9</td>
<td>106.5</td>
<td>14.15</td>
<td>123.7</td>
</tr>
<tr>
<td></td>
<td>Cu</td>
<td>SEM</td>
<td>0.52</td>
<td>9.4</td>
</tr>
<tr>
<td></td>
<td>10.5</td>
<td>10.2</td>
<td>0.62</td>
<td>11.3</td>
</tr>
<tr>
<td></td>
<td>9.4</td>
<td>0.52</td>
<td>1.03</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>0.76</td>
<td>0.03</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>Zn</td>
<td>SEM</td>
<td>3.44</td>
<td>80.8</td>
</tr>
<tr>
<td></td>
<td>80.8</td>
<td>78.3</td>
<td>2.88</td>
<td>82.9</td>
</tr>
<tr>
<td></td>
<td>80.8</td>
<td>78.3</td>
<td>3.44</td>
<td>3.44</td>
</tr>
<tr>
<td></td>
<td>Fe</td>
<td>SEM</td>
<td>15.22</td>
<td>106.8</td>
</tr>
<tr>
<td></td>
<td>106.8</td>
<td>110.6</td>
<td>15.22</td>
<td>110.6</td>
</tr>
<tr>
<td></td>
<td>10.6</td>
<td>0.85</td>
<td>15.22</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>0.13</td>
<td>0.88</td>
<td>0.85</td>
<td>0.13</td>
</tr>
<tr>
<td>Fetal Liver⁶</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cu⁶</td>
<td>669</td>
<td>307</td>
<td>55</td>
<td>563</td>
</tr>
<tr>
<td>Zn</td>
<td>835</td>
<td>521</td>
<td>103</td>
<td>479</td>
</tr>
<tr>
<td>Fe</td>
<td>5,481</td>
<td>5,000</td>
<td>638</td>
<td>4,233</td>
</tr>
<tr>
<td></td>
<td>Cu</td>
<td>SEM</td>
<td>98</td>
<td>413</td>
</tr>
<tr>
<td></td>
<td>605</td>
<td>371</td>
<td>58</td>
<td>605</td>
</tr>
<tr>
<td></td>
<td>413</td>
<td>98</td>
<td>0.002</td>
<td>413</td>
</tr>
<tr>
<td></td>
<td>371</td>
<td>98</td>
<td>0.002</td>
<td>371</td>
</tr>
<tr>
<td></td>
<td>Zn</td>
<td>SEM</td>
<td>111</td>
<td>527</td>
</tr>
<tr>
<td></td>
<td>829</td>
<td>527</td>
<td>0.08</td>
<td>829</td>
</tr>
<tr>
<td></td>
<td>527</td>
<td>111</td>
<td>0.08</td>
<td>527</td>
</tr>
<tr>
<td></td>
<td>Fe</td>
<td>SEM</td>
<td>686</td>
<td>4,782</td>
</tr>
<tr>
<td></td>
<td>4,782</td>
<td>4,798</td>
<td>0.19</td>
<td>4,782</td>
</tr>
<tr>
<td></td>
<td>4,798</td>
<td>686</td>
<td>0.19</td>
<td>4,798</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹+Cu, Cu-adequate (n = 7); -Cu, Cu-deficient (n = 8).
²Angus (n = 7); Simm, Simmental (n = 8).
³Males (n = 8); Females (n = 7).
⁴Values expressed as milligrams per kilogram DM.
⁵Cu × breed (P > 0.15).
⁶Cu × breed (P = 0.07); Cu × gender (P > 0.15).
Table 9. Effect of dietary copper and breed on mRNA expression of copper regulatory proteins in the duodenum and liver of pregnant Angus and Simmental cows

<table>
<thead>
<tr>
<th>Dietary Cu1</th>
<th>Breed2</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cu</td>
<td>Breed</td>
</tr>
<tr>
<td>Duodenum3,4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctrl</td>
<td>1.63</td>
<td>2.46</td>
</tr>
<tr>
<td>Atox1</td>
<td>2.89</td>
<td>0.95</td>
</tr>
<tr>
<td>Atp7a</td>
<td>1.18</td>
<td>1.67</td>
</tr>
<tr>
<td>Atp7b</td>
<td>2.41</td>
<td>1.02</td>
</tr>
<tr>
<td>Cox17</td>
<td>1.09</td>
<td>1.22</td>
</tr>
<tr>
<td>Sod1</td>
<td>1.64</td>
<td>1.01</td>
</tr>
<tr>
<td>Mt1a</td>
<td>1.49</td>
<td>4.27</td>
</tr>
<tr>
<td>Liver3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ctrl</td>
<td>1.12</td>
<td>1.02</td>
</tr>
<tr>
<td>Atox1</td>
<td>1.55</td>
<td>0.88</td>
</tr>
<tr>
<td>Atp7a</td>
<td>1.45</td>
<td>1.16</td>
</tr>
<tr>
<td>Atp7b</td>
<td>1.10</td>
<td>1.02</td>
</tr>
<tr>
<td>Commd1</td>
<td>1.44</td>
<td>1.05</td>
</tr>
<tr>
<td>Cp</td>
<td>2.46</td>
<td>1.19</td>
</tr>
<tr>
<td>Cox17</td>
<td>0.99</td>
<td>1.19</td>
</tr>
<tr>
<td>Sod1</td>
<td>1.37</td>
<td>1.00</td>
</tr>
<tr>
<td>Mt1a</td>
<td>0.92</td>
<td>1.98</td>
</tr>
</tbody>
</table>

1+Cu, Cu-adequate (n = 7); -Cu, Cu-deficient (n = 8).
2Angus (n = 7); Simm, Simmental (n = 8).
3Values are relative expression of target genes normalized to the housekeeping gene, TATA box binding protein, Tbp.
4Cu × breed (P > 0.15).
5Values are relative expression of target genes normalized to the housekeeping gene, ribosomal protein subunit 9, Rps9.
Table 10. Effect of dietary copper and breed on mRNA expression of copper regulatory proteins in fetal liver collected from pregnant Angus and Simmental cows.\(^1\)\(^2\)

<table>
<thead>
<tr>
<th>Dietary Cu(^3)</th>
<th>Breed of Dam(^4)</th>
<th>(P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+Cu</td>
<td>-Cu</td>
</tr>
<tr>
<td>Ctrl</td>
<td>1.10</td>
<td>1.06</td>
</tr>
<tr>
<td>Atox1</td>
<td>0.61</td>
<td>2.34</td>
</tr>
<tr>
<td>Atp7a</td>
<td>1.18</td>
<td>0.93</td>
</tr>
<tr>
<td>Atp7b</td>
<td>1.05</td>
<td>1.26</td>
</tr>
<tr>
<td>Commd1</td>
<td>0.68</td>
<td>1.41</td>
</tr>
<tr>
<td>Cp</td>
<td>1.41</td>
<td>1.35</td>
</tr>
<tr>
<td>Cox17</td>
<td>0.91</td>
<td>1.22</td>
</tr>
<tr>
<td>Sod1</td>
<td>0.92</td>
<td>1.11</td>
</tr>
<tr>
<td>Mt1a</td>
<td>1.76</td>
<td>1.13</td>
</tr>
</tbody>
</table>

\(^1\)Values are relative expression of target genes normalized to the housekeeping gene, ribosomal protein subunit 9, \(Rps9\).

\(^2\)Cu × breed (\(P > 0.15\))

\(^3\)+ Cu, Cu-adequate (\(n = 7\)); -Cu, Cu-deficient (\(n = 8\)).

\(^4\)Angus (\(n = 7\)); Simm, Simmental (\(n = 8\)).
Table 11. Effect of gender on mRNA expression of copper transporter 1 and metallothionein 1a in bovine fetal liver

<table>
<thead>
<tr>
<th></th>
<th>Males</th>
<th>Females</th>
<th>SEM</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ctr1</em></td>
<td>1.28</td>
<td>0.89</td>
<td>0.106</td>
<td>0.03</td>
</tr>
<tr>
<td><em>Mt1a</em></td>
<td>2.41</td>
<td>0.48</td>
<td>0.504</td>
<td>0.03</td>
</tr>
</tbody>
</table>

1 Values are relative expression of target genes normalized to the housekeeping gene, ribosomal protein subunit 9, *Rps9*.
2 Males (n = 8); Females (n = 7)
Figure 1. Effect of dietary Cu and breed on expression of duodenal copper transporter 1 (CTR1) and copper chaperone for SOD1 (CCS) protein in pregnant Angus and Simmental cows. +Cu, Cu-adequate (n = 7); -Cu, Cu-deficient (n = 8); A, Angus (n = 7); S, Simmental (n = 8). CTR1; Cu ($P = 0.21$), Breed ($P = 0.10$). CCS; Cu ($P = 0.43$), Breed ($P = 0.26$).
Figure 2. Effect of dietary Cu and breed on expression of hepatic copper transporter 1 (CTR1) and copper chaperone for SOD1 (CCS) protein in pregnant Angus and Simmental cows. +Cu, Cu-adequate (n = 7); -Cu, Cu-deficient (n = 8); A, Angus (n = 7); S, Simmental (n = 8). CTR1; Cu (P = 0.28), Breed (P = 0.67). CCS; Cu (P = 0.81), Breed (P = 0.90).
Figure 3. Effect of dietary Cu, breed of dam, and fetal gender on expression of copper transporter 1 (CTR1) protein in fetal liver. +Cu, Cu-adequate (n = 7); -Cu, Cu-deficient (n = 8); A, Angus (n = 7); S, Simmental (n = 8); M, Males (n = 8); F, Females (n = 7). Cu ($P=0.81$). Breed ($P=0.81$). Gender ($P=0.08$).
Figure 4. Effect of dietary Cu, breed of dam, and fetal gender on expression of copper chaperone for SOD1 (CCS) protein in fetal liver. +Cu, Cu-adequate (n = 7); -Cu, Cu-deficient (n = 8); A, Angus (n = 7); S, Simmental (n = 8); M, Males (n = 8); F, Females (n = 7). Cu ($P = 0.94$). Breed ($P = 0.61$). Gender ($P = 0.97$).
CHAPTER 4

Gene expression of copper regulatory proteins in the small intestine of weanling pigs is modulated by level and source of dietary copper, and modulation is associated with changes in digesta and mucosal copper concentrations

R. Scott Fry, Melissa S. Ashwell, William L. Flowers, Kara R. Stewart, Karen E. Lloyd, Audrey T. O’Nan, and Jerry W. Spears*

Department of Animal Science, North Carolina State University, Raleigh, NC, 27695

*Corresponding author: Campus Box 7621, Raleigh, NC, 27695; ph: 919-515-4008; fax: 919-515-4463; email: jerry_spears@ncsu.edu
Abstract

A study was conducted to determine the effects of level and source of dietary copper (Cu) on Cu concentrations and mRNA expression of Cu regulatory proteins in the small intestine of weanling pigs. Thirty weanling male pigs, approximately 21 d of age, were stratified by weight and assigned to one of the following dietary treatments: 1) control (6.7 mg Cu/kg diet), 2) 225 mg supplemental Cu/kg diet from Cu sulfate (CuSO₄), or 3) 225 mg supplemental Cu/kg from tribasic Cu chloride (Cu₂(OH)₃Cl). Pigs were harvested following an 8 h fast and 8 h refeeding on d 35 and 36. Soluble Cu and mucosal Cu concentrations in duodenum, proximal jejunum, and ileum were higher (P < 0.001) in Cu-supplemented pigs versus controls. Duodenal Cu was higher (P = 0.04) in CuSO₄ vs. Cu₂(OH)₃Cl-supplemented pigs. However, proximal jejunum mucosal Cu was higher (P = 0.0001) in Cu₂(OH)₃Cl vs. CuSO₄ pigs, but did not differ in ileal mucosa. As determined via qRT-PCR, duodenal copper transporter 1 (Ctr1) was lower in Cu₂(OH)₃Cl compared to CuSO₄ (P = 0.03) and controls (P = 0.01). In the proximal jejunum, Ctr1 mRNA was lower in CuSO₄ (P = 0.02) and Cu₂(OH)₃Cl (P = 0.003) pigs versus controls; while antioxidant 1 (Atox1) mRNA was higher (P = 0.03) in Cu-supplemented pigs. Ileal Ctr1 was lower (P = 0.03) in CuSO₄ versus controls. These data demonstrate that both Cu level and source affect uptake and mRNA expression of Cu regulatory proteins throughout the small intestine of weanling pigs.

Introduction

The copper (Cu) requirement of the young pig is 5 mg/kg Cu (NRC, 1998). However, it has been well documented that when weanling pigs are fed Cu (100 to 250 mg/kg diet) well above their requirement a pharmacological response is often observed where growth and
feed intake are increased (Drouliscos et al., 1970; Cromwell et al., 1998; Armstrong et al., 2004). Although several physiological changes have been observed in response to high dietary Cu (Zhou et al., 1994; Li et al., 2008), definitive mechanisms responsible for this pharmacological effect remain unknown. While pigs are tolerant to high dietary Cu, it is unknown how the young pig regulates high dietary Cu intake to prevent toxicity. The duodenum and proximal jejunum are the primary sites for Cu absorption (Linder and Hazegh-Azam, 1996), and thus the small intestine serves as a key regulatory organ in Cu acquisition and utilization, as absorption of Cu is affected by level of dietary Cu intake (Turnlund et al., 1998). Intracellular Cu is tightly regulated by transport and chaperone proteins that have been identified in rodents to regulate Cu absorption (Kim et al., 2008). We have previously characterized these Cu regulatory proteins at the mRNA level in porcine duodenum and proximal jejunum under conditions of dietary iron (Fe) deprivation and excess (Fry et al., 2010a; Fry et al., 2010b). However, to date these pathways have not been examined in pigs fed pharmacological levels of dietary Cu.

Two inorganic sources of Cu that are used in swine diets as growth promotants are Cu sulfate (CuSO₄) and tribasic Cu chloride (Cu₂(OH)₃Cl). These sources differ greatly in their in vitro water and acid solubility. Miles et al. (1998) demonstrated that Cu from CuSO₄ was very soluble (99%) in water whereas very little (<1%) was soluble from Cu₂(OH)₃Cl. Pang and Applegate (2007) reported that in the absence of the Cu antagonist phytate, the in vitro solubility of Cu₂(OH)₃Cl at pH 2.5 was equal to that of CuSO₄, but at pH of 5.5 and 6.5, its solubility was much lower. The objectives of the present study were to determine the effects of level and source of dietary Cu on digesta soluble Cu concentrations, mucosal mineral
concentrations, and regulation of mRNA of Cu transport and chaperone proteins in different sections of the small intestine of weanling pigs.

Materials and Methods

Animals and experimental design

Prior to the initiation of the study, animal care, handling and sampling was approved by the North Carolina State University Animal Care and Use Committee. Thirty weanling barrows (castrated males) weighing 6.33 ± 0.23 kg were weaned at approximately 21 d of age and stratified by weight and litter. Subsequently, pigs were randomly assigned to one of the following dietary treatments: 1) control (analyzed mean of 6.7 mg Cu/kg diet), 2) 225 mg supplemental Cu/kg from CuSO₄ or 3) 225 mg supplemental Cu/kg from Cu₂(OH)₃Cl. Pigs were housed two pigs per pen with ad libitum access to water and feed. To transition pigs from their mother’s milk to a solid diet, diets were fed in three phases (Table 1). Phase 1 diet was a complex formulated diet containing milk proteins and was fed from d 0 to 6. The phase 2 diets, decreasing in milk proteins, were fed from d 7 to 20. Phase 3 diets were fed from d 21 until harvest. Diets were formulated to meet or exceed NRC requirements and the control diet met the pig’s nutritional requirement for Cu (NRC, 1998).

Sample collection and analytical procedures

Prior to harvest on d 35 and 36, pigs were fasted for 8 h then refed for 8 h. Three pens representing all dietary treatments were euthanized via captive bolt within a specific time frame. As each pig was euthanized the abdominal cavity was lacerated vertically for sample collection. To prevent the flow of digesta contents out of the intestine, the ileal-cecal junction and proximal duodenum were quickly ligated with umbilical tape with minimal movement of
the gastrointestinal tract to prevent unwanted flow of digesta contents within the tract.

Subsequently, 60 cm proximal to the ileal-cecal junction represented the ileum and was ligated at the proximal end to retain luminal contents. Sixty cm from the proximal duodenum was measured and at that point was ligated with umbilical tape to represent the duodenum. To obtain proximal jejunum, 152 cm from the distal duodenum was measured and from that point a 60 cm section was obtained by ligating both the proximal and distal ends with umbilical tape. As each of the aforementioned sections were removed, digesta contents were collected into a 50 mL conical tube and pH measurements were immediately recorded. The digesta contents were then placed on ice for determination of soluble Cu concentrations.

Each section of the intestine was then opened and rinsed with 1 X PBS to clean the intestine epithelium of digesta and other debris. Subsequently, chilled microscope slides were used to obtain two sequential mucosal scrapes from each section. One mucosal scrape was placed into a 50 mL conical tube containing RNAlater (Ambion) as per manufacturer’s instructions, while the other was placed on ice in a 15 mL conical tube for mineral analysis.

**Digesta and mucosal mineral analysis**

Immediately following sample collection, digesta samples were taken to the laboratory and processed for subsequent determination of soluble Cu concentrations. From each section, digesta samples were filtered through cheesecloth to separate the solid and liquid fractions. The liquid fraction was then centrifuged at 15,000 × g for 10 min to separate out any remaining solid particles and frozen at -20°C until Cu determination.

Approximately 1 g of mucosal scrapes from the duodenum, proximal jejunum, and ileum was dried in a forced-air oven (60°C: Imperial II; Lab-Line Instruments). Mucosal and
feed samples were prepared for mineral analysis by wet ashing using a Microwave (Mars 5; CEM) procedure described by Gengelbach et al. (1994). Mineral analysis was conducted via atomic absorption spectrophotometry (Shimazu, Japan) and a NIST certified liver standard was included in the analysis to validate instrument accuracy. Supernatant from digesta samples was aspirated directly into the flame.

**RNA isolation and Quantitative Real-Time PCR**

Total RNA was isolated from the duodenum, proximal jejunum, and ileum via the Qiagen RNeasy kit with an on-column DNase digestion (Qiagen). Quantity and integrity of RNA was determined via NanoDrop-1000 spectrophotometer and integrity of 28s and 16s ribosomal subunits was captured on a 1.2% agarose gel. Subsequently, 1 µg of total RNA was used to synthesize cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc.) as per manufacturer’s instructions. Quantitative real-time PCR was ran in duplicate (Biorad) and each reaction contained 50 ng cDNA, 10 µM each of forward and reverse primers, and 1X SYBR Green Master Mix (Ambion). The cycling program was as reported by Hansen et al. (2009a). Real-time primers were designed with Beacon Designer software (Premier Biosoft Intl.) to be compatible with SYBR Green I (Supplemental Table). One amplicon from each reaction was sequence validated to confirm primer specificity. Primer specificity was also confirmed by melt curve analysis and determination of product size via agarose gel electrophoresis. Primers for divalent metal transporter 1 (Dmt1) and hephaestin (Heph) were taken from published reports by Hansen et al. (2009b, 2010). Numerous housekeeping genes, taken from Nygard et al. (2007), were assessed for their stability in each section of the intestine. Of those assessed, Rpl4, Tbp,
*Hprt1*, and *β-actin* were found to be the most stable in all sections. Within each section, the geometric mean of the aforementioned housekeeping genes was determined for target normalization. Relative expression of target genes was determined via the $2^{-\Delta\Delta C_T}$ method (Lavak and Schimittgen, 2001).

**Statistical analysis**

Data analysis was performed by ANOVA using the MIXED procedure of SAS (SAS Institute). The model for all data included the fixed effect, treatment, and the random effects, replicate and block (harvest day). Experimental unit was animal. Within each dietary treatment group, the main effect of intestinal section on mineral concentrations and mRNA expression of Cu regulatory proteins was determined using ANOVA. Significance was declared at $P \leq 0.05$. Data are presented as the least square mean (LSM) ± SEM.

**Results**

**Soluble Cu and mucosal mineral concentrations**

Soluble Cu concentrations in the duodenal digesta were higher ($P < 0.0001$) in Cu-supplemented pigs compared to the controls, but did not differ significantly ($P = 0.08$) between Cu source. Copper-supplemented pigs had markedly higher ($P < 0.0001$) Cu concentrations in the duodenal mucosa than controls. Mucosal Cu in the duodenum was also higher ($P = 0.04$) in CuSO$_4$ than in Cu$_2$(OH)$_3$Cl pigs. In the proximal jejunum, soluble Cu concentrations in the digesta were much higher ($P < 0.001$) in Cu-supplemented pigs, but did not differ ($P = 0.33$) among Cu sources. Mucosal Cu concentrations in proximal jejunum were lower ($P = 0.02$) in control pigs compared with those supplemented with 225 mg Cu/kg
diet, and pigs supplemented with Cu$_2$(OH)$_3$Cl had higher ($P = 0.0001$) mucosa Cu concentrations than those supplemented with CuSO$_4$. Soluble Cu in the ileal digesta was much higher ($P < 0.0001$) in Cu-supplemented pigs compared to controls but did not differ among the Cu sources. Mucosal Cu concentrations were higher ($P = 0.004$) in CuSO$_4$ and even more so ($P < 0.001$) in Cu$_2$(OH)$_3$Cl pigs compared to controls (Table 2).

Level of Cu did not affect ($P \leq 0.12$) duodenal Fe concentrations (Table 3). However, Cu$_2$(OH)$_3$Cl-supplemented pigs had lower ($P = 0.003$) duodenal Fe concentrations than those supplemented with CuSO$_4$. In the proximal jejunum and ileum, mucosal Fe concentrations were not affected by level or source of dietary Cu. Duodenal and proximal jejunum Zn concentrations were not affected by level or source of dietary Cu. However, ileal mucosal Zn concentrations were lower ($P = 0.01$) in Cu-supplemented pigs compared to controls.

**Copper effects on cellular Cu metabolism in small intestine**

Relative expression of duodenal copper transporter 1 (Ctr1) mRNA was lower in Cu$_2$(OH)$_3$Cl pigs compared to controls ($P = 0.01$) and those supplemented with CuSO$_4$ ($P = 0.03$; Figure 1). In the proximal jejunum, Ctr1 mRNA was down-regulated in CuSO$_4$ ($P = 0.02$) and Cu$_2$(OH)$_3$Cl ($P = 0.003$) pigs compared to controls and was not affected by Cu source (Figure 1). Ileal expression of Ctr1 was down-regulated ($P = 0.03$) in CuSO$_4$ pigs compared to controls and was similar ($P = 0.06$) among controls and Cu$_2$(OH)$_3$Cl pigs (Figure 1).

Expression of antioxidant 1 (Atox1) mRNA was not affected by level or source of dietary Cu in the duodenum or ileum (Figure 2). However, Atox1 mRNA was up-regulated ($P$
Expression of Atp7a mRNA was not affected by level or source of dietary Cu in the duodenum, proximal jejunum, or ileum (Figure 3).

Duodenal metallothionein 1a (Mt1a) mRNA was markedly higher ($P = 0.0003$) in CuSO$_4$-supplemented pigs compared to control and Cu$_2$(OH)$_3$Cl pigs, but did not differ ($P = 0.08$) among Cu$_2$(OH)$_3$Cl pigs and control pigs (Figure 4). In the proximal jejunum and ileum, Mt1a mRNA was not affected by level or source of dietary Cu (Figure 4). Expression of Dmt1 mRNA was not affected by level or source of dietary Cu throughout the small intestine (Figure 5). Copper level and source did not affect mRNA expression of cytochrome c oxidase assembly protein 17 (Cox17), Heph, or Atp7b in any of the sections analyzed (Supplemental Figures).

**Main effects of small intestine section**

Digesta pH was not affected by Cu level or source, but was different ($P < 0.0001$) between all sections of the intestine. Duodenal pH ($5.7 \pm 0.08$) was lower ($P < 0.0001$) than that of the proximal jejunum ($6.8 \pm 0.08$) and ileum ($6.5 \pm 0.08$). Digesta pH was also lower ($P = 0.01$) in ileum compared to the proximal jejunum. In control animals, soluble Cu concentrations in duodenal digesta was lower than that in the proximal jejunum ($P = 0.0002$) and ileum ($P = 0.002$), but did not differ ($P = 0.32$) between the proximal jejunum and ileum. Duodenal mucosal Cu concentrations were higher ($P < 0.0001$) than Cu concentrations in the proximal jejunum and ileum, but was not different ($P = 0.61$) between the latter portions. In pigs supplemented with CuSO$_4$, soluble Cu concentrations in duodenal digesta were much lower than that of digesta collected from the proximal jejunum ($P = 0.0002$) and ileum ($P <
0.0001). However, there were no differences \( (P = 0.18) \) between the proximal jejunum and ileum. Mucosal Cu in duodenum of CuSO\(_4\) supplemented pigs was much higher \( (P < 0.0001) \) than that found in their proximal jejunum and ileum, but did not differ \( (P = 0.75) \) between proximal jejunum and ileum. Duodenal digesta from pigs fed Cu\(_2\)(OH)\(_3\)Cl had much lower soluble Cu concentrations than Cu concentration digesta from the proximal jejunum \( (P < 0.0001) \) and ileum \( (P = 0.001) \), but there were no difference \( (P = 0.32) \) between concentrations in the proximal jejunum and ileum. Mucosal Cu in duodenum of Cu\(_2\)(OH)\(_3\)Cl was much higher \( (P < 0.0001) \) than that found in the proximal jejunum and ileum, but was not different \( (P = 0.69) \) between the latter portions.

Regardless of dietary treatment, mucosal Fe concentrations in the duodenum was much higher \( (P < 0.0001) \) than that in the proximal jejunum and ileum, and did not differ \( (P > 0.9) \) between the aforementioned sections. Mucosal Zn concentrations in the duodenum of control animals did not differ from the proximal jejunum, but were lower \( (P = 0.001) \) than that determined in the ileum. Ileal Zn concentrations were also higher \( (P = 0.01) \) than that found in the proximal jejunum of these animals. In pigs supplemented with CuSO\(_4\), mucosal Zn concentrations did not differ across different portions of the small intestine. Furthermore, duodenal Zn concentrations in Cu\(_2\)(OH)\(_3\)Cl pigs were similar to concentrations in the proximal jejunum \( (P = 0.06) \) and ileum \( (P = 0.10) \), and did not differ between the last two sections.

Section of the small intestine had no effect on transcript levels of any of the transporters and chaperones evaluated, except for \( Mtl\alpha \). Relative expression of \( Mtl\alpha \) was not different throughout the small intestine of control pigs. However, CuSO\(_4\) and Cu\(_2\)(OH)\(_3\)Cl
pigs had considerable changes in transcript levels of Mt1a throughout the small intestine. In CuSO4 pigs, duodenal Mt1a was approximately 21 fold higher ($P = 0.002$) than transcript levels in the proximal jejunum and 14 fold higher ($P = 0.001$) than transcript levels in the ileum. However, transcript levels of Mt1a did not differ between the proximal jejunum and ileum. In pigs supplemented Cu$_2$(OH)$_3$Cl, duodenal Mt1a expression was approximately 4.5 fold higher ($P = 0.008$) than transcript levels in the proximal jejunum and approximately 6 fold higher ($P = 0.004$) than ileal Mt1a expression.

**Discussion**

Weanling pigs are quite tolerant to high levels of dietary Cu, and concentrations ranging from 100 to 250 mg Cu/kg diet are supplemented to obtain a pharmacological response (Cromwell et al., 1998; Armstrong et al., 2004). In the present study, we supplemented 225 mg Cu/kg diet from two inorganic sources of Cu whose *in vitro* water and acid solubility differ to examine how level and source affect Cu metabolism in the small intestine of weanling pigs. As expected, Cu-supplemented pigs had higher digesta and mucosal Cu concentrations in the duodenum, proximal jejunum, and ileum than control pigs. These data are in agreement with earlier data in pigs supplemented with 250 mg Cu/kg diet from CuSO4 (Drouliscos et al., 1970).

Copper transport and chaperone proteins are essential for Cu acquisition, intracellular distribution and utilization (Kim et al., 2008). Copper transporter 1 is located on the apical membrane of the enterocyte (Nose et al., 2010) and is essential for intestinal Cu absorption (Nose et al., 2006). Intestinal deletion of Ctr1 in mice results in severe Cu deprivation, as
well as perturbations in growth, Fe metabolism, and cardiac function (Nose et al., 2006). Once Cu has entered the cell, Cu can be delivered to the Cu-dependent ATPase, ATP7A via ATOX1 (Hamza et al., 2003). Antioxidant 1 is an integral component of the secretory pathway of Cu metabolism as shown in Atox1 knock-out mice. These mice are characterized by clinical signs of Cu deficiency as a result of compromised Cu efflux (Hamza et al., 2001). In the present study, expression of Ctr1 and Atox1 mRNA was not affected by level of dietary Cu in the duodenum, where soluble Cu in the digesta was quite low relative to mucosa Cu. However, in the proximal jejunum of Cu-supplemented pigs, Ctr1 mRNA was down-regulated 2-fold while Atox1 mRNA was up-regulated 2-fold. These changes may have occurred in response to saturation, as soluble Cu in the digesta from the proximal jejunum of Cu-supplemented pigs was relatively higher than that of their mucosal Cu concentrations. Previous research in humans has shown that high Cu intake decreases Cu absorption (Turnlund et al., 1989), and more recent work in cell culture has shown that Cu uptake via CTR1 is saturable (Lee et al., 2002). There is a limited body of in vivo studies examining effects of high Cu on gene products involved in Cu metabolism. Previous data in Cu-loaded rats demonstrated that ATP7A protein was most abundant in the proximal jejunum, with much less present in the ileum and intermediate levels in the duodenum (Nyasa et al., 2007). These authors suggested that the pattern in ATP7A protein levels likely reflected sites in which Cu was delivered into the bloodstream. Although Atp7a mRNA in our study was not affected, it is plausible that up-regulation of Atox1 mRNA likely occurred as a response to increase Cu efflux. This suggests that Atox1, and not Atp7a, may be the limiting factor in regard to Cu efflux. If Cu utilization into the bloodstream is indeed occurring in the proximal
jejenum, then reciprocal modulation of *Ctr1* and *Atox1* in Cu-supplemented pigs would make sense. Recent work in Hek293T cells demonstrated that CTR1 protein levels decreased within 4 h when cells were cultured in high Cu media (Nose et al., 2010).

Although source of dietary Cu did not affect soluble Cu concentrations in the digesta, mucosal Cu concentrations in the duodenum and proximal jejunum were affected by Cu source. It is unclear why CuSO$_4$-supplemented pigs had much higher duodenal Cu concentrations than their Cu$_2$(OH)$_3$Cl-supplemented counterparts, and why the opposite occurred in the proximal jejunum. Absorption of a mineral is determined by the extent to which the mineral becomes soluble in the stomach and the extent of mineral interactions with various ligands that occur during digestion (Powell et al., 1994). Copper from Cu$_2$(OH)$_3$Cl may not become completely solubilized in the stomach and may gradually become soluble as the digesta descends down the tract. Another possibility is that these Cu sources may interact differently with exogenous ligands such as phytate. Previous studies have examined the effects of these Cu sources on *in vitro* solubility and *in vitro* phytate hydrolysis (Miles et al., 1998; Pang and Applegate, 2006). However, we do not have an understanding of how solubility of the Cu sources is affected when these sources are supplemented to diets containing ingredients such as corn and soybean meal. Further research in this area is warranted.

Differences in duodenal Cu among Cu sources may be explained, to some degree, by the differences in *Ctr1* and *Mtl1a* mRNA. Transcript levels of *Ctr1* were approximately 2-fold lower in Cu$_2$(OH)$_3$Cl pigs than in CuSO$_4$ pigs. If transcript levels are a reflection of the abundance of protein present, then this likely explains why their mucosal Cu concentrations
were lower than those fed CuSO$_4$. This may also explain why transcript levels of $Mt1a$ were not significantly higher than that of CuSO$_4$ and controls pigs. Metallothionein is a cytosolic protein that provides protection from toxicity of metals such as Cu, Zn, and cadmium (Cousins et al., 1985). Abundance of $Mt1a$ mRNA was much higher in pigs supplemented CuSO$_4$ relative to Cu$_2$(OH)$_3$Cl and control pigs. This large difference in $Mt1a$ mRNA may have occurred as a result of $Ctr1$ mRNA in CuSO$_4$-supplemented pigs being practically equal to that of the controls. In agreement with the mRNA $Mt1a$ results in the present study, Naziripour and Klassing (unpublished data) has shown that duodenal MT protein was much higher in broilers supplemented 150 mg/kg Cu from CuSO$_4$ than in those supplemented the same level from Cu$_2$(OH)$_3$Cl.

Changes in Cu concentrations in the digesta and mucosa between intestinal sections disagree with previous data by Drouliscos et al. (1970). These authors reported that in control pigs receiving no supplemental Cu, mucosal Cu did not differ between sections and Cu in duodenal contents was higher than that collected further down the tract. Furthermore, authors reported that mucosal Cu in pigs supplemented with 250 mg Cu/kg diet from CuSO$_4$ was higher in the jejunum than in duodenum with the ileum being intermediate. Digesta Cu in these pigs did not differ between sections (Drouliscos et al., 1970). The reason for these discrepancies is unclear, but may be due to the fact that pigs in the study by Drouliscos et al. (1970) were not fasted and refed prior to harvest. Pigs in the present study were fasted for 8 h prior to an 8 h refeeding, thus all animals were in the same fed state. It is likely that changes in the small intestine in the present study more closely resembled regulation that occurs following the consumption of a diet containing high levels of Cu.
Relative expression of *Mt1a* mRNA was much higher in the duodenum of Cu-supplemented pigs compared to transcript levels in their proximal jejunum and ileum. This is in agreement with previous data in broilers illustrating that MT protein was higher in the duodenum of Cu-supplemented birds than in distal portions of their small intestine (Naziripour and Klassing, unpublished data). If transcription of *Mt1a* in the present study is reflective of the amount of protein present, then it is likely that a major portion of duodenal Cu is sequestered to MT. Copper bound to MT can be lost via endogenous losses such as sloughing of enterocytes (Hoogenraad, 2006). This may be a way in which pharmacological levels of dietary Cu are regulated in the small intestine of weanling pigs.

In conclusion, our findings demonstrate that level of dietary Cu increases the amount of soluble Cu in the digesta and the mucosa of the duodenum, proximal jejunum, and ileum. Level of dietary Cu did not affect mRNA expression of Cu regulatory proteins in the duodenum and minimal effects were noted in the ileum. In the proximal jejunum, down-regulation of *Ctr1* and up-regulation of *Atox1* may have occurred in response to Cu saturation. Source effects on duodenal *Ctr1* and *Mt1a* provide some reasoning as to why mucosal uptake was higher in CuSO₄-supplemented pigs. Higher mucosal Cu in the proximal jejunum of Cu₂(OH)₃Cl pigs suggests that Cu from Cu₂(OH)₃Cl may gradually become soluble further down the tract. Mucosa Cu concentrations were the highest in the duodenum, while soluble Cu concentrations were the lowest in this section compared to the proximal jejunum and ileum. In Cu-supplemented pigs, levels of *Mt1a* were much higher in the duodenum than in the proximal jejunum and ileum. This suggests that the duodenum is likely sequestering much of the dietary Cu and may be providing protection from excessive Cu
efflux. These data provide a better understanding of how the young pig regulates pharmacological levels of Cu in the small intestine. Because this study only analyzed gene expression of Cu regulatory proteins, future work is needed in evaluating possible translational and posttranslational modifications that may occur under similar dietary conditions.

References


Nose, Y., B.E. Kim, and D.J. Thiele. 2006. Ctr1 drives intestinal copper absorption and is essential for growth, iron metabolism, and neonatal cardiac function. Cell Metab. 4: 235-244.


Table 1. Composition of nursery diets

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Phase 1</th>
<th>Phase 2</th>
<th>Phase 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn, ground</td>
<td>376.4</td>
<td>451.4</td>
<td>608.4</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>220.0</td>
<td>290.0</td>
<td>340.0</td>
</tr>
<tr>
<td>Dried whey</td>
<td>275.0</td>
<td>175.0</td>
<td>---</td>
</tr>
<tr>
<td>Plasma protein</td>
<td>40.0</td>
<td>30.0</td>
<td>---</td>
</tr>
<tr>
<td>Fish meal</td>
<td>45.0</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Poultry fat</td>
<td>20.0</td>
<td>20.0</td>
<td>20.0</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>4.0</td>
<td>15.0</td>
<td>14.0</td>
</tr>
<tr>
<td>Vitamin mix</td>
<td>7.5</td>
<td>7.5</td>
<td>7.5</td>
</tr>
<tr>
<td>Limestone</td>
<td>7.0</td>
<td>7.0</td>
<td>7.0</td>
</tr>
<tr>
<td>Salt</td>
<td>4.5</td>
<td>3.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Trace mineral mix</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
</tr>
</tbody>
</table>

1 Basal diet in each phase provided (per kilogram of diet): 6.8 mg Cu, 258.8 mg Fe, 144.3 mg Zn (phase 1); 6.6 mg Cu, 288.9 mg Fe, 141.4 mg Zn (phase 2); 6.7 mg Cu, 221.5 mg Fe, 129.1 mg Zn (phase 3).
2 Provided (per kilogram of vitamin mix): vitamin A 20,568,918 IU, vitamin D3 2,932,118 IU, vitamin E 117,505 IU, vitamin B12 73 mg, riboflavin 14,698 mg, niacin 88,184 mg, d-pantothenic acid 58,790 mg, menadione 9,700 mg, folic acid 4,409, biotin 589 mg.
3 Provided (per kilogram of diet): 80 mg Fe as FeSO4, 0.3 mg Se as Na2SeO3, 80 mg Zn as ZnSO4, 25 mg Mn as MnO, and 0.5 mg I as Ca(IO3)2(H2O).
Table 2. Level and source of dietary Cu affects soluble Cu concentrations in digesta and mucosa in the small intestine of weanling pigs\textsuperscript{1,2}

<table>
<thead>
<tr>
<th>Soluble Cu, mg/L</th>
<th>Control</th>
<th>CuSO\textsubscript{4}</th>
<th>Cu\textsubscript{2}(OH)\textsubscript{3}Cl</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenum</td>
<td>0.41\textsuperscript{a}</td>
<td>10.1\textsuperscript{b}</td>
<td>7.9\textsuperscript{b}</td>
<td>0.820</td>
</tr>
<tr>
<td>Proximal Jejunum</td>
<td>0.67\textsuperscript{a}</td>
<td>62.2\textsuperscript{b}</td>
<td>77.3\textsuperscript{b}</td>
<td>10.200</td>
</tr>
<tr>
<td>Ileum</td>
<td>0.48\textsuperscript{a}</td>
<td>77.8\textsuperscript{b}</td>
<td>63.7\textsuperscript{b}</td>
<td>6.760</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mucosal Cu, mg/kg DM</th>
<th>Control</th>
<th>CuSO\textsubscript{4}</th>
<th>Cu\textsubscript{2}(OH)\textsubscript{3}Cl</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenum</td>
<td>14.4\textsuperscript{a}</td>
<td>130.3\textsuperscript{b}</td>
<td>104.3\textsuperscript{c}</td>
<td>8.23</td>
</tr>
<tr>
<td>Proximal Jejunum</td>
<td>10.5\textsuperscript{a}</td>
<td>22.1\textsuperscript{b}</td>
<td>44.7\textsuperscript{c}</td>
<td>3.23</td>
</tr>
<tr>
<td>Ileum</td>
<td>9.8\textsuperscript{a}</td>
<td>23.8\textsuperscript{b}</td>
<td>32.8\textsuperscript{b}</td>
<td>3.15</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Data are means per treatment (n = 9-10).
\textsuperscript{2}Means in row without common superscript differ $P < 0.05$. 

130
Table 3. Level and source of dietary Cu affects Fe and Zn concentration in the small intestine of weanling pigs\textsuperscript{1,2}

<table>
<thead>
<tr>
<th>Dietary Treatment</th>
<th>Control</th>
<th>CuSO\textsubscript{4}</th>
<th>Cu\textsubscript{2}(OH)\textsubscript{3}Cl</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mucosal Fe, mg/kg DM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duodenum</td>
<td>830.1\textsuperscript{a}</td>
<td>1080.1\textsuperscript{a,b}</td>
<td>559.1\textsuperscript{a,c}</td>
<td>110.67</td>
</tr>
<tr>
<td>Proximal Jejunum</td>
<td>86.7</td>
<td>75.7</td>
<td>76.9</td>
<td>4.36</td>
</tr>
<tr>
<td>Ileum</td>
<td>77.3</td>
<td>75.3</td>
<td>73.4</td>
<td>3.15</td>
</tr>
<tr>
<td><strong>Mucosal Zn, mg/kg DM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duodenum</td>
<td>104.8</td>
<td>106.7</td>
<td>107.6</td>
<td>2.11</td>
</tr>
<tr>
<td>Proximal Jejunum</td>
<td>114.4</td>
<td>114.1</td>
<td>118.2</td>
<td>3.08</td>
</tr>
<tr>
<td>Ileum</td>
<td>137.5\textsuperscript{a}</td>
<td>115.9\textsuperscript{b}</td>
<td>115.9\textsuperscript{b}</td>
<td>5.55</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Data are means per treatment (n = 9-10).
\textsuperscript{2}Means in row without common superscript differ $P < 0.05$. 


### Supplemental table 1. Real-time polymerase chain reaction primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Accession #</th>
<th>Primer sequence (5’- 3’‡)</th>
<th>Product length</th>
</tr>
</thead>
</table>
| β-actin† | DQ845171 | F: CACGCCATCTCGGTCTGGA  
R: AGCACCCTGTGGCGTACAG | 100 |
| Rpl4† | DQ845176 | F: CAAGAGTAACACTAGGCTTT | 122 |
| Tbp† | DQ845178 | F: AACAGTTTGATATATGACGAG  
R: AGATGTTTCAAAACGCTTCG | 153 |
| Hprt1† | DQ845175 | F: GGAATTTGATCTTGTTG  
R: CAGATGGTTTCAAAACTCAG | 91 |
| Ctr1 | NM214100.2 | F: ATGATGTAGTGCATCTATGCC  
R: GATGCTGACTTTGGGACCTTG | 150 |
| Atox1 | NM001167641.1 | F: CCGAAGCAGAGTTCTCC  
R: TGTGGGCAAGTCAATGTC | 109 |
| Cox17 | NM001190922.1 | F: GAGCAGTGTGGACACCTAATTGAG  
R: TCACAAGCAGACACCATC | 86 |
| Atp7a | AB271958.1 | F: AAGGAGGAGAAGAAGACTTCATC  
R: CGGATTAAACTGCTATCATCAG | 200 |
| Atp7b | XM001925351.1 | F: TCATCAAGAAGCTTGGAAAG  
R: ATGGGTGCTTTGGACATTC | 148 |
| Mt1a | NM001001266.1 | F: TCCTGCTCACTGGTGAA  
R: AAATACACCCTCCTCAAC | 65 |
| Heph‖ | TC328481 | F: GCCCCAACGAGGACTTATC  
R: GCCCTGCTGGGAAGTACATTC | 100 |
| Dmt1§ | GI:190360608 | F: AGGATCTAGGCATGTGTTG  
R: CCACATGCTCAGGAAAGCAT | 124 |

‡F: Forward; R: Reverse  
†Taken from Nygard et al. (2007)  
‖Taken from Hansen et al. (2009, 2010)  
§Contains 3’ iron responsive element
Figure 1. Relative expression of copper transporter 1 (Ctr1) in duodenum, proximal jejunum, and ileum of weanling pigs. Values are means (n = 9-10) ± SEM. Treatments without common letter differ $P < 0.05$. 
Figure 2. Relative expression of antioxidant 1 (*Atox1*) in duodenum, proximal jejunum, and ileum of weanling pigs. Values are means (n = 9-10) ± SEM. Treatments without common letter differ $P < 0.05$. 
Figure 3. Relative expression of the Cu-dependent ATPase \textit{Atp7a} in duodenum, proximal jejunum, and ileum of weanling pigs. Values are means (n =9-10) ± SEM.
Figure 4. Relative expression of metallothionein 1a ($Mt1a$) in duodenum, proximal jejunum, and ileum of weanling pigs. Values are means (n = 9-10) ± SEM. Treatments without common letter differ $P < 0.05$. 
Figure 5. Relative expression of divalent metal transporter 1 (Dmt1) in duodenum, proximal jejunum, and ileum of weanling pigs. Values are means (n = 9-10) ± SEM.
Supplemental Figure 1. Relative expression of the cytochrome c oxidase assembly protein 17 (Cox17) in duodenum, proximal jejunum, and ileum of weanling pigs. Values are means (n = 9-10) ± SEM
Supplemental Figure 2. Relative expression of the Cu-dependent ATPase *Atp7b* in duodenum, proximal jejunum, and ileum of weanling pigs. Values are means (n = 9-10) ± SEM.
Supplemental Figure 3. Relative expression of the Cu-dependent ferroxidase, hephaestin (Heph) in duodenum, proximal jejunum, and ileum of weanling pigs. Values are means (n = 9-10) ± SEM.
CHAPTER 5

Level and source of dietary copper affect small intestine morphology, duodenal lipid peroxidation, and hepatic oxidative stress in weanling pigs, and also affects mRNA expression of hepatic copper regulatory proteins¹


North Carolina State University, Raleigh, NC, USA, 27695

²Corresponding author: jerry_spears@ncsu.edu
**Abstract**

Thirty weanling, crossbred barrows were utilized to determine the effect of level and source of dietary copper (Cu) on small intestine morphology and lipid peroxidation, hepatic oxidative stress, and hepatic Cu homeostasis. At 21 d of age pigs were stratified by weight (6.33 ± 0.23 kg) and allocated to one of the following dietary treatments: 1) control (no supplemental Cu; 6.7 mg Cu/kg), 2) 225 mg supplemental Cu/kg diet from Cu sulfate (CuSO₄), or 3) 225 mg supplemental Cu/kg diet from tribasic Cu chloride (Cu₂(OH)₃Cl). Pigs were housed 2 pigs per pen and were fed a three phase diet regime until d 35 or 36 of the study. Prior to harvest pigs were fasted for 8 h and subsequently re-fed for 8 h. During harvest bile and liver were obtained for mineral analysis, and liver samples were also obtained for analysis of liver glutathione and mRNA expression of Cu regulatory proteins. Segments of duodenum, proximal jejunum and ileum were obtained from one pig per pen to examine mucosal morphology. Mucosal scrapes were collected from all pigs for analysis of malondialdehyde (MDA). Duodenal villus height was reduced in CuSO₄ pigs compared to controls (P = 0.001) and Cu₂(OH)₃Cl (P = 0.03) pigs. Villus height in the proximal jejunum of CuSO₄ pigs was reduced (P = 0.03) compared to controls, while ileal villus height was not affected by treatment. Duodenal malondialdehyde concentrations were higher (P = 0.03) in CuSO₄ vs. controls, but concentrations were similar (P = 0.10) among controls and Cu₂(OH)₃Cl pigs. Liver Cu was higher (P = 0.01) in CuSO₄ vs. control pigs, but only tended (P = 0.07) to be higher in Cu₂(OH)₃Cl pigs than in controls. Bile Cu concentrations were higher (P < 0.001) in CuSO₄ and Cu₂(OH)₃Cl vs. controls and were also higher (P = 0.04) in Cu₂(OH)₃Cl vs. CuSO₄ pigs. Total liver glutathione concentrations were lower (P = 0.02) in
pigs supplemented with CuSO₄ vs. controls but did not differ between controls and Cu₂(OH)₃Cl pigs. As determined via quantitative Real-time PCR, hepatic mRNA of cytochrome c oxidase assembly protein 17 was lower ($P = 0.01$) in CuSO₄ vs. control pigs. Expression of antioxidant 1 mRNA was higher ($P = 0.04$) in Cu₂(OH)₃Cl pigs compared to controls and tended ($P = 0.06$) to be higher in CuSO₄ vs. control pigs. Differences in solubility between Cu sources may explain differences in small intestine morphology and lipid peroxidation, as well as differences in hepatic Cu metabolism and oxidative stress.

**Introduction**

In weanling pigs, a breadth of literature has indicated that pig performance is enhanced when dietary copper (Cu) is supplemented well above their NRC requirement of 5 mg Cu/kg diet (Cromwell et al., 1998; Armstrong et al., 2004). However, definitive mechanism(s) responsible for this pharmacological response remains unknown. Copper absorption primarily occurs in the duodenum and upper jejunum while the liver serves as the major storage organ for Cu (Linder and Hazegh-Azam, 1996). As demonstrated in rodents, intracellular Cu ions are tightly regulated by numerous transport and chaperones proteins that are responsible for Cu acquisition, intracellular distribution, and excretion (Kim et al., 2008). We have previously examined the effects of the Cu antagonist iron (Fe) on mRNA expression of hepatic Cu transporters and chaperones in weanling pigs (Fry et al., 2010a). However, it is unknown how these mechanisms are affected by level and source of dietary Cu.

Although Cu is an essential component of superoxide dismutase, an important
enzyme in the antioxidant defense system, it can also act in a prooxidant manner. Copper ions are actively oxidized and reduced, and are able to catalyze the formation of hydroxyl radicals, which can lead to lipid peroxidation (Bremner, 1998). Miles et al. (1998) demonstrated that poultry diets supplemented with Cu sulfate (CuSO₄) had greater prooxidant activity during storage that those supplemented with tribasic Cu chloride (Cu₂(OH)₃Cl). Both CuSO₄ and Cu₂(OH)₃Cl are inorganic sources of Cu that are efficacious in promoting growth in weanling pigs (Cromwell et al., 1998). To date it is unknown how these Cu sources affect Cu homeostasis and other physiological responses in weanling pigs. The objectives of the present study were to determine the effects of level and source (CuSO₄ vs. Cu₂(OH)₃Cl) of dietary Cu on 1) small intestine histology and lipid peroxidation, 2) hepatic oxidative stress, and 3) mRNA expression of Cu regulatory proteins involved in hepatic Cu homeostasis.

Materials and Methods

Animals and Experimental Design

Prior to the initiation of the study, animal care, use, and sampling procedures were approved by the North Carolina State University Animal Care and Use Committee. At approximately 21 d of age, thirty weanling, crossbred barrows (6.33 ± 0.23 kg) were stratified by weight and assigned to one of the following dietary treatments: 1) control (no supplemental Cu), 2) 225 mg supplemental Cu/kg from CuSO₄, and 3) 225 mg supplemental Cu/kg from Cu₂(OH)₃Cl. Pigs had *ad libitum* access to feed and water and were housed two pigs per pen. Diets were in mash form and were fed in three phases. Phase 1 diets were fed
from d 0 to 6, phase 2 diets were fed from d 7 to 21, and phase 3 diets were fed from d 22 until harvest (Table 1). Diets were formulated to meet or exceed NRC requirements and the control diet met the pig’s nutritional requirement for Cu (NRC, 1998).

**Performance Measurements and Blood Sampling**

At the conclusion of each phase performance data were obtained, and phase 3 performance was concluded on d 33 of the study. At the conclusion of each phase feed samples were obtained for determination of mineral concentrations. Jugular blood samples were obtained in 6 mL trace element grade heparinized tubes (BD Vacutainer, Franklin Lakes, NJ) from each pig on d 33 for determination of plasma Cu and holo(active)-ceruloplasmin (holo-Cp). Plasma Cu was prepared and analyzed as described by Hansen et al. (2008) and holo-Cp was analyzed via the procedure described by Houchin (1958). Feed samples were dried in a forced air oven and wet ashed as described by Gengelbach et al. (1994). Mineral concentrations in feed and plasma were determined using atomic absorption spectrophotometry (AA-6701F, Shimadzu, Japan) and a certified liver standard was included to validate instrument accuracy.

**Intestine and Liver Sampling Procedures**

On d 35 and 36 pigs were harvested for sample collection following an 8 h fast and 8 h re-feeding. To properly balance harvest order, three pens representing each of the dietary treatments were randomly assigned to a fast and re-feeding period, and thus a harvest order. Pigs were humanely euthanized one at a time via captive bolt. Subsequently, the abdominal
A cavity was lacerated vertically to collect bile, liver, and intestinal samples from the duodenum, proximal jejunum, and ileum. The duodenum represented the first 60 cm distal the pyloric value. Approximately 152 cm from the distal end of the duodenum was measured and at that point a 60 cm section was obtained to represent the proximal jejunum. Lastly, 60 cm proximal the ileal-cecal junction represented the ileum. Duodenal scrapings were collected from both pigs per pen as described by Hansen et al. (2009) and snap frozen in liquid nitrogen for subsequent analysis of malondialdehyde (MDA) concentrations as per manufacturer’s instructions (Oxford Biomedical, Oxford, MI). To examine mucosal histology in the small intestine, a 1 cm section of tissue from one randomly selected pig per pen was obtained from the proximal end of all the aforementioned sections. Samples were fixed in 10% formalin for 24 h and subsequently embedded in paraffin for hematoxylin and eosin staining. Villus height, crypt depth, and villus width were examined via a light microscope and measurements were obtained using the Spot Advance software (Diagnostic Instruments, Inc., Sterling Heights, MI). Within each section and animal, 12 villus height, crypt depth, and villus width measurements were taken and averaged to provide equal morphological representation.

Bile samples were collected in 5 mL conical tubes and placed on ice for subsequent Cu analysis as described by Armstrong et al. (2000). Three portions of liver were obtained from the lobe adjacent to gallbladder and rinsed in 0.01 M phosphate buffered saline (pH 7.4) for mineral analysis, total glutathione (GSH) determination, and mRNA analysis of Cu regulatory proteins. Liver for mineral analysis was prepared and analyzed for minerals as described earlier for feed samples. Liver for total GSH determination was snap frozen in
liquid nitrogen and subsequently analyzed as per manufacturer’s instructions (Trevigen, Gaithersburg, MD). The portion of liver collected for mRNA analysis was placed in a 50 mL tube containing RNAlater as per manufacturer’s instructions (Ambion, Austin, TX).

**RNA Isolation and Real-time PCR**

Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) with an on-column DNase digestion. Integrity and quantity of RNA was determined via the Nanodrop-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Furthermore, RNA was ran on a 1.2% agarose gel to capture images of the 28s and 16s ribosomal subunits to determine their integrity. One µg of total RNA was used to synthesize cDNA via the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc., United States) as per manufacturer’s instructions. Real-time primers were designed with Beacon Designer software (Premier Biosoft Intl., Palo Alto, CA) to be compatible with SYBR Green I (Table 2). Proposed function(s) of each of the target genes analyzed are noted in Table 3. One amplicon from each reaction was sequence validated to confirm primer specificity. Primer specificity was also confirmed by melt curve analysis and examination of product size via agarose gel electrophoresis. Quantitative real-time PCR reactions were ran in duplicate (Bio-Rad, Hercules, CA) and each reaction contained 50 ng cDNA, 10 µM each of forward and reverse primers, and 1X SYBR Green Master Mix (Ambion, Austin, TX). The cycling program was as reported by Hansen et al. (2009a). Primer sequences for divalent metal transporter 1 (*Dmt1*) were obtained from published data with swine (Hansen et al., 2009b). Numerous housekeeping genes, taken from Nygard et al. (2007), were accessed for their
stability in liver. Of those assessed, ribosomal protein L4 (\textit{Rpl4}), TATA box binding protein (\textit{Tbp}), hypoxanthine phosphoribosyltransferase 1 (\textit{Hprt1}), and beta-actin (\textit{\beta}-\textit{actin}) were found to be the most stable. The geometric mean of the aforementioned housekeeping genes was determined for target normalization. Relative expression of target genes was determined via the $2^{-\Delta\Delta C_T}$ method (Lavak and Schimittgen, 2001).

**Statistical Analysis**

All data were analyzed as ANOVA using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC) with pen as the experimental unit. The model included the fixed effect of treatment and the random effects of replicate and block, in which block represented harvest day. All possible interactions were tested and removed if not significant, $P \geq 0.15$. Treatment effects were separated using least square means. Simple Pearson correlations for duodenal MDA concentrations and villus height, as well as liver Cu and total GSH concentrations were generated using the CORR procedure in SAS. Data are expressed as least square means ± SEM. Significance was declared at $P \leq 0.05$ and tendencies are discussed at $P < 0.10$.

**Results**

**Performance and Plasma Measurements**

During phase 1, ADG ($P = 0.01$) and G:F ($P = 0.02$) were higher in pigs supplemented with Cu$_2$(OH)$_3$Cl compared to those supplemented with CuSO$_4$. Average daily feed intake was not affected by Cu level or source, and performance of controls did not differ from Cu-supplemented pigs. Phase 2 ADG ($P = 0.01$) and ADFI ($P = 0.04$) were higher,
while G:F tended \( (P = 0.06) \) to higher in CuSO\(_4\) pigs compared to controls. Neither phase 3, nor overall performance was affected by level or source of dietary Cu (Table 4). Plasma Cu was not affected by source, but was higher \( (P = 0.01) \) in Cu-supplemented pigs than in controls. Holo-Cp was not affected by level or source of dietary Cu (Table 5).

**Liver Mineral, Bile Copper, and Total Glutathione Concentrations**

Bile Cu concentrations were affected by level and source of dietary Cu. When compared to the controls, CuSO\(_4\) pigs \( (P = 0.0004) \) and Cu\(_2\)(OH)\(_3\)Cl pigs \( (P < 0.0001) \) had higher bile Cu concentrations. Furthermore, bile Cu was higher \( (P = 0.04) \) in Cu\(_2\)(OH)\(_3\)Cl vs. CuSO\(_4\) pigs (Table 5). Pigs supplemented with CuSO\(_4\) had higher \( (P = 0.01) \) liver Cu than controls. However, liver Cu in Cu\(_2\)(OH)\(_3\)Cl supplemented pigs only tended \( (P = 0.07) \) to be higher than controls. Liver Cu did not differ among Cu sources (Table 6). Liver Zn tended \( (P = 0.07) \) to be higher in CuSO\(_4\) pigs compared to controls, but did not differ among the other dietary treatment groups. Liver Fe concentrations were lower \( (P = 0.04) \) in Cu\(_2\)(OH)\(_3\)Cl pigs compared to controls, but did not differ between other dietary treatment groups (Table 6).

Concentrations of total liver GSH were lower \( (P = 0.02) \) in CuSO\(_4\) vs. controls, but did not differ among other dietary treatments (Table 7). There was a negative relationship between liver Cu and total GSH concentrations \( (R^2 = -0.60; \ P = 0.02) \) regardless of dietary treatment.

**Mucosal Histology and Duodenal MDA Concentrations**

Pigs supplemented with CuSO\(_4\) had higher \( (P = 0.03) \) duodenal MDA concentrations (Table 7). Duodenal MDA concentrations in Cu\(_2\)(OH)\(_3\)Cl pigs did not differ from controls or
CuSO₄ pigs. Crypt depth and villus width were not affected by level or source of dietary Cu in any section of the small intestine (data not shown). Duodenal villus height was reduced in CuSO₄ supplemented pigs compared to control ($P = 0.001$) and Cu₂(OH)₃Cl supplemented ($P = 0.03$) pigs, while villus height was similar ($P = 0.11$) among Cu₂(OH)₃Cl and control pigs. Villus height in the proximal jejunum was reduced ($P = 0.03$) in CuSO₄-supplemented pigs compared to controls, but did not differ among other dietary treatments (Figure 1). When only considering MDA concentration in animals used for mucosal histology, there was a strong negative ($R^2 = -0.72; P = 0.002$) relationship between MDA concentrations and duodenal villus height.

**Gene Expression of Hepatic Cu Regulatory Proteins**

Neither level nor source of dietary Cu significantly affected mRNA expression of hepatic Cu transporter 1 (Ctr1; Table 8). However, antioxidant 1 (Atox1) mRNA was higher ($P = 0.04$) in pigs supplemented with Cu₂(OH)₃Cl and tended ($P = 0.06$) to be higher in CuSO₄-supplemented pigs compared to controls. The hepatic Cu-dependent ATPase, Atp7b, tended ($P = 0.09$) to be lower in Cu₂(OH)₃Cl-supplemented pigs compared to controls but was not differ ($P = 0.13$) between CuSO₄ and controls. Relative expression of Cp mRNA was lower ($P = 0.03$) in Cu₂(OH)₃Cl pigs compared to controls, but did not differ between CuSO₄ pigs and controls. Relative expression of Cu metabolism MURR1 domain 1 (Commd1) tended to be lower in CuSO₄ ($P = 0.09$) and Cu₂(OH)₃Cl ($P = 0.06$) supplemented pigs compared to the controls. Cytochrome c oxidase assembly protein 17 (Cox17) mRNA was down-regulated ($P = 0.01$) in CuSO₄ pigs and tended ($P = 0.08$) to be lower in Cu₂(OH)₃Cl
pigs relative to controls. Expression of *Atp7a* mRNA was not affected by level or source of dietary Cu. Divalent metal transporter 1 (*Dmt1*) mRNA tended (*P* = 0.06) to be lower in CuSO₄ pigs compared to controls, but was not different (*P* = 0.15) between Cu₂(OH)₃Cl and controls and Cu sources (*P* = 0.61). Metallothionein 1a (*Mt1a*) mRNA tended to be higher in CuSO₄ (*P* = 0.09) and Cu₂(OH)₃Cl (*P* = 0.06) pigs compared to controls but did not differ between source (Table 8).

**Discussion**

Since Braude’s first observation that weanling pigs licking Cu pipes grew faster than those without access to Cu pipes (Braude, 1948), an abundance of research has been conducted examining the efficacy of different sources of dietary Cu in regard to promoting growth (Droushilcos et al., 1970; Cromwell et al., 1998; Armstrong et al., 2004). Studies have been conducted to elucidate possible modes of action of pharmacological levels of dietary Cu (Zhou et al., 1994; Li et al., 2008), yet definitive mechanisms remain unclear. Copper can act as a prooxidant (Bremner, 1998) and little research has examined possible detrimental effects of pharmacological levels of Cu on lipid peroxidation and oxidative stress. Previous data has shown that CuSO₄ is more reactive than Cu₂(OH)₃Cl in stored poultry diets (Miles et al., 1998) and that greater oxidation of vitamin E also occurs (Luo et al., 2005). Results of the present study suggests that supplementing pharmacological levels of CuSO₄ as opposed to Cu₂(OH)₃Cl has a more detrimental effect on intestinal mucosa and hepatic oxidative status relative to providing no supplemental Cu.

Previous data in swine has demonstrated that pharmacological levels of CuSO₄ affect
small intestine morphology in weanling pigs. Shurson et al. (1990) reported that 283 mg Cu/kg diet from CuSO₄ reduced duodenal villus height compared to control pigs receiving no supplemental Cu. In the present study, villus height in the duodenum and proximal jejunum was reduced by supplementing 225 mg Cu/kg diet from CuSO₄, but not 225 mg Cu/kg diet from Cu₂(OH)₃Cl. Duodenal villi were also affected by source in which villus height was reduced in CuSO₄-supplemented pigs compared to those supplemented with Cu₂(OH)₃Cl. Much of the dietary Cu consumed is absorbed in the duodenum and proximal jejunum (Linder and Hazegh-Azam, 1996), and the source effect on villus height likely occurred as a result of mucosal Cu concentrations in the duodenum of CuSO₄-supplemented pigs being much higher than that of Cu₂(OH)₃Cl-supplemented pigs (Fry et al., 2010b). Mucosal Cu concentrations were much lower in the proximal jejunum than in the duodenum of Cu-supplemented pigs (Fry et al., 2010b), yet villus height in the proximal jejunum of CuSO₄-supplemented pigs remained shorter than that of controls. Moreover, villus height in the proximal jejunum of Cu₂(OH)₃Cl fed pigs was not reduced even though their mucosal Cu concentrations in the proximal jejunum were higher than that of CuSO₄-supplemented pigs (Fry et al., 2010b).

Malondialdehyde is widely used experimentally as an indicator of lipid peroxidation (Halliwell and Gutteridge, 1999). Duodenal MDA did not differ among Cu sources, but increased to a greater magnitude in CuSO₄ pigs than in Cu₂(OH)₃Cl pigs and consequently was higher in CuSO₄ pigs than in controls, but not in Cu₂(OH)₃Cl pigs. A strong negative correlation between duodenal villus height and duodenal MDA concentrations existed in the present study, which suggests that CuSO₄ supplementation reduced surface area in the
intestine by increasing lipid peroxidation. Weaning poses a great deal of stress on the small intestine of newly weaned pigs (Moser et al., 2007), and the addition of pharmacological levels of CuSO₄ right after weaning may have been detrimental to pig performance. If the aforementioned Cu effects on the small intestine occurred early on, this may explain why performance was lower in CuSO₄ fed pigs compared to Cu₂(OH)₃Cl-supplemented pigs during phase 1 feeding.

Liver Cu increased to a greater magnitude in CuSO₄ pigs than in pigs supplemented with Cu₂(OH)₃Cl. This is in agreement with Cromwell et al. (1998) demonstrating that the magnitude in which liver Cu increased in weanling pigs supplemented with 200 mg Cu/kg diet from CuSO₄ was greater than in pigs supplemented Cu₂(OH)₃Cl at the same level. A recent study reported that liver Cu was highest in broilers supplemented 250 mg Cu/kg diet from CuSO₄, while liver Cu in birds supplemented 250 mg Cu/kg diet from Cu₂(OH)₃Cl was intermediate to that of CuSO₄ birds and control birds receiving no supplemental Cu (Xiang-Qi et al., 2009). The major route of Cu excretion is via the bile (Mahoney et al., 1955), and once excreted bile-Cu is poorly reabsorbed (Linder and Hazegh-Azam, 1996). Armstrong et al. (2000) reported that weanling pigs supplemented with 225 mg Cu/kg diet from CuSO₄ had much higher bile Cu concentrations than their unsupplemented counterparts. In our study, bile Cu was affected by both level and source of dietary Cu, with bile Cu concentrations being intermediate in CuSO₄ supplemented pigs. Higher bile Cu in Cu₂(OH)₃Cl compared to CuSO₄ pigs may explain why liver Cu concentrations were less elevated in pigs supplemented with pharmacological levels of Cu from Cu₂(OH)₃Cl.
Furthermore, higher bile Cu and lower liver Cu may also explain why hepatic total GSH concentrations were not affected in Cu$_2$(OH)$_3$Cl pigs. Glutathione is an efficient Cu chelator that detoxifies Cu soon after cellular entry. In addition to being a major substrate for the antioxidant enzyme glutathione peroxidase, glutathione also protects membranes from various aldehydes that arise during peroxidation (Halliwell and Gutteridge, 1999). The negative relationship between total GSH and liver Cu concentrations suggests that the robust increase in liver Cu in CuSO$_4$-supplemented pigs resulted in a greater demand for intracellular detoxification and consequently reduced an important antioxidant. Lower concentrations of total GSH in CuSO$_4$ fed pigs is also consistent with the increased lipid peroxidation that occurred in the duodenum.

Over the past decade many advances have been made utilizing rodents and cell lines to better understand the roles of Cu transport and chaperone proteins. These Cu proteins are essential in regulating intracellular Cu ions to prevent toxicity as well as deficiency (Kim et al., 2008). This study is the first to examine mRNA expression of Cu regulatory proteins in the liver of weanling pigs fed pharmacological levels of dietary Cu. Neither level nor source of dietary Cu affected Ctr1 mRNA, a high affinity Cu transporter that is the primary route for hepatic Cu uptake (Kim et al., 2009). Once Cu is taken up by the liver, it must be bound to Cu chaperone proteins or the Cu storage protein, MT1. Previous studies have demonstrated that Cu loading increases both MT1 protein and Mt1 mRNA (Mehra and Bremner, 1984; Bauerly et al., 2005), and in the present study its transcription tended to be up-regulated in Cu-supplemented pigs regardless of source. One of the several Cu chaperones to which Cu
must be bound to is COX17. This protein is a cytosolic and mitochondrial protein that delivers Cu to the terminal oxidase in the electron transport chain. Transcript levels of Cox17 were lower in CuSO4 pigs and tended to be lower in Cu2(OH)3Cl pigs compared to controls. It is unclear why Cox17 mRNA was down-regulated, but this may have occurred to promote Cu efflux as Atox1 mRNA tended to be higher in CuSO4 pigs and was higher in Cu2(OH)3Cl pigs. Antioxidant 1 is a Cu chaperone involved in the secretory pathway of Cu metabolism whose primary role is to deliver Cu to the Cu-dependent ATPase, ATP7B. Biliary Cu excretion relies on the transport proteins, ATP7B and COMMD1. Humans with Wilson disease suffer from Cu toxicosis as a result of mutation in the Atp7b gene (Cox and Moore, 2002), and Bedlingtion terriers serve as a useful animal model for Cu toxicity as these animals suffer from a mutation in the Commd1 gene (Klomp et al., 2003). Although bile Cu concentrations were affected, mRNA levels of Atp7b and Commd1 were not up-regulated as we initially hypothesized. In fact, transcript levels of both tended to be down-regulated to some degree in a source dependent manner. It is unclear why this occurred and it is also unclear why Cp mRNA was down-regulated in Cu2(OH)3Cl pigs. In addition to biliary Cu excretion, Atp7b is also involved in synthesizing holo-Cp, a Cu-dependent ferroxidase that represents much of the Cu in circulation. Lack of up-regulation of Atp7b mRNA may explain why small increases in plasma Cu occurred in pigs fed 225 mg Cu/kg diet in the present study and in studies reported by others (Armstrong et al., 2000).

In conclusion, these data demonstrate for the first time that pharmacological levels of CuSO4 may promote lipid peroxidation and oxidative stress to a greater magnitude than Cu2(OH)3Cl when supplemented to weanling pigs at pharmacological concentrations. Copper
source affected duodenal villus height with CuSO₄-supplemented pigs having shorter villi than both controls and those supplemented with Cu₂(OH)₃Cl. Pigs supplemented with CuSO₄ had increased duodenal lipid peroxidation and reduced total GSH concentrations in liver when compared to controls. Higher bile Cu concentrations in Cu₂(OH)₃Cl-supplemented pigs may explain why liver Cu increased to a smaller degree in this treatment group compared to those supplemented with CuSO₄. Copper from Cu₂(OH)₃Cl may gradually become soluble throughout the small intestine as Cu ions disperse in the digesta contents (Fry et al., 2010). This may explain why Cu₂(OH)₃Cl is less detrimental in the intestine. Gene expression of hepatic Cu regulatory proteins demonstrates that Cu level has a greater impact than does Cu source on the modulation of Cu transporters and chaperones at the transcription level. Analysis of these Cu regulatory proteins at the level of translation would provide further insight as to how level and source of dietary Cu affects hepatic Cu metabolism in the young pig. Further research is warranted to better understand the physiological impacts of level and source of dietary Cu in weanling pigs.

References


<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Phase 1</th>
<th>Phase 2</th>
<th>Phase 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corn, ground</td>
<td>37.64</td>
<td>45.14</td>
<td>60.84</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>22.00</td>
<td>29.00</td>
<td>34.00</td>
</tr>
<tr>
<td>Dried whey</td>
<td>27.50</td>
<td>17.50</td>
<td>---</td>
</tr>
<tr>
<td>Plasma protein</td>
<td>4.00</td>
<td>3.00</td>
<td>---</td>
</tr>
<tr>
<td>Fish meal</td>
<td>4.50</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Poultry fat</td>
<td>2.00</td>
<td>2.00</td>
<td>2.00</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td>0.40</td>
<td>1.50</td>
<td>1.40</td>
</tr>
<tr>
<td>Vitamin mix&lt;sup&gt;3&lt;/sup&gt;</td>
<td>0.75</td>
<td>0.75</td>
<td>0.75</td>
</tr>
<tr>
<td>Limestone</td>
<td>0.70</td>
<td>0.70</td>
<td>0.70</td>
</tr>
<tr>
<td>Salt</td>
<td>0.45</td>
<td>0.35</td>
<td>0.25</td>
</tr>
<tr>
<td>Trace mineral mix&lt;sup&gt;4&lt;/sup&gt;</td>
<td>0.06</td>
<td>0.06</td>
<td>0.06</td>
</tr>
</tbody>
</table>

<sup>1</sup>All diets were fed in meal form.

<sup>2</sup>Phase 1 basal diet provided 6.8 mg Cu, 258.8 mg Fe, 144.3 mg Zn/kg diet. Phase 2 basal diet provided 6.6 mg Cu, 288.9 mg Fe, 141.4 mg Zn/kg diet. Phase 3 basal diet provided 6.7 mg Cu, 221.5 mg Fe, 129.1 mg Zn/kg diet.

<sup>3</sup>Provided (per kilogram of vitamin mix): vitamin A 20,568,918 IU, vitamin D<sub>3</sub> 2,932,118 IU, vitamin E 117,505 IU, vitamin B<sub>12</sub> 73 mg, riboflavin 14,698 mg, niacin 98,184 mg, pantothenic acid 58,790 mg, menadione 9,700 mg, folic acid 4,409, biotin 589 mg.

<sup>4</sup>Provided (per kilogram of diet): 80 mg Fe as FeSO<sub>4</sub>, 0.3 mg Se as Na<sub>2</sub>SeO<sub>3</sub>, 80 mg Zn as ZnSO<sub>4</sub>, 25 mg Mn as MnO, and 0.5 mg I as Ca(IO<sub>3</sub>)<sub>2</sub>(H<sub>2</sub>O).
<table>
<thead>
<tr>
<th>Primer</th>
<th>Accession #</th>
<th>Primer sequence (5’ - 3’)$^1$</th>
<th>Product size</th>
</tr>
</thead>
</table>
| $\beta$-actin$^2$ | DQ845171 | F: CACGCCATCTCCTGCTGGA  
R: AGCACCCTGTTGGCAGTAGAG | 100 |
| Rpl4$^2$ | DQ845176 | F: CAAAGATACATACACCTTC  
R: GAACCTCAAGGATATCTTC | 122 |
| Tbp$^2$ | DQ845178 | F: AACAGTTGAGTATATGAGCCAGA  
R: AGATGTTCTCAACAGCTTGG | 153 |
| Hprt1$^2$ | DQ845175 | F: GACCTGAATCATTTGTTGTTG  
R: CAGATTCTCAACACTCAAC | 91 |
| Ctr1 | NM214100.2 | F: ATGATGATGATGCTATGACC  
R: GATGTGACTTGGGGACTTG | 150 |
| Atox1 | NM001167641.1 | F: CCGAAGACAGAGTTCTCC  
R: TGGTGGCAGGTCAATGTC | 109 |
| Cox17 | NM001190922.1 | F: GAGCACGTGGAGACCCATTGAG  
R: TCACAACGCAGACCCATTTC | 86 |
| Atp7a | AB271958.1 | F: AAGGAGGAGCAAAAGACTTATC  
R: CCGATTCAACTCTCATCAAG | 200 |
| Atp7b | XM001925351.1 | F: TCACATTAGAGCCTGGAAAG  
R: ATGGGTGCTTTGACATC | 148 |
| Mt1a | NM001001266.1 | F: TCCTGCTCCACCTGTAAG  
R: TAAATACCTCCCTCCTC | 65 |
| Commd1 | TC315951 | F: TGAGGTCAGATGCAACCAAG  
R: CTTCAGCAACTCCTCCTCATC | 100 |
| Ccs | NM001001866.1 | F: TCTTCAGAGTGAGGAGT  
R: CGGTGATTTGGGATAGG | 116 |
| Sod1 | NM001190422.1 | F: GGAGACCTGGGGGATATGG  
R: TACTTTCTCTATCCTCCTGT | 160 |
| Cp | XM001925237.1 | F: TCGTGGTGAGTGAAAATCTC  
R: GCCTGTATTGGTATTAGC | 267 |
| Dmt1$^3$ | GI:190360608 | F: AGGTAGTTAGGGCAGTAGGAGT  
R: CCACAGTCAGGAAGGACAT | 124 |

$^1$F: Forward; R: Reverse  
$^2$Taken from Nygard et al. (2007)  
$^3$Taken from Hansen et al. (2009); Isoform containing 3’ iron responsive element.
### Table 3. Proposed function of copper regulatory gene products

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Acronym</th>
<th>Proposed function(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper transporter 1</td>
<td>Ctr1</td>
<td>Plasma membrane uptake</td>
</tr>
<tr>
<td>Antioxidant 1</td>
<td>Atox1</td>
<td>Delivers Cu to Atp7a/7b</td>
</tr>
<tr>
<td>Cytochrome c oxidase assembly protein 17</td>
<td>Cox17</td>
<td>Delivers Cu to mitochondria for incorporation into cytochrome c oxidase</td>
</tr>
<tr>
<td>Cu chaperone for Cu/Zn superoxide dismutase</td>
<td>CCS</td>
<td>Delivers Cu to Sod1</td>
</tr>
<tr>
<td>Cu/Zn superoxide dismutase</td>
<td>Sod1</td>
<td>Scavenges superoxide ion</td>
</tr>
<tr>
<td>Cu transporting alpha-polypeptide Atpase</td>
<td>Atp7a</td>
<td>Function in liver is unknown</td>
</tr>
<tr>
<td>Cu transporting beta-polypeptide Atpase</td>
<td>Atp7b</td>
<td>Incorporation of Cu into Cp liver and Cu biliary excretion</td>
</tr>
<tr>
<td>Metallothionein 1a</td>
<td>Mt1a</td>
<td>Copper storage protein and may also function as a chaperone protein</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>Cp</td>
<td>Copper dependent ferroxidase secreted from liver</td>
</tr>
<tr>
<td>Copper metabolism MURR domain 1</td>
<td>Commd1</td>
<td>Interacts with Atp7b to excrete Cu in bile</td>
</tr>
<tr>
<td>Divalent metal transporter 1</td>
<td>Dmt1</td>
<td>Imports Fe</td>
</tr>
</tbody>
</table>
Table 4. Effect of level and source of dietary copper performance of weanling pigs

<table>
<thead>
<tr>
<th>Dietary Treatment</th>
<th>Control</th>
<th>CuSO₄</th>
<th>Cu₂(OH)₃Cl</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase 1, d 0-6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADG, g</td>
<td>97.6ᵃᵇ</td>
<td>75.9ᵇ</td>
<td>142.4ᵃ</td>
<td>15.59</td>
</tr>
<tr>
<td>ADFI, g</td>
<td>209.2</td>
<td>229.5</td>
<td>213.1</td>
<td>14.58</td>
</tr>
<tr>
<td>G:F</td>
<td>0.47ᵃᵇ</td>
<td>0.35ᵇ</td>
<td>0.66ᵃ</td>
<td>0.081</td>
</tr>
<tr>
<td>Phase 2, d 7-20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADG, g</td>
<td>225.8ᵃ</td>
<td>351.5ᵇ</td>
<td>297.3ᵃᵇ</td>
<td>19.13</td>
</tr>
<tr>
<td>ADFI, g</td>
<td>385.3ᵃ</td>
<td>468.0ᵇ</td>
<td>411.8ᵃᵇ</td>
<td>22.11</td>
</tr>
<tr>
<td>G:F</td>
<td>0.67ᶜ</td>
<td>0.75ᵈ</td>
<td>0.72ᶜᵈ</td>
<td>0.024</td>
</tr>
<tr>
<td>Phase 3, d 21-33</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADG, g</td>
<td>579.4</td>
<td>525.6</td>
<td>586.7</td>
<td>30.35</td>
</tr>
<tr>
<td>ADFI, g</td>
<td>977.6</td>
<td>944.1</td>
<td>1014.8</td>
<td>42.04</td>
</tr>
<tr>
<td>G:F</td>
<td>0.59</td>
<td>0.56</td>
<td>0.58</td>
<td>0.016</td>
</tr>
<tr>
<td>Overall, d 0-33</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADG, g</td>
<td>355.7</td>
<td>357.9</td>
<td>383.1</td>
<td>18.12</td>
</tr>
<tr>
<td>ADFI, g</td>
<td>588.1</td>
<td>596.9</td>
<td>613.3</td>
<td>24.79</td>
</tr>
<tr>
<td>G:F</td>
<td>0.61</td>
<td>0.60</td>
<td>0.62</td>
<td>0.016</td>
</tr>
</tbody>
</table>

ᵃᵇMeans (n = 5) within row with different superscript differ $P < 0.05$.
ᶜᵈMeans (n = 5) within row with different superscript differ $P < 0.10$. 
Table 5. Effect of level and source of dietary copper on plasma Cu, holo-ceruloplasmin, and bile Cu concentrations in weanling pigs

<table>
<thead>
<tr>
<th>Dietary Treatment</th>
<th>Control</th>
<th>CuSO₄</th>
<th>Cu₂(OH)₃Cl</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Cu, mg/L</td>
<td>2.04ᵃ</td>
<td>2.33ᵇ</td>
<td>2.28ᵇ</td>
<td>0.051</td>
</tr>
<tr>
<td>Holo-Cp, mg/100 mL</td>
<td>79.2</td>
<td>83.2ᵇ</td>
<td>78.6</td>
<td>3.966</td>
</tr>
<tr>
<td>Bile Cu, mg/L</td>
<td>0.65ᵃ</td>
<td>3.21ᵇ</td>
<td>4.24ᶜ</td>
<td>0.285</td>
</tr>
</tbody>
</table>

ᵃᵇMeans (n = 5) within row with different superscript differ P < 0.05.
Table 6. Effect of level and source of dietary copper on liver mineral concentrations in weanling pigs\(^1\)

<table>
<thead>
<tr>
<th></th>
<th>Dietary Treatment</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>CuSO(_4)</td>
<td>Cu(_2)(OH)_3Cl</td>
<td>SEM</td>
</tr>
<tr>
<td>Cu</td>
<td>34.9(^a)</td>
<td>268.2(^b)</td>
<td>188.6(^{a,b})</td>
<td>49.21</td>
</tr>
<tr>
<td>Zn</td>
<td>206.9(^c)</td>
<td>252.8(^d)</td>
<td>237.8(^{c,d})</td>
<td>15.27</td>
</tr>
<tr>
<td>Fe</td>
<td>456.9(^a)</td>
<td>421.9(^{a,b})</td>
<td>321.4(^b)</td>
<td>38.58</td>
</tr>
</tbody>
</table>

\(^{a,b}\)Means (n = 5) within row with different superscript differ \(P < 0.05\).

\(^{c,d}\)Means (n = 5) within row with different superscript differ \(P < 0.10\).

\(^1\)Means expressed as mg/kg DM.
Table 7. Effect of level and source of dietary copper on duodenal malondialdehyde and hepatic total glutathione concentrations in weanling pigs

<table>
<thead>
<tr>
<th></th>
<th>Dietary Treatment</th>
<th></th>
<th></th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>CuSO₄</td>
<td>Cu₂(OH)₃Cl</td>
<td></td>
</tr>
<tr>
<td>MDA/mg protein</td>
<td>0.33ᵃ</td>
<td>0.57ᵇ</td>
<td>0.4⁹ᵇ</td>
<td>0.06₁</td>
</tr>
<tr>
<td>Total GSH, pmole/well</td>
<td>6722ᵃ</td>
<td>5688ᵇ</td>
<td>6034ᵇ</td>
<td>258.9</td>
</tr>
</tbody>
</table>

ᵃᵇMeans (n = 5) within row with different superscript differ P < 0.05.
Table 8. Effect of level and source of dietary Cu on mRNA expression of Cu regulatory proteins in liver of weanling pigs

<table>
<thead>
<tr>
<th>Dietary Treatment</th>
<th>Control</th>
<th>CuSO₄</th>
<th>Cu₂(OH)₃Cl</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl1</td>
<td>0.89</td>
<td>1.50</td>
<td>1.46</td>
<td>0.273</td>
</tr>
<tr>
<td>Atox1</td>
<td>0.90ᵃ,ᶜ</td>
<td>1.78ᵈ</td>
<td>1.86ᵇ</td>
<td>0.272</td>
</tr>
<tr>
<td>Atp7a</td>
<td>1.04</td>
<td>1.22</td>
<td>1.06</td>
<td>0.148</td>
</tr>
<tr>
<td>Atp7b</td>
<td>1.52ᶜ</td>
<td>0.91</td>
<td>0.89ᵈ</td>
<td>0.219</td>
</tr>
<tr>
<td>Commd1</td>
<td>1.36ᶜ</td>
<td>0.96ᵈ</td>
<td>0.93ᵈ</td>
<td>0.137</td>
</tr>
<tr>
<td>Cp</td>
<td>1.47ᵃ</td>
<td>1.09</td>
<td>0.77ᵇ</td>
<td>0.186</td>
</tr>
<tr>
<td>Cox17</td>
<td>1.07ᵃ,ᶜ</td>
<td>0.81ᵇ</td>
<td>0.90ᵈ</td>
<td>0.050</td>
</tr>
<tr>
<td>Ccs</td>
<td>1.12</td>
<td>1.15</td>
<td>0.94</td>
<td>0.140</td>
</tr>
<tr>
<td>Sod1</td>
<td>1.24</td>
<td>1.03</td>
<td>0.79</td>
<td>0.160</td>
</tr>
<tr>
<td>Dmt1</td>
<td>1.31ᶜ</td>
<td>0.85ᵈ</td>
<td>0.97</td>
<td>0.151</td>
</tr>
<tr>
<td>Mt1a</td>
<td>1.01ᶜ</td>
<td>5.14ᵈ</td>
<td>6.23ᵈ</td>
<td>1.557</td>
</tr>
</tbody>
</table>

Means are relative expression of target gene normalization to geometric mean of the housekeeping genes ribosomal protein L4 (Rpl4), TATA box binding protein (Tbp), hypoxanthine phosphoribosyltransferase 1 (Hprt1), and beta-actin (β-actin).

ᵃᵇ Mean (n = 5) in a row without common superscript differ P < 0.05.
ᶜᵈ Mean (n = 5) in a row without common superscript differ P < 0.10.
Figure 1. Effects of level and source of dietary Cu on villus height in the duodenum (Duod), proximal jejunum (ProxJej), and ileum of weanling pigs. A) Values are means (n = 5) ± SEM expressed as microns. a,b Means within section without common letter differ $P < 0.05$. B) Selected images from duodenum of a control, CuSO$_4$-supplemented, and Cu$_2$(OH)$_3$Cl-supplemented pig to illustrate changes in villus height due to level and source of dietary Cu. Arrows indicate intestinal villi.
CHAPTER 6

Dietary iron modulates mechanisms involved in copper acquisition and utilization in porcine liver

R.S. Fry, J.W. Spears*, S.L. Hansen¹, and M.S. Ashwell

Department of Animal Science, North Carolina State University, Raleigh, NC, USA 27695

*Corresponding author: Telephone; 919-515-4008. Fax; 919-515-4463. (J.W. Spears)
Abstract

It has been well documented that the metabolism of iron (Fe) and copper (Cu) are closely related. The effect of dietary Fe on Cu transporters and chaperones has not been evaluated in the porcine model, nor have these mechanisms been identified in the pig. Thus, weanling male pigs were used to determine the effect of dietary Fe on mRNA expression of duodenal and hepatic Cu transporters and chaperones. For 33 days pigs received diets that were deficient (20 mg Fe/kg), adequate (120 mg Fe/kg; control), or high (520 mg Fe/kg) in Fe. Increasing dietary Fe increased Fe concentrations in proximal duodenum and liver. Liver Cu was higher in Fe-deficient and high-Fe pigs than in control pigs, but duodenal Cu did not differ among treatments. Expression of Atp7b, the Cu-ATPase involved in secretion of Cu from liver, was down regulated in Fe-deficient and high-Fe pigs relative to controls. Additionally, copper transporter 1 (Ctr1) and ceruloplasmin (Cp) were up-regulated in liver of Fe-deficient vs. control pigs. Minimal effects of dietary Fe on cellular Cu metabolism were noted in the duodenum. This study characterized Cu transporters and chaperones for the first time in the porcine model, which closely resembles the digestive physiology and morphology of humans, to provide a better understanding of the interactions that occur at the cellular level between Fe and Cu.

Introduction

Iron (Fe) deficiency is the most prevalent mineral deficiency in the world in humans and is the primary cause of anemia (Baltussen et al., 2004). On the other hand, individuals suffering from Fe overload, such as aceruloplasminemia, progressively accumulate Fe in many of their tissues (Hellman and Gitlin, 2002). The metabolism of the essential trace
elements Fe and copper (Cu) are intimately related (Fox, 2003). To date only one clear
relationship exists at the cellular level between these elements as the multicopper
ferroxidases, hephaestin (Hp) and ceruloplasmin (Cp) are needed for proper Fe export in the
intestine and liver, respectively (Sharp, 2004).

Both Fe and Cu must be tightly regulated intracellularly to prevent toxicity as well as
deficiency (Kim et al., 2008). The high affinity Cu transporter, copper transporter 1 (Ctr1) is
responsible for Cu uptake in intestinal epithelium and liver (Nose et al., 2006; Kim et al.,
2009). Once Cu enters the cell, the ion must be bound to one of several chaperones to prevent
toxicity. Cytochrome c oxidase assembly protein 17 (Cox17) delivers Cu to the mitochondria
for Cu incorporation into cytochrome c oxidase, the terminal enzyme in the electron transport
chain. Another key chaperone, CCS, delivers Cu to superoxide dismutase (Sod1), an
important component in the oxidative defense system. Another option for intracellular Cu is
to be sequestered in the intestine or stored in the liver by metallothionein 1 (Mt1).
Antioxidant 1 (Atox1) is a key component to the secretory pathway and delivers Cu to the
Cu-ATPases, Atp7a (intestine) and Atp7b (liver) located in the trans golgi network.
Basolateral Cu export from the enterocyte is carried out via Atp7a, which is also involved in
incorporating Cu into cuproenzymes. Menkes patients have a mutated $Atp7a$ and, as a result,
these individuals are Cu deficient and this disease often results in death (Cox and Moore,
2002).

One key difference in Fe and Cu metabolism is there is not a primary route of
excretion of absorbed Fe, whereas Cu is primarily excreted via the bile by Atp7b and
Commd1. Wilson disease patients have a mutated $Atp7b$ that results in hyperaccumulation of
liver Cu, resulting in decreased holo-Cp synthesis and liver damage due to lipid peroxidation (Kim et al., 2008). Bedlington terrier dogs have an inherent defect in Commd1, thus biliary Cu excretion is markedly reduced (Klomp et al., 2003). Dietary Fe has been shown to affect hepatic Cu metabolism. Biliary excretion of Cu decreased in a linear fashion in rats as dietary Fe increased from deficient to high (Yu et al., 1994). Furthermore, numerous studies have documented that Fe deficiency increases liver Cu concentrations (Lahey et al., 1952; Yu et al., 1994; Ravia et al. 2005) while high dietary Fe in rodents has minimal effects on liver Cu concentrations. To date it is unclear the mechanisms responsible for these changes in Cu metabolism when subjects consume diets deficient or high in Fe.

Studies have evaluated the effect of dietary Fe on some of the aforementioned Cu transporters and chaperones in cell culture and rodents to elucidate the antagonistic effects of dietary Fe on Cu, but to date they have not been characterized in the pig. While rodents have been extensively used as a model for humans, a recent review by Patterson et al. (2008) documented marked differences in digestive physiology and morphology betweens rodents and humans. Furthermore, this review made a strong argument in favor of the porcine model over rodents in evaluating mineral transporters for application to trace mineral metabolism in humans as the physiology of the pig more closely resembles the human. The objective of the present study was to characterize Cu transporters and chaperones in porcine duodenum and liver and to determine the effects of dietary Fe on mechanisms involved in Cu acquisition, distribution, and utilization in these tissues.
Materials and methods

Animals and diets

Experimental sampling procedures, care, and handling of animals were approved by the North Carolina State University Animal Care and Use Committee. Weanling male pigs (n = 24) from a previous study (Hansen et al., 2009) evaluating the effects of dietary iron (Fe) on Fe transporters and manganese metabolism were further utilized to characterize and evaluate cellular Cu metabolism in these animals. Briefly, at 21 days of age, pigs began receiving dietary treatments containing: 1) 20 mg Fe/kg (Fe-deficient), 2) 120 mg Fe/kg (control), or 3) 520 mg Fe/kg (high-Fe). Dietary Fe was provided as FeSO₄ and diets were formulated to meet or exceed NRC (1998) dietary requirements with the exception of Fe.

Sample collection, RNA isolation, and Quantitative RT-PCR

Briefly, animals received dietary treatments for 33, 34, 35, or 36 days, in which two pigs per treatment were harvested on each of these days. Duodenal mucosa and liver samples were obtained from each pig and snap frozen in liquid nitrogen and stored at -80°C until subsequent isolation. Further detail in regard to sampling is described elsewhere (Hansen et al., 2009).

Total RNA was isolated using the RNeasy Mini kit with an on-column DNase digestion as per manufacturer’s instruction (Qiagen). One microgram of total RNA was used to synthesize complementary DNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc., Foster City, CA, USA) following manufacturer’s instructions.

Primers for target genes were designed to be compatible with SYBR Green I using Beacon Designer software (Premier Biosoft Intl., Palo Alto, CA, USA; Supplemental Table).
Specificity of each PCR reaction was assessed using melt curves and one amplicon from each primer pair was sequenced to confirm the identity of the PCR product. Nygard et al. (2007) assessed numerous candidate housekeeping genes throughout many porcine tissues. Thus, we utilized this panel of housekeeping genes to assess the most stable housekeeping gene(s) in our experimental samples. In the duodenum, *Ywaz*, *Rpl4*, and *Tbp* were found to be the most stable (Supplemental Table). In the liver, *Rpl4*, *Ywaz*, *Tbp*, and *Hprt1* were most stable across dietary treatments (Supplemental Table). In each of these tissues the geometric mean of these housekeeping genes were calculated for target gene normalization. Relative expression of target genes was determined via the $2^{-\Delta \Delta Ct}$ method (Lavak and Schmittgen, 2001).

**Statistical Analysis**

Relative expression means of target genes in duodenum and liver were compared using one-way ANOVA using the MIXED procedure in SAS (SAS Institute, Inc.). Treatment effects were considered significant at $P \leq 0.05$ and tendencies will be discussed at $P \leq 0.10$. Means are shown with standard errors of the mean (SEM).

**Results**

**Tissue mineral concentrations**

Duodenal Fe concentrations were 108, 624, and 1240 mg Fe/kg DM for Fe-deficient, controls, and high-Fe pigs, respectively. Duodenal Cu concentrations were similar between dietary treatments (12.5, 12.4, and 13.5 mg Cu/kg DM for Fe-deficient, controls, and high-Fe pigs, respectively). Liver Fe concentrations increased linearly and were 72, 228, and 411 mg Fe/kg DM for Fe-deficient, controls, and high-Fe pigs, respectively. Liver Cu concentrations
were higher in Fe-deficient (35.3 mg Cu/kg DM) and high-Fe (36.7 mg Cu/kg DM) pigs than in controls (25.4 mg Cu/kg DM). These data are in a previous report from our laboratory (Hansen et al., 2009).

**Duodenal mRNA expression profiles**

Duodenal *Atox1* tended (*P* = 0.10) to be higher in Fe-deficient vs. control pigs (Figure 1). Transcript levels of *Ctr1* (Figure 1), *Mt1a*, or *Atp7a* (Figure 2) were not affected by dietary Fe. Additionally, *Cox17*, *CCS*, *Sod1*, and *Atp7b* were not affected by dietary treatment in porcine duodenum (Supplemental Figures).

**Hepatic mRNA expression profiles**

*Ctr1* was 1.4 fold higher (*P* = 0.01) in Fe-deficient vs. control pigs (Figure 3). Expression of *Atp7b* was lower (*P* = 0.05) in Fe-deficient and high-Fe pigs vs. controls (Figure 4). *Cp* was differentially expressed (*P* < 0.001) among all dietary treatments (Figure 3). Iron-deficient pigs had 2.2 fold higher (*P* < 0.001) *Cp* expression than their control counterparts. *Cp* was 1.6 fold higher in (*P* = 0.01) high-Fe vs. controls, and was 1.3 fold higher (*P* = 0.02) in Fe-deficient compared with high-Fe pigs. *Commd1* expression across dietary treatments followed a similar pattern to that of *Atp7b*, but only tended (*P* = 0.09) to be lower in Fe-deficient vs. control pigs (Figure 4). Transcript levels of *Atox1*, *Cox17*, *CCS*, *Sod1*, *Mt1a*, and *Atp7a* were not affected by dietary Fe (Supplemental Figures).

**Discussion**

Some of the first studies evaluating routes for mammalian Cu excretion, as well as metabolic interactions between Fe and Cu, were conducted in pigs (Gubler et al., 1952; Lahey et al., 1952; Mahoney et al., 1955). In fact, the role of Cp in Fe metabolism was
characterized in the porcine model (Roeser et al., 1970). The present study demonstrates for the first time the presence of Cu transporters and chaperones in porcine duodenal mucosa and liver. Characterization of these molecular mechanisms in the porcine model is significant in the realm of human nutrition, as Patterson et al. (2008) recently provided an extensive review indicating that the pig more closely resembles the physiology and morphology of the human gastrointestinal tract than do rodents.

Our data suggests that dietary Fe has minimal effects on Cu homeostasis in porcine duodenum. In contrast, Ravia et al. (2005) reported that duodenal Cu was higher, while Collins et al. (2005, 2009) found greater abundances of Ctr1, Atp7a, and Mt1a in rats fed a diet severely deficient in Fe than in controls receiving 198 mg Fe/kg diet. Species differences (pigs vs. rats) may have contributed to these discrepancies; however, other differences between the present study and previous studies in rats may also explain the discrepancies. In the rodent studies, diets were extremely low (3 mg Fe/kg) in Fe relative to requirements (NRLA, 1995) and rats were very anemic (Ravia et al., 2005). Iron deficiency in the present study, and thus the degree of anemia was less severe and more similar to that typically observed in humans (Looker et al., 1997; Totin et al., 2002). Another key difference between the rat studies and the present pig study relate to the complexity of the diets. Rats were fed a semi-purified diet containing negligible phytate. Pigs in our study were fed a more complex diet that contained the Fe-antagonist, phytate (in corn), that can form stable complexes with Fe and reduce its absorption (Hurrell and Egli, 2010). If more Fe is bound to phytate, the negative effect of Fe on Cu absorption is reduced (Wapnir, 1998).

This is the first work to demonstrate that dietary Fe may affect Atox1 mRNA in vivo.
Duodenal *Atox1* mRNA tended to be up-regulated in Fe-deficient pigs. This may have occurred in response to the lower duodenal Fe concentrations and Fe indices in Fe-deficient pigs compared to controls (Hansen et al., 2009). Duodenal Cu concentrations of pigs used in the present study were not affected by dietary Fe (Hansen et al., 2009), suggesting that *Atox1* mRNA increased in response to the Fe status of Fe-deficient pigs and not their Cu status. Moreover, Hansen et al. (2009) reported that Hp protein expression was lower in Fe-deficient pigs compared to controls suggesting that ATOX1 may be a link between Cu and Fe metabolism.

Growth of pigs fed the Fe-deficient diet was reduced and by day 32, membranes of these pigs were pale white and hemoglobin values were low (≤ 70 g/L; Hansen et al., 2009), indicating that these animals were anemic and possibly hypoxic (NRC, 1998; Widmaier et al., 2004). Hepcidin mRNA was markedly lower and ferroportin mRNA was up-regulated in the liver of Fe-deficient pigs (Hansen et al., 2009), which are cellular events that occur due to increased hypoxia inducible factor 1-α (HIF-1α) activity during a hypoxic state (Peyssonnaux et al., 2007). Iron deficiency induces HIF-1α (Mukhopadhyay et al., 2000) and research in cell culture and rodents has demonstrated increased expression of *Cp* mRNA and *Ctr1* protein as a result (Martin et al., 2005; White et al., 2009). Although changes in liver mRNA likely did not occur solely as a result of hypoxia, it is important to be mindful of the effects hypoxia can have on *Ctr1* and *Cp* expression.

This is the first demonstration of increased *Ctr1* mRNA expression in liver of Fe-deprived animals. Collins et al. (2009) reported that *Ctr1* was up-regulated in the duodenum of Fe-deficient rats and work in macrophages has shown that *Ctr1* protein is increased by
hypoxia with a concomitant increase in Cu uptake (White et al., 2009). Thus, it seems plausible that up-regulation of hepatic Ctr1 contributed to the increase in liver Cu concentrations in Fe-deficient compared with control pigs. Furthermore, up-regulation of Ctr1 in Fe-deficient pigs may explain the higher expression of Cp mRNA, as greater uptake of Cu would allow more Cu to be delivered for Cp synthesis. This likely occurred in response to the low liver Fe concentrations of these pigs (Hansen et al., 2009), as Cp is involved in the oxidation of Fe^{2+} to Fe^{3+} for proper Fe transport (Roeser et al., 1970). Our porcine Cp mRNA results are in contrast to the report by Tran et al. (2002) in which Cp mRNA was not affected when rats received diets deficient or high in Fe. In the present study, up-regulation of Cp mRNA in high-Fe pigs relative to controls may have occurred in response to inflammation as a result of accumulated hepatic Fe. Intracellular Fe is actively involved in production of hydroxyl radicals which can cause tissue damage and inflammation (Hallimell and Gutteridge, 1990). Ceruloplasmin is an acute phase protein that combats oxidative damage (Fox et al., 2000), and a previous report by Gitlin (1988) illustrated that Cp mRNA increases in response to inflammation in hepatocytes.

This is also the first report to demonstrate that dietary Fe alters Atp7b expression and that dietary Fe may affect Commd1 in vivo. Transcript levels of Atp7b were lower in high-Fe pigs compared to controls and may explain their increased liver Cu. Although bile Cu was not examined in high-Fe pigs, it is possible that Atp7b down-regulated as a means to decrease biliary Cu efflux. Rats supplemented with high-Fe had lower bile Cu concentrations than controls (Yu et al., 1994). In the present study, down-regulation of Atp7b and a tendency for Commd1 to be lower in Fe-deficient pigs can explain their increased liver Cu relative to
controls. The increase in liver Cu in Fe-deficient pigs is in agreement with data in Fe-deprived rats (Yu et al., 1994). Recently, Ravia et al. (2005) reported that liver Cu was markedly higher (~6 fold on average) in Fe-deficient rats compared to controls, but Atp7b expression was not affected. Liver Cu in the present study was only 1.4 fold higher in Fe-deficient and in high-Fe pigs, illustrating a robust response by Atp7b mRNA to changes in liver Cu homeostasis. As demonstrated in the Bedlington terrier, a function of Commd1 is to excrete Cu via the bile. Although bile Cu concentrations were not determined in the present study, modulation of Atp7b and Commd1 suggests that bile Cu concentrations may have been lower in Fe-deficient pigs relative to controls. Moreover changes in transcription may have occurred to provide protection from excessive Cu efflux via the bile, allowing for more Cu incorporation into Cp. Markedly increased Cp mRNA in Fe-deficient pigs in the present study agrees with this theory.

Prior to this study no attention had been garnered to the porcine model for evaluating Cu transporters and chaperones to better understanding Cu homeostasis in human nutrition and health. Our data not only characterizes for the first time Cu transporters and chaperones in the porcine model, but also provides novel information in regard to Fe and Cu interactions at the cellular level in a model that closely resembles the physiology of humans. For example, pigs have a gallbladder, while rats do not, and it is unknown what affect the presence of a gallbladder has on these mechanisms involved in Cu metabolism as bile is the major route of Cu excretion. We conclude that when a complex diet (non-purified) is fed to deliver varying levels of dietary Fe there are minimal effects on cellular Cu metabolism in porcine duodenum and much greater changes in the liver. Our data suggest that with
relatively small changes in liver Cu concentrations, mRNA expression of key components in
the secretory pathway of Cu metabolism modulate to maintain liver Cu within normal
concentrations found in the pig (Armstrong et al., 2000).

References

copper source (cupric citrate vs. cupric sulfate) and level on growth performance and copper

Baltussen, R., C. Knai, and M. Sharan. 2004. Iron fortification and iron supplementation are
cost-effective interventions to reduce iron deficiency in four subregions of the world. J. Nutr.
134: 2678-2684.

differentially expressed genes in response to dietary iron deprivation in rat duodenum. Am. J.
Physiol. Gastrointest. Liver Physiol. 288: 964-971.

Liver Physiol. 297: 695-707.


16: 9-40.

Fox, P.L., B. Mazumder, E. Ehrenwald, and C.K. Mukhopadhyay. 2000. Ceruloplasmin and

Gitlin, J.D. 1988. Transcriptional regulation of ceruloplasmin gene expression during

on copper metabolism. III. The metabolism of iron in copper deficient swine. Blood 7: 1075-
1092.

Halliwell, B. and J.M.C. Gutteridge. 1990. Role of free radicals and catalytic metal ions in


Nose, Y., B.E. Kim, and D.J. Thiele. 2006. Ctrl1 drives intestinal copper absorption and is essential for growth, iron metabolism, and neonatal cardiac function. Cell Metab. 4: 235-244.


Figure 1. Relative expression of duodenal *Ctr1* and *Atox1* in weanling male pigs normalized to the geometric mean of *Rpl4*, *Ywaz*, and *Tbp*. Samples were assayed in duplicate and values are expressed as mean relative expression ± SEM. *Atox1*; Fe-deficient vs. Control $P = 0.10$. 

![Graph showing relative expression of Ctr1 and Atox1](image-url)
Figure 2. Relative expression of duodenal *Atp7a* and *Mt1a* in weanling male pigs normalized to the geometric mean of *Rpl4*, *Ywaz*, and *Tbp*. Samples were assayed in duplicate and values are expressed as mean relative expression ± SEM.
Figure 3. Relative expression of hepatic *Ctr1* and *Cp* in weanling male pigs normalized to the geometric mean of *Rpl4, Hprt1, Ywaz,* and *Tbp.* Samples were assayed in duplicate and values are expressed as mean relative expression ± SEM. *Ctr1;* Fe-deficient vs. Control, *P* = 0.01. *Cp;* Fe-deficient vs. Control, *P* < 0.0001; High-Fe vs. Control, *P* = 0.01; Fe-deficient vs. High-Fe, *P* = 0.02.
Figure 4. Relative expression of hepatic *Atp7b* and *Commd1* in weanling male pigs normalized to the geometric mean of *Rpl4*, *Hprt1*, *Ywaz*, and *Tbp*. Samples were assayed in duplicate and values are expressed as mean relative expression ± SEM. *Atp7b*; Fe-deficient and High-Fe vs. Control, *P* = 0.05. *Commd1*; Fe-deficient vs. Control, *P* = 0.09.
## Supplemental table 1. Real-time polymerase chain reaction primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Accession #</th>
<th>Primer sequence (5' - 3')‡</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ywaz†,*</td>
<td>DO845179</td>
<td>F: TGATGATAAAGAAAGGATGGG R: GATGGGATGTCATTAC 203</td>
<td></td>
</tr>
<tr>
<td>Rpl4†,*</td>
<td>DO845176</td>
<td>F: CAAGAGTAATACCACTTC R: GAACTCTACGGATGAATTC 122</td>
<td></td>
</tr>
<tr>
<td>Tbp†,*</td>
<td>DO845178</td>
<td>F: AGACATCGATGTTATGAGCCAGA R: AGATTTCCTCAACGGCTC 153</td>
<td></td>
</tr>
<tr>
<td>Hprt1†</td>
<td>DO845175</td>
<td>F: GACTGAAATCATGTGTTGT R: CAGATGTTTCCAACACTCAAC 91</td>
<td></td>
</tr>
<tr>
<td>Ctr1†,*</td>
<td>NM214100.2</td>
<td>F: ATGATGATGGCTATGACC R: GATGCTGACTTGGGACTTG 150</td>
<td></td>
</tr>
<tr>
<td>Atox1†,*</td>
<td>NM001167641.1</td>
<td>F: CCGAAGCACAGAGTTCCTCC R: TGTTGGCACCGTCAATGTC 109</td>
<td></td>
</tr>
<tr>
<td>Cox17†,*</td>
<td>NM001190922.1</td>
<td>F: GAGCAGCTGTGGGACACCTAATTGAG R: TCACAACGCAGACCACCACTTC 86</td>
<td></td>
</tr>
<tr>
<td>Ccs†,*</td>
<td>NM001001866.1</td>
<td>F: TCTTCAGAGATTGGGATGGATG R: CGTGACTTGGGATAAGG 116</td>
<td></td>
</tr>
<tr>
<td>Sod1†,*</td>
<td>NM001190422.1</td>
<td>F: GAGAGACTGGGCAATGTG R: TCTGGTCTCCACCTTCTGG 160</td>
<td></td>
</tr>
<tr>
<td>Atp7a†,*</td>
<td>AB271958.1</td>
<td>F: AAGGAGGAGACCAAGACCTTCAC R: CGGATTAACCTGCTATCATCAAG 200</td>
<td></td>
</tr>
<tr>
<td>Atp7b†,*</td>
<td>XM001925351.1</td>
<td>F: TCACCTAGGAACCTGGGAAG R: ATGGGGTCCTTGGGACATC 148</td>
<td></td>
</tr>
<tr>
<td>Commd1†</td>
<td>TC315951</td>
<td>F: TGGACTGAAAGGCTGATCC R: CTTCAGCAACTCCATCTCAAC 100</td>
<td></td>
</tr>
<tr>
<td>Cp†</td>
<td>XM001925237.1</td>
<td>F: TCTGTGGTGGATGAAAATCTC R: CGCGGTAGATTTATTAGC 267</td>
<td></td>
</tr>
<tr>
<td>Mt1a†,*</td>
<td>NM001001266.1</td>
<td>F: TCCTGCTCCACTGGAAG R: AAATACACACTTCTCCAAAC 65</td>
<td></td>
</tr>
</tbody>
</table>

‡F: Forward; R: Reverse  
*Analyzed in duodenum; †Analyzed in liver
Supplementary Figure 1. Relative expression of duodenal \textit{Cox17} and \textit{Atp7b} in weanling male pigs normalized to the geometric mean of \textit{Rpl4}, \textit{Ywaz}, and \textit{Tbp}. Samples were assayed in duplicate and values are expressed as mean relative expression ± SEM.

![Graph showing relative expression of Cox17 and Atp7b in different Fe conditions]
Supplementary Figure 2. Relative expression of duodenal *Ccs* and *Sod1* in weanling male pigs normalized to the geometric mean of *Rpl4*, *Ywaz*, and *Tbp*. Samples were assayed in duplicate and values are expressed as mean relative expression ± SEM.
Supplementary Figure 3. Relative expression of hepatic *Ccs* and *Sod1* in weanling male pigs normalized to the geometric mean of *Rpl4*, *Hprt1*, *Ywaz*, and *Tbp*. Samples were assayed in duplicate and values are expressed as mean relative expression ± SEM.
Supplementary Figure 4. Relative expression of hepatic Cox17 in weanling male pigs normalized to the geometric mean of Rpl4, Hprt1, Ywaz, and Tbp. Samples were assayed in duplicate and values are expressed as mean relative expression ± SEM.
Supplementary Figure 5. Relative expression of hepatic Mt1a in weanling male pigs normalized to the geometric mean of Rpl4, Hprt1, Ywaz, and Tbp. Samples were assayed in duplicate and values are expressed as mean relative expression ± SEM.
Supplementary Figure 6. Relative expression of hepatic *Atox1* and *Atp7a* in weanling male pigs normalized to the geometric mean of *Rpl4*, *Hprt1*, *Ywaz*, and *Tbp*. Samples were assayed in duplicate and values are expressed as mean relative expression ± SEM.
CHAPTER 7

High dietary iron does not antagonistically affect expression of duodenal or hepatic Copper Transporter 1, but increases copper efflux and hepatic \textit{Atp7b} mRNA expression in the young ruminant

R.S. Fry, M.S. Ashwell, S.L. Hansen, and J.W. Spears*
Department of Animal Science, North Carolina State University, Raleigh, NC, USA

*Corresponding Author: Campus Box 7621, Raleigh, NC 27695; jerry_spears@ncsu.edu
Abstract

An experiment was conducted to determine the effects of high dietary iron (Fe) on molecular mechanisms involved in copper (Cu) homeostasis in bovine duodenum and liver. Fourteen Holstein bull calves (intact males) were fed a control diet, adequate in Fe (50 mg Fe/kg DM) or the control diet supplemented with 750 mg Fe/kg diet (high Fe diet). Jugular blood samples were obtained on days 0, 35, and 56 for plasma Cu analysis and determination of ceruloplasmin (Cp) activity. On days 57 and 58 of the study, calves were euthanatized and tissues were collected for mineral analysis and analysis of mRNA and protein of Cu transporters and chaperones. By day 35, plasma Cu and Cp was higher in high Fe vs. control calves and remained statistically higher for the duration of the study. Duodenal and heart Cu was not affected by treatment, but liver Cu tended to be lower in high Fe calves. Protein and mRNA expression of duodenal and liver Ctr1 was not affected by dietary Fe. As determined by quantitative real-time PCR, duodenal Cox17 and hepatic Atp7b mRNA were higher in high Fe vs. controls. In conclusion, it is likely that increases in plasma Cu and Cp are a result of increased Atp7b mRNA expression. These data suggest that high dietary Fe decreases liver Cu by increasing Cu efflux from the liver. Furthermore, the increase in Cox17 mRNA in high Fe calves likely occurred in response to oxidative stress, but a definitive explanation is unknown.

Introduction

Copper (Cu) deficiency is a problem in ruminant diets and many feedstuffs consumed by ruminants (i.e. alfalfa, soyhulls, and corn silage; NRC, 1996; Kerr et al., 2008) often contain high levels of iron (Fe), which can greatly affect Cu status. In cattle, liver Cu
(Humphries et al., 1983) and plasma Cu (Gengelbach et al., 1994) were reduced by
superfluous amounts of dietary Fe to concentrations below thresholds indicative of Cu
deficiency (20 mg Cu/kg of liver dry matter (DM) and 0.5 mg/L of plasma; Underwood and
Suttle, 1999).

Free or unbound Cu ions are toxic to the cell and numerous Cu transport and
chaperone proteins have been identified in model species that are essential for proper Cu
homeostasis. The high affinity copper transporter, copper transporter1 (CTR1) is essential for
proper Cu uptake (Nose et al., 2006; Kim et al., 2009; Kim et al., 2010), thus it is plausible
that excess dietary Fe could hinder Cu uptake via this transporter. Proper Cu export relies on
functional Cu-ATPases in the enterocyte (ATP7A) and liver (ATP7B). These export proteins
receive Cu\(^{+}\) from the Cu chaperone, antioxidant 1 (ATOX1). Humans with a mutated Atp7a
suffer from Menkes’ disease, a state of deficiency, in which basolateral Cu efflux and
synthesis of cuproenzymes are markedly hindered. Wilson’s disease patients suffer from Cu
toxicity as a result of a mutated Atp7b, in which Cu incorporation into ceruloplasmin (Cp)
and biliary excretion of Cu are markedly decreased. Each of the aforementioned molecules
makes up the secretory pathway of Cu metabolism, and it is plausible that Cu metabolism
could be affected by high Fe by increasing efflux from the liver.

We have previously characterized many of the mechanisms involved in Cu transport
and utilization in bovine tissues during a state of Cu deficiency (Fry et al., 2009). However,
the molecular mechanisms involved in the interactions between Cu and Fe have not been
evaluated in ruminants. Therefore, a study was conducted to determine the effect of high
dietary Fe on mechanisms involved in Cu acquisition, distribution, and utilization in bovine

duodenum and liver to provide a better understanding of how Fe antagonistically affects Cu metabolism.

Materials and Methods

Animals and Experimental Design

Prior to initiation of the study, animal care, handling, and sampling procedures were approved by the North Carolina State University Animal Care and Use Committee. Experimental design and animal handling has been described in detail elsewhere (Hansen et al., 2010). Briefly, fourteen Holstein bull (intact males) calves of 1 to 7 days of age were placed in individual calf hutches and received whole milk until 8 weeks of age. During this period, calves were fed a starter diet ad libitum (Table 1). One week prior to weaning, calves were placed into individual pens (~ 3 m²). At weaning, calves were then randomly allocated to one of two dietary treatments: 1) control diet adequate in Fe (50 mg Fe/kg DM) or 2) control diet supplemented with 750 mg Fe/kg DM (high Fe). Iron was provided as ferrous sulfate (FeSO₄). All diets were formulated to meet or exceed all NRC recommendations for growing calves (Table 1; NRC, 2001). Copper was supplemented as copper sulfate (CuSO₄), in combination with Cu naturally present in feed ingredients to meet the NRC requirement. Diets analyzed 17.2, 15.5, and 15.7 mg Cu/kg DM for the starter, grower 1, and grower 2 diets, respectively.

Sample Collection

Jugular blood samples were obtained from each calf on days 0, 35, and 56 for plasma Cu analysis and determination of Cp activity. Six calves per treatment group were selected at random to be humanely euthanized for tissue collection for Cu analysis and mRNA and
protein expression of Cu transporters and chaperones. On days 57 and 58, three calves per
treatment were sedated via an i.v. dose of xylazine (100 mg) and subsequently injected with
an i.v. overdose of sodium pentobarbital (Hansen et al., 2010). Mucosal scrapes were
conducted as described by Hansen et al. (2009), and removal and storage of duodenum, heart,
and liver for mineral analysis have been previously described (Hansen et al., 2010). Mucosal
scrapes and liver samples for mRNA and protein analysis were placed into a 50 mL tube
containing RNAlater preservative (Ambion) as per manufacturer's instructions for
subsequent analysis.

Analytical Procedures

Ceruloplasmin activity was determined using the methods described by Houchin et al.
(1958), which determines the amount of holo- (active) Cp present in circulation. Tissues and
feed samples for Cu analysis were dried and wet ashed via microwave digestion (Mars 5,
CEM Corp., Matthews, NC) as described by Gengelbach et al. (1994). Copper content in
feed samples and tissues were determined by flame atomic absorption spectroscopy
(Shimadzu Scientific Instruments, Japan), and a bovine liver standard was used to validate
instrumental accuracy.

Protein Extraction and Immunoblotting Procedures

Isolation of duodenal and liver proteins and SDS-PAGE analysis was conducted as
described by Hansen et al. (2009). Within each tissue homogenate, a pooled sample was
generated by obtaining equal molar proportions from each sample for normalization of
unknown samples. This pooled sample represented an experimental pool. Approximately 65
μg of duodenal protein and 81 μg of liver protein were loaded into sample wells. Samples for
both duodenal and liver CTR1 were heated for 10 min at 70°C prior to electrophoresis. After SDS-PAGE analysis, membranes were stained with Ponseau S Stain (Sigma) to determine equal loading. Membranes were then blocked for one hour at room temperature with tris-buffered saline (TBS; pH 7.4) containing 3% (w/v) bovine serum albumin (BSA-TBS). After blocking, membranes were probed for 1 h with a polyclonal rabbit anti-CTR1 at a dilution of 1:1,000 (vol/vol) in TBS containing 0.05% (vol/vol) tween-20 and 3% (wt/vol) BSA (BSA-TTBS; anti-CTR1 was kindly provided by Dr. Dennis J. Thiele). Subsequently, an anti-rabbit alkaline phosphatase-linked secondary antibody was diluted at 1:10,000 (vol/vol) in BSA-TTBS and incubated for 30 min. Membranes were briefly washed and incubated with Lumi-Phos™ WB substrate as per manufacturer’s instructions (Thermo Scientific) prior to autoradiography film exposure. Band densities on the captured film were detected using Image Quant TL software (Amersham Biosciences, Piscataway, NJ, USA). Experimental pool samples were run in duplicate on each gel, one at each end of the gel. Band densities of experimental replicates were averaged and expressed as a ratio to the mean density of the pooled samples within the sample gels. Thus results were expressed as a ratio to the experimental pool.

**Isolation of RNA and Quantitative Real-Time PCR**

Total RNA from duodenum and liver were isolated using the RNeasy Mini Kit as per manufacturer’s instructions with an on-column DNase digestion (Qiagen). Following isolation, quantity and integrity of RNA was determined via a Nanodrop-100 Spectrophotometer and agarose gel analysis. Real-time primers were designed to be compatible with SYBR Green I using Beacon Designer Software. Primer sequences for target
genes are indicated in Table 2. Ribosomal protein subunit 9 (Rps9) was utilized as an internal housekeeping as previous research in cattle (Janovick-Guretzky et al., 2007) has demonstrated that this gene is unaffected by nutritional manipulations and high dietary Fe (Hansen et al., 2010). Primer specificity was determined by generating melting curves for each PCR reaction.

One microgram of total RNA was used to synthesize complementary DNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Inc., Foster City, CA, USA) as per manufacturer’s instructions. Real-time PCR was performed as described in detail by Hansen et al. (2009). Relative expression of target genes was determined using the $2^{-\Delta\Delta C_t}$ method (Lavak and Schmittgen, 2001).

**Statistical Analysis**

All data was analyzed by ANOVA using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC). Day 0 was used as a covariate for day 35 and 56 Cp values. Animal was the experimental unit and significance was declared at $P \leq 0.05$ and tendencies are discussed at $P \leq 0.10$. To determine correlations between liver Cu and liver gene expression, PROC CORR in SAS was utilized.

**Results**

**Copper Status**

Plasma Cu and Cp activity were affected by treatment ($P < 0.001$; Table 3). By day 35 calves receiving high Fe had higher ($P = 0.04$) plasma Cu than controls, and on day 56 plasma Cu tended ($P = 0.07$) to be higher in high Fe vs. control calves. On day 0, Cp was significantly different between treatments and was used as a covariate for day 35 and 56 data.
Plasma Cp activity tended to be higher \((P = 0.06)\) on day 35 and was higher \((P = 0.05)\) on day 56 in high Fe vs. controls. After 56 days of receiving dietary treatments, Cu concentrations in duodenum and heart were not affected by treatment; however, liver Cu tended \((P = 0.10)\) to be lower in high Fe vs. controls (Table 4).

**Protein and mRNA expression of Cu transporters and chaperones**

Duodenal and hepatic CTR1 protein migrated to an apparent molecular weight of \(~30\) kDa, which agrees with data in rodents (Nose et al., 2006). Protein expression of CTR1 was not affected by high dietary Fe in either tissue (Figure 1) nor was *Ctr1* mRNA (Figure 1 & 2). In the duodenum, mRNA expression of *Atox1*, *Atp7a*, or *Atp7b* were not affected by high Fe. However, *Cox17* expression tended \((P = 0.06)\) to be higher in high Fe vs. control calves (Figure 2). In the liver, mRNA expression of *Atox1*, *Cox17*, *Sod1*, *Atp7a*, *Commd1*, *Mt1*, and *Cp* was not affected by high Fe. However, *Atp7b* expression was 1.9 fold higher \((P = 0.01)\) in calves fed high Fe diets compared to controls (Figure 3). Furthermore, expression of *Atp7b* tended \((P = 0.07)\) to be negatively correlated \((R^2 = -0.54)\) with liver Cu regardless of dietary treatment, but was not significantly correlated within dietary treatment.

**Discussion**

It has been well documented that high dietary Fe can greatly reduce Cu status in ruminants (Humphries et al., 1983; Gengelbach et al., 1994), but to date interactions at the cellular level between these ions has not garnered attention. Sources of excess dietary Fe include feedstuffs naturally high in Fe (i.e. corn silage, soyhulls, alfalfa; NRC, 1996; Kerr et al., 2008) and soil contamination, which most likely occurs during harvest. Recent work by Hansen and Spears (2009) demonstrated that soil contamination prior to the ensiling process
of corn silage resulted in markedly higher soluble Fe concentrations following \textit{in vitro}
simulated digestion. Ferrous sulfate was used as the source of supplemental Fe in the present
study and is readily available (Ammerman, 1976). Excess dietary Fe (600 mg Fe/kg DM; as
Fe carbonate) supplemented to calves reduced plasma Cu below levels indicative of Cu
deficiency (\(< 0.5 \mu\text{g/mL}; \) Gengelbach et al., 1994). Humphries et al. (1983) supplemented
saccharated Fe carbonate at 800 mg Fe/kg DM to calves and reported that liver Cu
concentrations decreased to concentrations less than 20 mg Cu/kg DM, a threshold used to
diagnosis Cu deficiency (Underwood and Suttle, 1999). While the aforementioned \textit{in vivo}
studies reported marked changes in Cu status, the possible mechanistic alterations
responsible for these metabolic changes are unknown.

As indicated by others (Humphries et al., 1983; Yu et al., 1994), two sites exist in
which Fe could have the most dramatic effect, at the site of absorption in the small intestine
or efflux from the liver. High dietary Fe in sheep (Suttle and Peter, 1985) and rats (Yu et al.,
1994) has decreased apparent Cu absorption. More recently, a molecular approach taken by
Lee et al. (2002) demonstrated that Hek-293 cells transfected with human CTR1 had lower
$^{64}\text{Cu}$ uptake when incubated with 50 molar excess of Fe. These authors suggested that high
Fe may be a reversible inhibitor of the human CTR1 protein. In the present study,
supplementing 750 mg Fe/kg DM did not affect duodenal Cu concentrations (11.7 vs. 11.3
mg Cu/kg DM); nor did it affect duodenal or hepatic CTR1 protein and mRNA expression.
Lack of an Fe affect on mRNA and protein expression of CTR1 suggests that high dietary Fe
did not antagonize or inhibit Cu metabolism at the level of absorption in the duodenum and
liver in the present study. When animals in the present study were harvested, a marked
darkening of the rumen epithelium was observed (Hansen et al., 2010) compared to control animals. This observation is in line with reports by others (Hamada et al., 1969; Standish et al., 1971; McGuire et al., 1985) when ruminants were fed high levels of Fe from FeSO₄ or consumed feedstuffs contaminated with soil. As speculated by Hansen et al. (2010), the rumen is likely providing a buffering effect thereby protecting the intestinal epithelium from high levels of soluble Fe, and may be the reason why CTR1 and even DMT1 were not affected by diet.

Liver Cu tended to be lower, while liver Fe was higher (152.9 vs. 116.8 mg Fe/kg DM; Hansen et al., 2010) in H-Fe calves compared to their control counterparts. An approximately 2 fold increase in mRNA expression of the hepatic Cu efflux pump, Atp7b in H-Fe calves likely explains the decrease in liver Cu. This hepatic Cu exporter is responsible for biliary Cu excretion and incorporation of Cu into the ferroxidase, Cp (Kim et al., 2008). To our knowledge this is the first study to demonstrate that high dietary Fe increases Atp7b mRNA in ruminants as well as in model species. We have recently documented that high dietary Fe increases porcine Atp7b mRNA with concomitant increases in biliary Cu (Fry et al., 2010). Biliary Cu concentrations were not determined in the present study. However, plasma Cu and Cp were higher or tended to be higher in H-Fe calves vs. controls, suggesting that high Fe increased hepatic Cu efflux. Lack of an Fe effect on Cp mRNA at the conclusion of the study suggests that high dietary Fe is affecting the activity of the enzyme and not its transcription. Tran et al. (2002) reported that supplementing excess dietary Fe to rats did not affect protein, mRNA, or oxidase (holo-Cp) activity of Cp. In the present study, regardless of diet, liver Cu and Atp7b mRNA tended to be negatively correlated. Along with ATP7B,
COMMD1 plays a critical role in proper biliary Cu excretion (Klomp et al., 2003). Commd1 mRNA was not affected by diet, suggesting that biliary Cu excretion may not be affected in H-Fe calves. Determination of biliary Cu and ATP7B and COMMD1 protein expression would provide more information in this regard.

Hepatic expression of Atox1, Cox17, Sod1, and Mt1 were not affected by high dietary Fe. Lack of an effect on Atox1 mRNA further demonstrates that in the present study high Fe is solely affecting transcription of Atp7b and not other components of the secretory pathway. SOD1 plays a role in scavenging superoxide ions and metallothionein (MT1) is the main Cu storage protein in the liver.

Liver Cu concentrations in calves in the present study were well above adequate (≥ 100 mg Cu/kg DM; Underwood and Suttle, 1999). Concentrations are relatively high because of the relatively young age of these animals (~4 months), as a significant amount of Cu accumulates in fetal liver during pregnancy to supply newborns with a source of Cu following parturition because milk is quite low in Cu (Underwood and Suttle, 1999). Relatively high liver Cu in the present study likely explains why plasma Cu and Cp were higher in H-Fe calves. Several studies have demonstrated that high dietary Fe markedly decreased plasma Cu and Cp (Standish et al., 1969; Standish et al., 1971; Humphries et al., 1983; Gengelbach et al., 1994), but these studies were conducted in cattle with lower liver Cu concentrations at the beginning of the trial and were fed high Fe diets for a longer time period. Humphries et al. (1983) depleted liver Cu to approximately 100 mg Cu/kg DM in young beef calves prior to feeding experimental diets and subsequently supplemented 800 mg Fe/kg DM. High Fe-supplemented calves had liver Cu concentrations below 20 mg Cu/kg
DM by day 56 of the study and by day 224, mean liver Cu was further reduced to 3.6 mg Cu/kg DM. These authors also reported that by day 112, Fe supplemented calves had plasma Cu and Cp values below thresholds indicative of Cu deficiency (0.5 µg/ml and 15 mg/100mL, respectively; Underwood and Suttle; 1999). Thus it seems very plausible that had our study continued for a longer time period, liver Cu in H-Fe calves would have likely been at or approaching concentrations indicative of Cu deficiency. Furthermore, plasma Cu and Cp would not have been increased by high dietary Fe; they would have very likely been lower.

COX17 is present in both the cytoplasm and the intermembrane space of the mitochondria and this chaperone is responsible for Cu delivery to the mitochondria for Cu incorporation into cytochrome c oxidase, the terminal enzyme in electron transport (Kim et al., 2008). It is unclear why an increase in duodenal Cox17 mRNA expression in H-Fe calves occurred. This is novel, as we are unaware of any published reports demonstrating modulation of Cox17 mRNA due to dietary Fe. However, unpublished work from our laboratory demonstrated that Cox17 mRNA is up-regulated in intestine, liver, and heart of pigs receiving high dietary Fe (797 mg Fe/kg; ~8 fold excess of Fe requirement) even though tissue Cu concentrations were not affected by dietary Fe (Fry et al., unpublished data). Very little published data exists evaluating dietary effects on expression of Cox17 in mammalian tissues. However, work in Arabidopsis has demonstrated that Cox17 mRNA increased followed induction of hydrogen peroxide (Balandin and Castresana, 2002). From animals used in the present study, Hansen et al. (2010) reported that gut permeability was higher in H-Fe calves as indicated by lower transepithelial resistance and higher mannitol flux. Iron is
known to be an oxidant stressor and concentrations of hydrogen peroxide along with other active oxygen species may have been higher in the duodenal mucosa of H-Fe calves, thus it is quite plausible that up-regulation of Cox17 mRNA occurred as a result of oxidative stress or tissue damage. Duodenal Atox1 and Atp7a mRNA were not affected by diet, suggesting that efflux of Cu from the enterocyte is not affected by high dietary Fe.

In conclusion, mucosa Cu concentrations were not affected in H-Fe calves, and supplementing dietary Fe in excess of 15 fold of their NRC requirement did not affect protein or mRNA expression of the high-affinity importer, Ctr1, in the duodenal mucosa or liver. The increase in Cox17 mRNA that occurred in H-Fe calves is novel and may have occurred in response to oxidative stress, as suggested by the impaired mucosa integrity in H-Fe calves reported by Hansen et al. (2010). Liver Cu tended to be lower, while plasma Cu and Cp were higher in H-Fe calves. These changes in Cu concentrations likely occurred as a result of increased Atp7b mRNA expression. Analysis of ATP7B protein expression and biliary Cu excretion, and even COMMD1 protein data would provide further information in regard to efflux, but at any rate these data suggest that high dietary Fe antagonizes Cu metabolism in ruminants with a relatively high Cu status by increasing efflux of Cu from the liver rather than decreasing intestinal absorption. It is unclear if an increase in Atp7b mRNA would have occurred had liver Cu concentrations been much lower. Additionally it is unknown if expression of Ctr1 would have been affected in these animals had their Cu status been lower. Further investigations in regard to Fe and Cu interactions at the cellular are warranted to provide a better understanding as to how Cu status and even species may affect modulation of Cu transporters and chaperones when animals receive superfluous dietary Fe.
References


Nose, Y., B.E. Kim, and D.J. Thiele. 2006. Ctr1 drives intestinal copper absorption and is essential for growth, iron metabolism, and neonatal cardiac function. Cell Metab. 4: 235-244.


Table 1. Ingredient composition of diets

<table>
<thead>
<tr>
<th>Ingredient, % DM</th>
<th>Starter</th>
<th>Grower 1</th>
<th>Grower 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cottonseed hulls</td>
<td>15.0</td>
<td>30.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Ground corn</td>
<td>57.1</td>
<td>49.0</td>
<td>47.8</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>24.0</td>
<td>13.0</td>
<td>15.8</td>
</tr>
<tr>
<td>Casein</td>
<td>--</td>
<td>1.75</td>
<td>--</td>
</tr>
<tr>
<td>Limestone</td>
<td>1.5</td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Rumensin(^2)</td>
<td>1.25</td>
<td>1.25</td>
<td>1.25</td>
</tr>
<tr>
<td>White salt</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Urea</td>
<td>--</td>
<td>0.45</td>
<td>0.6</td>
</tr>
<tr>
<td>Phosphoric acid(^3)</td>
<td>0.3</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Trace mineral premix(^4)</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Vitamin premix(^5)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Sucram(^6)</td>
<td>0.04</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Corn supplement(^7)</td>
<td>--</td>
<td>2.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

\(^1\)Starter diet was provided in addition to milk through weaning and had an analyzed content of 60 mg of Fe/kg dry matter; grower 1 diet had an analyzed Fe content of 67 mg of Fe/kg dry matter and was fed from day 0 to 38; grower 2 diet had an analyzed Fe content of 62 mg of Fe/kg dry matter and was fed from day 38 to 56.

\(^2\)Provided 275 mg of Rumensin per head per day (Rumensin for Dairy, Renaissance Nutrition, Roaring Spring, PA).

\(^3\)Food grade and provided by Potash Corp., Aurora, NC.

\(^4\)Starter provided per kilogram of diet: 40 mg of Zn as ZnSO\(_4\); 40 mg of Mn as MnSO\(_4\); 0.25 mg of I as Ca(IO\(_3\))\(_2\)(H\(_2\)O); 0.2 mg of Se as Na\(_2\)SeO\(_3\); and 0.1 mg of Co as CoCO\(_3\). Grower 1 and 2 provided per kilogram of diet: 40 mg of Zn as ZnSO\(_4\); 0.25 mg of I as Ca(IO\(_3\))\(_2\)(H\(_2\)O); 0.2 mg of Se as Na\(_2\)SeO\(_3\); and 0.1 mg of Co as CoCO\(_3\); 28 mg of Mn as MnSO\(_4\); and 4 mg of Cu as CuSO\(_4\).

\(^5\)Provided per kilogram of diet: 9,912 IU of vitamin A; 2,203 IU of vitamin D\(_3\); and 4.4 IU of vitamin E.

\(^6\)Flavoring agent provided by Pancosma, Geneva, Switzerland.

\(^7\)Provided dietary Fe treatment.
Table 2. Real-time polymerase chain reaction primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Accession #</th>
<th>Primer sequence (5’- 3’)‡</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rps9§</td>
<td>DT860044</td>
<td>F:CCTCGACCAAGAGCTGAAG</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:CCTCCAGACCTCACGTTGTTC</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F:AAGAGTCCTGGAGGTTGTG</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:GGTCAGATGAAAGTTGTTC</td>
<td></td>
</tr>
<tr>
<td>Ctr1</td>
<td>NM001100381.1</td>
<td>F:CGGAAGCAGAGTTCTCC</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:TGTGAGCGAGGCTAATGTC</td>
<td></td>
</tr>
<tr>
<td>Atox1</td>
<td>XM877831.2</td>
<td>F:GAGCAGACCCACCTTCATTC</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:GCCAAGCGGCAATAATG</td>
<td></td>
</tr>
<tr>
<td>Cox17</td>
<td>AF031558.1</td>
<td>F:GAAGATTATAGTATGGCTCAACC</td>
<td>190</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:CCTCTGCTGTGATAAGATGG</td>
<td></td>
</tr>
<tr>
<td>Atp7a</td>
<td>XM615430.3</td>
<td>F:AAGTCCTTGCCACGTCCTCC</td>
<td>109</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:GAGATGGATGTCGAGGAAACC</td>
<td></td>
</tr>
<tr>
<td>Atp7b</td>
<td>XM596258.3</td>
<td>F:GGATGGAGTAGAAATGGATGG</td>
<td>275</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:TCAGCACGATGATAGATG</td>
<td></td>
</tr>
<tr>
<td>Cp</td>
<td>XM592003.4</td>
<td>F:CCTCCGTGGGATTAATGGATGG</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:GACTCTCTCGGATTGATTGTTATG</td>
<td></td>
</tr>
<tr>
<td>Commd1</td>
<td>NM001046384.1</td>
<td>F:ATGGACCCGACTGTGTCTG</td>
<td>182</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:GCCAAGCGGCTGACTTGTCCG</td>
<td></td>
</tr>
<tr>
<td>Mt1</td>
<td>NM001040492.1</td>
<td>F:AGATACAGTCGTGGTAAC</td>
<td>125</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R:ACAGAGATTTAGATGTCGGG</td>
<td></td>
</tr>
</tbody>
</table>

‡F, Forward; R, Reverse
§Reported by Janovick-Guretzky et al., 2007.

*rp99, ribosomal protein S9; ctr1, copper transporter 1; atox1, antioxidant 1; cox17, cytochrome c oxidase assembly protein 17; atp7a, copper transporting, alpha polypeptide; atp7b, copper transporting, beta polypeptide; cp, ceruloplasmin; commd1, copper metabolism domain containing 1; mt1, metallothionein 1.*
Table 3. Effect of adequate or high dietary iron on ceruloplasmin and plasma copper concentrations in young calves.

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment</th>
<th>Control</th>
<th>High Fe</th>
<th>SEM&lt;sup&gt;1&lt;/sup&gt;</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma Cu, mg/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 0</td>
<td></td>
<td>0.96</td>
<td>1.07</td>
<td>0.059</td>
<td>0.20</td>
</tr>
<tr>
<td>day 35</td>
<td></td>
<td>0.96</td>
<td>1.11</td>
<td>0.048</td>
<td>0.04</td>
</tr>
<tr>
<td>day 56</td>
<td></td>
<td>0.81</td>
<td>0.93</td>
<td>0.042</td>
<td>0.07</td>
</tr>
<tr>
<td>Ceruloplasmin, mg/100 mL&lt;sup&gt;2&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>day 35</td>
<td></td>
<td>23.2</td>
<td>30.2</td>
<td>2.15</td>
<td>0.06</td>
</tr>
<tr>
<td>day 56</td>
<td></td>
<td>16.8</td>
<td>21.9</td>
<td>1.51</td>
<td>0.05</td>
</tr>
</tbody>
</table>

<sup>1</sup>Pooled standard error of the mean (n = 7 per treatment)

<sup>2</sup>Day 0 ran as covariate
Table 4. Effect of adequate or high dietary iron on duodenal, liver, and heart Cu concentrations in young calves.

<table>
<thead>
<tr>
<th>Item</th>
<th>Treatment</th>
<th>Control</th>
<th>High Fe</th>
<th>SEM$^1$</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenal Cu, mg/kg DM</td>
<td>Control</td>
<td>11.7</td>
<td>11.3</td>
<td>1.09</td>
<td>0.82</td>
</tr>
<tr>
<td>Liver Cu, mg/kg DM</td>
<td>Control</td>
<td>363.8</td>
<td>293.2</td>
<td>27.41</td>
<td>0.10</td>
</tr>
<tr>
<td>Heart Cu, mg/kg Cu</td>
<td>Control</td>
<td>14.9</td>
<td>14.2</td>
<td>0.72</td>
<td>0.50</td>
</tr>
</tbody>
</table>

$^1$Pooled standard error of the mean (n = 7 per treatment)
Figure 1. Effect of high dietary Fe on duodenal and hepatic CTR1 protein expression in young calves. ROI, relative optical intensity. Vertical bars indicate standard error of each target protein within dietary treatment. P-values are as follows: Duodenal CTR1 (P = 0.81). Hepatic CTR1 (P = 0.99).
Figure 2. Effect of high dietary Fe on relative mRNA expression of duodenal Cu transporters and chaperones in young calves. Vertical bars indicate standard error of each target gene within dietary treatment. P-values are as follows: Ctrl (P = 0.36), Atox1 (P = 0.93), Cox17 (P = 0.06), Atp7a (P = 0.41), Atp7b (P = 0.15).
Figure 3. Effect of high dietary Fe on relative mRNA expression of hepatic Cu transporters and chaperones in young calves. Vertical bars indicate standard error of each target gene within dietary treatment. P-values are as follows: Ctrl ($P = 0.20$), Atox1 ($P = 0.43$), Cox17 ($P = 0.29$), Atp7a ($P = 0.47$), Atp7b ($P = 0.01$), Cp ($P = 0.78$), Commd1 ($P = 0.86$), Sod1 ($P = 0.56$), Mti ($P = 0.74$).