ABSTRACT

HANSEN, STEPHANIE LAURA. Nutritional Interrelationships between Iron, Copper and Manganese in Domestic Livestock. (Under the direction of Jerry W. Spears).

Oftentimes the diets of cattle and pigs contain levels of iron well beyond the nutritional requirement of the animal. This superfluous iron may come from feedstuffs naturally high in iron, or from the consumption of soil, though bioavailability of iron from soil is unknown. Additionally, excess iron in cattle diets has been shown to negatively impact the metabolism of manganese and copper, though the molecular mechanism behind this interaction is unclear. The purpose of this research was threefold: 1) to determine the effect of ensiling on bioaccessibility of iron from soil contamination of corn greenchop, 2) to identify proteins important in iron metabolism in bovine and swine, and 3) to determine if these proteins are affected by dietary iron concentration. The results reported herein suggest that bioaccessibility of iron from soil contamination is greatly increased when soil undergoes a prolonged exposure to a low pH environment, such as that found with fermenting forages. These data indicate that very little iron from soil is available to the animal if no prior exposure to an acidic environment occurs. Also, for the first time we report that several proteins known to be essential to iron metabolism in rodents are present in bovine small intestine and liver. Specifically, the iron importer divalent metal transporter 1, the iron exporter ferroportin, and the multi-copper ferroxidase hephaestin were all present in bovine duodenum. In the bovine, reduced iron status, as induced by a primary deficiency of copper, resulted in increased gene expression of divalent metal transporter 1 and ferroportin in duodenum and decreased expression of the ferroportin regulatory hormone hepcidin and divalent metal transporter 1 in liver. Protein expression of ferroportin and hephaestin were

also increased in duodenum due to reduced iron status. The addition of excessive amounts of iron to the diets of young calves also appeared to regulate protein expression of transporters important in iron metabolism. Specifically, high iron tended to decreased duodenal protein levels of divalent metal transporter 1 and reduced ferroportin protein levels, though no effect on hephaestin was observed. We also examined iron metabolism in the young pig, in order to examine the effect of an iron deficient diet on expression of these proteins. We found that hephaestin protein in the duodenum was lowered by feeding a high iron diet, and levels of both ferroportin and divalent metal transporter 1 tended to be reduced by high dietary iron compared to pigs fed a low iron diet. Additionally, we found that feeding a high iron diet to pigs negatively impacted liver concentrations of manganese. And feeding a high iron diet to either pigs or calves reduced duodenal concentrations of manganese, suggesting that high dietary iron reduces manganese absorption. Because high iron diets fed to both pigs and calves tended to reduce duodenal levels of divalent metal transporter 1, a protein known in rodents to transport both iron and manganese, it appears that the observed reductions in duodenal manganese concentrations may be a result of reduced transporter availability. Collectively, our data suggest that high dietary iron may negatively affect manganese absorption, and because the iron content of livestock diets is often high, further research is warranted.

Nutritional Interrelationships between Iron, Copper and Manganese in Domestic Livestock

by
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DEDICATION

This work is dedicated posthumously to two of the most influential people I have ever known, whom we lost during my time in graduate school. To my grandmother Marie Hansen, who was a card shark to the end and had the most amazing sense of humor. And to my grandfather Jack Powell, who instilled in me a strong appreciation for agriculture, hard work and the rolling hills of northwestern Iowa. I know they would both be proud to see me achieve this goal.

BIOGRAPHY

Stephanie Hansen was born and raised in Sergeant Bluff, Iowa. She is the daughter of Dennis and Susan Hansen and has one younger sister, Stacie. Stephanie was actively involved in 4-H in her youth, and enjoyed raising beef cattle on her grandparent's farm. She received her B.S. degree in Animal Science from Iowa State University in 2002. Stephanie earned her M.S. degree in Animal Science from North Carolina State University in 2005 and stayed with Dr. Jerry Spears to pursue her Ph.D. in Nutrition. Following completion of her degree Stephanie will be returning to the department of Animal Science at Iowa State University as an Assistant Professor of beef cattle nutrition. Beyond the halls of academia, her interests include photography, camping, hiking and canoeing.

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Thanks to the many graduate students who have become good friends over the past few years. Thanks Erinn, Heather, Lauren, Kelly, and Kyle for the entertaining road trips, numerous outdoor adventures and enjoyable friendships. Perhaps the biggest debt of gratitude is owed to my friend Mary Drewnoski, for fruitful discussions, scientific and otherwise, and for putting up with me as a roommate for the past three years. And finally, thank you to my parents, Dennis and Susan Hansen, and my sister Stacie for acquiescing to five years of long distance phone calls and twice a year visits so that I could pursue my career half way across the country. I am happy to be going home to Iowa, but I will greatly miss my friends in North Carolina.

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CHAPTER 1

Literature Review

Introduction

Ruminants are often exposed to levels of dietary iron (Fe) well beyond their nutritional requirement (Humphries et al., 1983). Among the potential sources of excessive dietary Fe in typical cattle production systems are drinking water, soil, and feedstuffs that naturally contain large amounts of Fe. Very little is known about the potential bioavailability of Fe in these feedstuffs. Iron is an essential trace mineral and serves several important functions in the body, including oxygen transport, oxidative phosphorylation and DNA synthesis. However, high amounts of dietary Fe may result in free radical production, which can lead to oxidative damage of tissues (Ganz and Nemeth, 2006). Iron absorption is generally low (3-10%), and the duodenum and upper jejunum of the small intestine are the primary sites of absorption (De Domenico et al., 2008). The absorption of Fe decreases with age and body Fe status and Fe metabolism in the body is carefully regulated (Frazer and Anderson, 2005). Both in vitro and in vivo studies with monogastric species have clearly demonstrated an antagonistic relationship between excessive dietary Fe and the intestinal absorption of copper (Cu) and manganese (Mn; Arredondo et al., 2003; Garrick et al., 2006a). In ruminants, the negative impacts of high dietary Fe on Cu status are well documented in the literature (Standish et al., 1969; Standish et al., 1971; Humphries et al., 1983; Phillipo et al. 1987), while interactions between Fe and Mn are less well studied. Copper and Mn are essential trace minerals which play important roles in enzymatic processes in the body, and adequate supplies of both are necessary for optimum growth of

animals. However, essentially no information exists concerning the molecular mechanisms behind the antagonism of Fe with Cu and Mn in ruminants. Therefore, the majority of the work addressed in this literature review is reflective of the enormous amount of information that has been gathered in the past decade concerning Fe metabolism using both rodent and in vitro models.

Overview of Iron Absorption

The absorption of Fe involves the concerted action of several unique proteins (Figure 1). Due to the essential and yet toxic nature of Fe, this process is closely regulated. Dietary Fe in the ferric form is reduced to the ferrous form in the acidic environment of the stomach or abomasum or through the action of intestinal reductases, such as duodenal cytochrome b reductase, found on the apical surface of enterocytes. Divalent Fe is then co-transported through the apical portion of the enterocyte along with H^+, via divalent metal transporter 1. While the exact mechanisms behind translocation of Fe across the cell are still unclear, cellular Fe has one of two potential destinations. In situations where Fe is not readily needed for export into plasma, Fe is bound to the storage protein ferritin. This stored Fe may eventually be released for export from the cell, or lost in the feces when the intestinal cell is sloughed. If Fe is needed by the body, ferrous Fe is exported by the Fe exporter, ferroportin. Ferrous Fe is then oxidized to ferric Fe by the actions of the membrane-bound cuproenzyme hephaestin. Ferric Fe then binds to one of two binding sites located on transferrin, the primary transport protein for Fe, and is moved through the blood to the liver or other tissues for use.

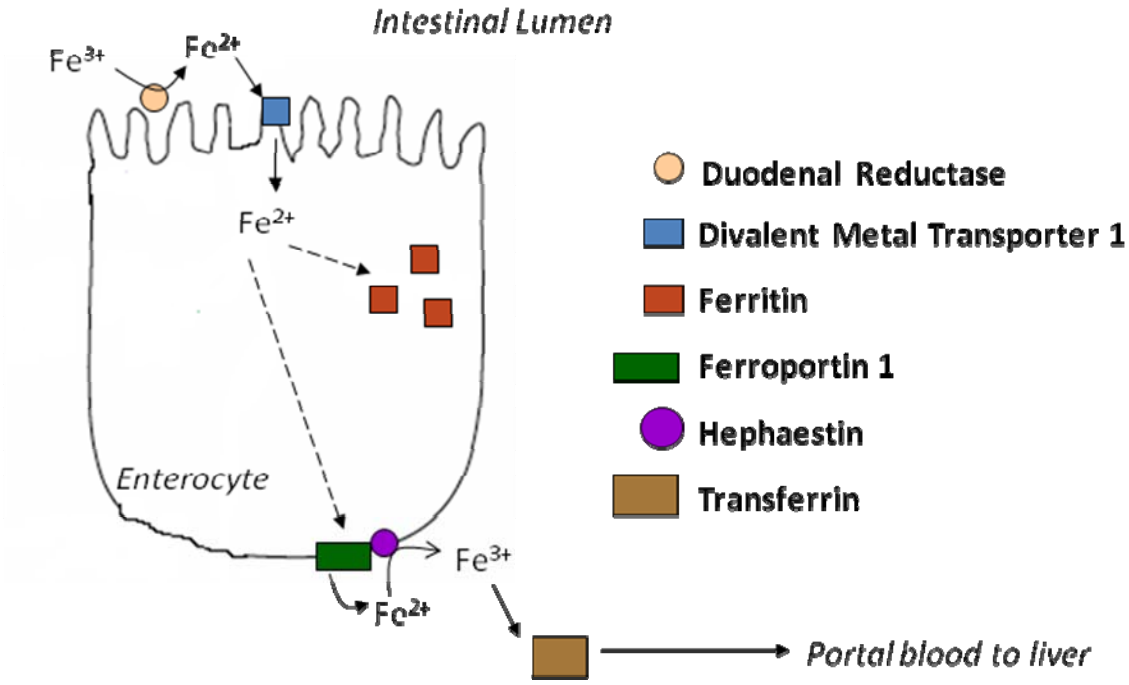

Figure 1. Model of intestinal absorption of iron in rodents

Proteins Involved in Iron Metabolism

Divalent Metal Transporter 1

Iron, Mn, Cu and several other trace metals share a common pathway for intestinal absorption, via a protein known as divalent metal transporter 1 (DMT1). Recognized in 1995, DMT1 is also referred to as natural resistance associated macrophage protein 2, divalent cation transporter 1, and solute carrier family 11, member 2. Gunshin et al. (1997) was one of the first to characterize the transporter, using a variety of molecular techniques to determine the structural nature of DMT1 and what metals it might transport. These authors injected *Xenopus laevis* oocytes with cDNA derived from duodenal mRNA isolated from rats fed an Fe deficient diet. They found that uptake of Fe^{55} was markedly increased in these

oocytes compared to controls injected with water. Eventually a single cDNA (for DMT1) was isolated, and found to increase Fe^{55} uptake by 200 fold. Using oocytes injected with DMT1 mRNA, the authors used voltage-clamp analysis to elucidate which metals invoked inward currents, suggesting movement of a divalent cation into the oocyte. In this in vitro study, DMT1 was found to have a broad range of specificity for divalent metals, including Fe, Mn, Zn, Co, Cd, Cu, Ni and Pb (Gunshin et al., 1997). The transport of divalent cations was also found to be pH dependent, with increased movement found at pH 5.5 compared to pH 7.5. This suggested that DMT1 was actually a proton coupled metal-ion transporter, facilitating movement of both H^+ and a divalent cation (Gunshin et al., 1997). Later studies revealed that ion transport via DMT1 is both pH and temperature dependent (Mackenzie et al. 2007).

DMT1 appears to play a role in both cellular and systemic control of Fe absorption. Systemically, DMT1 functions to import Fe across the apical membrane into duodenal enterocytes from the lumen of the gut, thereby contributing to whole system Fe status. At the cellular level, DMT1 serves as a route of non-transferrin bound iron uptake via the same mechanism that functions in enterocytes, while also mediating the transport of newly freed ferrous Fe out of the recycling endosome (Hubert and Hentze, 2002; Garrick et al., 2003).

Structurally, DMT1 is a predicted 12 transmembrane protein, with both the C and N termini orientated towards the cytosol, and a highly conserved fourth cytosolic loop. Like many proteins located on the apical surface of the enterocyte, DMT1 is highly glycosylated. Using site-directed mutagenesis to alter the binding site of N-glycans on DMT1, Tabuchi et al. (2002) found that glycosylation was not necessary for proper localization of the protein to

the intracellular endosome, but that the presence of N-glycans was necessary for migration of DMT1 to the apical plasma membrane in polarized renal epithelial MDCK cells.

In rats, DMT1 has a primary sequence length of 543 amino acids. There are currently 4 known isoforms of DMT1, with variations found in both the C and N termini encoding portion of the gene. The N terminal variants differ in the exon starting point of the gene, but these differences do not appear to be involved in the Fe responsiveness of the gene. The C terminal variants differ in the presence or absence of an iron responsive element (IRE) in the 3' untranslated region of the mRNA. Isoforms containing an IRE have a specific 18 amino acid sequence, while the non-IRE containing isoforms have a distinct 25 amino acid sequence in this region (Garrick et al., 2006b).

The presence of the IRE allows these isoforms to be highly responsive to changes in body Fe status. Iron regulatory proteins, released into the cytosol in response to low Fe stores, bind to the IRE resulting in increased stability of DMT1 mRNA, and therefore, increased concentrations of the protein (Garrick et al., 2003; Mackenzie and Hediger, 2004). Increased levels of DMT1 protein result in improved absorption of dietary Fe from the lumen of the small intestine, subsequently raising plasma Fe levels. While 4 distinct isoforms of DMT1 exist, Mackenzie et al. (2007) demonstrated equivalent efficiency of metal transport by the 4 isoforms. DMT1 is ubiquitously expressed in all body tissues tested thus far, however tissue distribution of DMT1 isoforms differ. Isoforms containing the IRE are expressed primarily by epithelial cells and are the primary isoforms found in the duodenum and kidney (Mackenzie and Hediger, 2004).

Ferroportin 1

Ferroportin is a 571 amino acid protein that is greater than 90% homologous among mice, rats and humans (Wessling-Resnick, 2006). Simultaneously discovered in several laboratories, ferroportin was initially also referred to as iron regulated transporter 1 or metal transporter protein 1. As the only known Fe exporter characterized to date, ferroportin serves a key role in Fe homeostasis. The protein is highly expressed by intestinal enterocytes and is important in the absorption of dietary Fe. Ferroportin has been shown to primarily localize along the basolateral membrane (McKie et al., 2000). In addition, Ferroportin is also strongly expressed in macrophages, where it plays an important role in recycling of heme Fe from senescent erythrocytes (Canonne-Hergaux et al., 2006). The exact membrane topology of ferroportin has been highly debated, and as a result ferroportin has been predicted to be a 9, 10 or 12 transmembrane protein by various laboratories (Oates, 2007).

Ferroportin is an N-linked glycoprotein, and proteins isolated from duodenum, liver and spleen have demonstrated slightly differing extents of glycosylation. Canonne-Hergaux et al. (2006) reported that fully glycosylated ferroportin proteins in mouse duodenum, spleen and liver weighed approximately 70, 65 and 63 kDa, respectively. When these samples were exposed to PNGase F, a glycosylase which preferentially cleaves N-linked sugars, ferroportin protein from the duodenum, spleen and liver migrated to smaller molecular weights of 61.5, 58 and 58 kDa, respectively, for the three tissues. As with DMT1, glycosylation of an integral membrane protein in the intestine has been theorized to allow for increased protection of the protein from intestinal proteases and the acidic nature of digesta, while also

potentially serving a role in trafficking of fully mature proteins to their appropriate membrane locations (Delacour and Jacob, 2006).

Translation of ferroportin mRNA is susceptible to regulation by iron regulatory protein (IRP), through binding to the IRE located in the 5'untranslated region of ferroportin mRNA. Binding of IRP to an IRE located in the 5' untranslated region of mRNA results in decreased stability of the protein and increased degradation, causing an overall decrease in the amount of ferroportin protein. This regulation appears to be limited to ferroportin present in tissues other than the small intestine, because multiple studies have demonstrated that Fe deficiency increases both transcript and protein levels of intestinal ferroportin (Chen et al, 2003; Canonne-Hergaux et al., 2006). As with DMT1, regulation of ferroportin by Fe status has been shown to be reciprocal in liver and duodenal tissues. In general, low dietary Fe results in increased duodenal expression of ferroportin and decreased liver expression, while high Fe results in decreased duodenal expression and increased liver expression of ferroportin (Abboud and Haile, 2000).

Hepcidin is a small, liver-derived peptide that has been shown to control levels of plasma Fe in response to inflammation and high hepatic Fe concentrations (Detivaud et al., 2006; Wrighting and Andrews, 2006). In an elegant series of experiments, Nemeth et al. (2004) demonstrated that ferroportin is actually the receptor for the hormone hepcidin. These authors used HEK-293 cells that were overexpressing ferroportin tagged with green fluorescent protein. When the cells were exposed to synthetic hepcidin, ferroportin was internalized and trafficked to acidic lysosomes for degradation. In cells that were not exposed to hepcidin, ferroportin remained localized to the plasma membrane. In addition,

increasing amounts of hepcidin added to the media of the HEK-293 cells resulted in increased intracellular ferritin concentrations, suggesting increased Fe concentrations in the cells (Nemeth et al., 2004).

Recently, De Domenico et al. (2007) endeavored to determine the exact sequence of events resulting in ferroportin internalization following exposure to hepcidin. These authors found that two tyrosine residues on ferroportin are phosphorylated following exposure to hepcidin. Tyrosine phosphorylation causes the protein to be targeted for ubiquination, and the protein is directed to the lysosome and subsequently degraded.

The complex regulation of ferroportin by hepcidin is an example of the intricate mechanisms the body has evolved in order to balance the essential and toxic nature of Fe. These experiments revealed that when hepcidin is released from the liver in response to high hepatic Fe stores, membrane-bound ferroportin concentrations in cells decrease, resulting in decreased export of Fe from enterocytes and macrophages and causing a reduction in plasma Fe concentrations. The combined action of hepcidin and ferroportin also serve to explain the hypoferremia of infection, as hepcidin is also produced as a part of the inflammatory response to limit Fe availability to bacterial invaders (De Domenico et al., 2007).

Ferroportin appears to function as an exporter for ferrous Fe, however, Fe must be in the trivalent state to bind to transferrin, the Fe transport protein found in the bloodstream. In the macrophage, ferroportin functions in conjunction with the multi-copper ferroxidase ceruloplasmin (Oates, 2007). In the intestine, ferroportin works in concert with a ceruloplasmin homolog, hephaestin, collectively functioning to export Fe from intestinal enterocytes into the blood stream (De Domenico et al., 2008). Ferroportin shuttles ferrous Fe

out of the enterocyte where fully functional hephaestin oxidizes Fe to the ferric form, which is then bound to the Fe transport protein transferrin, for transport within the bloodstream.

Hephaestin

Hephaestin is a homolog of the Cu-dependent ferroxidase, ceruloplasmin, the major Cu binding protein in the blood, with an amino acid sequence that is 50% identical and 68% similar to that of ceruloplasmin (Chen et al., 2003). An important component of this sequence conservation is the identical nature of resides involved in Cu binding and disulfide bond formation. Differences between the proteins do exist; ceruloplasmin is a cytosolic protein, while hephaestin is a membrane-bound protein which spans the plasma membrane in a single pass at the C-terminus (Anderson et al., 2002). Hephaestin functions as an electron transporter, accepting electrons from ferrous Fe and transporting them to oxygen, resulting in the production of ferric Fe and water in the following reaction: $4\ H^+ + 4\ Fe(II) + O_2 \rightarrow 2\ H_2O + 4\ Fe(III)$. Thus, the primary role of hephaestin in the intestine appears to be to oxidize Fe to allow for sufficient export of Fe from the basolateral side of the enterocyte into the bloodstream during the process of Fe absorption.

Hephaestin was first discovered as the candidate gene for an X-linked disorder known as sex linked anemia (*sla*). Mice displaying the disorder were characterized as suffering from microcytic, hypochromic anemia, which could be reversed through parenteral administration of Fe. However, oral administration of Fe was ineffective in resolving the anemia, leading researchers to conclude that intestinal absorption of Fe was somehow defective in these mice (Anderson et al., 2002). Further research using the *sla* mouse

revealed Fe loading of enterocytes, suggesting that Fe was adequately absorbed from the diet, but was not being effectively exported into the bloodstream, resulting in a buildup of Fe in the enterocyte (Chen et al., 2003). Genetic mapping led to the finding that the gene encoding hephaestin, named after Hephaestus, the Greek god of metals and the forge, was partially deleted in *sla* mice. The truncated version of hephaestin produced in these mice results in a dramatic reduction in the ferroxidase activity of the protein, which limits transport of Fe from the enterocytes to the bloodstream, resulting in the characteristic anemia.

The exact cellular location of hephaestin has been a controversial subject among laboratories, with arguments supporting localization within the cell and localization along the basolateral membrane of the enterocyte (Anderson et al., 2002; Nittis and Gitlin, 2004). Using confocal microscopy, Han and Kim (2007) examined the cellular localization of both ferroportin and hephaestin. Using fully differentiated Caco-2 cells, a well accepted model for intestinal Fe absorption, they determined that both hephaestin and ferroportin are located on the basolateral surface of the enterocyte. These findings were confirmed using Caco-2 cells overexpressing ferroportin tagged with green fluorescent protein. In addition, the authors used these overexpressing cells to demonstrate that hephaestin and ferroportin are colocalized on the basolateral surface of enterocytes. These findings also confirmed how important adequate level and activity of these two proteins are to normal Fe absorption.

Due to the important role that hephaestin plays in Fe absorption, transcript and protein levels of hephaestin both appear to be directly regulated by dietary Fe status of the animal. Chen et al. (2003) found that both enterocyte mRNA and protein levels of hephaestin were significantly increased in Fe-deficient wild type mice (consuming a diet

containing 12 mg Fe/kg DM) compared to control wild type mice consuming a normal diet of 50 mg Fe/kg DM. In addition, both transcript and protein levels of hephaestin were decreased in wild type mice consuming a high Fe diet (2% carbonyl iron supplemented in addition to the control diet) when compared to control wild type mice. These authors also included a homozygous *sla* mouse group in this study, and found that these mice produced a truncated hephaestin transcript, as expected, and had less intestinal hephaestin protein than control wild type mice (Chen et al., 2003). As a result of their findings, the authors suggested that hephaestin is likely regulated by signals of systemic Fe status rather than by local signals.

Because hephaestin is a cuproenzyme it was anticipated that transcript and proteins levels of hephaestin would be influenced by dietary Cu concentration. Chen et al. (2006) fed pregnant C57BL/6J mice diets containing 6 mg Cu/kg DM (Cu adequate) until parturition and then separated the mice into 2 groups, one fed the adequate diet and one fed a Cu-deficient diet (< 0.4 mg Cu/kg DM) for 3 weeks until weaning. After weaning, pups remained on the same diet as their dam for an additional 3 weeks. The pups were then euthanized and intestinal enterocytes and liver samples were collected. Using Western blot analysis, the authors found that intestinal hephaestin protein levels were significantly lower in mice receiving the Cu-deficient diet. Reeves et al. (2005) also reported that rats fed diets containing 0.25 mg Cu/kg DM (Cu deficient) for 19 days had only 30% as much hephaestin protein in the duodenum as rats fed Cu-adequate diets of 5 mg Cu/kg DM.

In a subsequent trial, Reeves and DeMars (2005) examined the effect of Cu restoration to rats fed a diet low in Cu on hephaestin protein content in the intestine. Male

Sprague-Dawley rats were divided into 3 groups; groups 1 and 2 received a Cu-deficient diet (0.25 mg Cu/kg DM) and group 3 received a Cu-adequate diet (5 mg Cu/kg DM). After 28 days on diets, rats in group 2 were switched to a repletion diet (Cu-adequate) for a period of 14 days, while rats in groups 1 and 3 remained on their respective dietary treatments. Rats were harvested and duodenal enterocytes were collected. Hephaestin protein was significantly lower in rats fed the Cu-deficient diet compared to those receiving the adequate diet; however, rats fed the Cu-repletion diet for the last 14 days actually had 3 times as much duodenal hephaestin protein as rats fed the Cu-adequate diet. These results suggest that while hephaestin protein is reduced during dietary Cu deficiency, the effect is reversible and supplementation with adequate dietary Cu results in a compensatory mechanism of increased hephaestin protein production (Reeves and DeMars, 2005).

In the study by Chen et al. (2006), the authors also measured ferroxidase activity in the intestine and found decreased activity, contributed to hephaestin, in the intestine of mice receiving the Cu-deficient diet compared to those receiving the Cu-adequate diet (Chen et al., 2006). This decrease in ferroxidase activity due to Cu deficiency is the likely cause of secondary Fe deficiency induced by Cu deficiency. Limited hephaestin activity results in impaired export of Fe from the enterocyte, resulting in a buildup of Fe in the cell, which provides negative feedback to DMT1, resulting in degradation of DMT1, and decreased Fe import into the intestine. Collectively, Cu deficiency results in impaired Fe absorption and thus results in systemic Fe-deficient anemia. Indeed, Cu-deficient mice in the study by Chen et al. (2006) displayed reduced serum Fe (0.27 mg/L) when compared to Cu-adequate mice

(0.49 mg/L). Copper deficient male rats also exhibited decreased serum Fe (0.51 mg/L) compared to rats receiving a diet adequate in Cu (0.89 mg/L; Reeves et al., 2005).

Transferrin and Uptake of Transferrin-Bound Iron

Transferrin is the major transport protein for Fe in the body. The 80 kDa glycoprotein contains 2 identical Fe binding sites, each capable of binding 1 molecule of ferric Fe. While transferrin has a very high affinity ($K_d = 10^{-23}$ M) for Fe, only 20-30% of binding sites typically contain Fe. As a result, free Fe in plasma is rapidly bound to transferrin for passage to tissues (DeDomenico, 2008).

Interestingly, when investigating the localization of ferroportin and hephaestin, Han and Kim (2007) also found that transferrin receptor was colocalized with these two proteins. The transferrin receptor serves as the primary method of transferrin-bound Fe acquisition by cells in the body. Iron laden transferrin binds to the transferrin receptor on the cell surface which is then internalized by the cell, where the Fe is released from transferrin in the acidic conditions within endosomes (De Domenico et al., 2008). The newly released apo-transferrin is then recycled to the cell surface to continue the cycle. While not yet corroborated through other work, Han and Kim (2007) speculated that colocalization of ferroportin, hephaestin and the transferrin receptor would allow for highly efficient recycling of recently released apo-transferrin to pick up newly oxidized Fe that has been shuttled out of the enterocyte via the ferroportin-hephaestin complex.

Ferritin

Iron is nearly always found bound to a support protein of some kind in the body. Storage of Fe is no exception, with the majority of Fe in tissues being bound by ferritin (Walters et al., 1973). Ferritin is a large protein, with a molecular weight of 450 kDa and is capable of binding up to 4500 atoms of Fe. Ferritin binds Fe, minimizing tissue damage due to free radical production, while maintaining Fe in a soluble and available form in the cytosol. Ferritin is formed by a series of 24 subunits, approximately 20 kDa in molecular weight, which combine to make up a protein coat surrounding a core of Fe stored as hydrous ferric oxide (Theil, 1987). There are 2 types of subunits, a heavy chain subunit weighing approximately 21 kDa, and a light chain, weighing between 19 and 20 kDa. The heavy chain subunit contains ferroxidase activity which is necessary for the acquisition of Fe by ferritin, while the light chain subunit appears to play important roles in the stability of ferritin and contains no ferroxidase activity (Orino et al, 2004). In humans, serum ferritin concentrations have been shown to be directly correlated with body Fe stores (Walters et al., 1973). Therefore, assays for serum ferritin may serve as a useful tool by which to diagnosis Fe overload in tissues.

Interactions between Iron and Copper

A close relationship exists between Cu and Fe. Adequate Cu is essential to proper metabolism of Fe, as Cu-dependent ferroxidases are necessary for Fe to be mobilized out of tissues (Osaki, 1971). Ceruloplasmin fulfills this role in the macrophage, hepatocyte and various other tissues such as certain cell types in the brain, while hephaestin is the

ferroxidase localized in the plasma membrane of intestinal enterocytes (Chen et al., 2006). Copper effect on the uptake and export of Fe from intestinal cells has been studied. Han and Wessling-Resnick (2002) recently demonstrated that when Caco-2 cells were incubated in the presence of 1 μM Cu for 3 hours, apical Fe^{55} uptake was increased by 32% and export into the basolateral chamber was increased by 59%. These authors also examined expression of several genes involved in Fe metabolism in Caco-2 cells following 8 days of 1 μM $CuCl_2$ exposure. Compared to non-Cu exposed cells, relative expressions of DMT1, ferroportin and hephaestin were significantly increased in the Cu exposed cells. In addition, expression of the transferrin receptor was greatly increased relative to controls, as would be expected in a limited Fe situation. Using this information, these authors speculated that Cu exposure results in increased Fe absorption through the following mechanism. Copper repletion causes increased hephaestin and ferroportin expression, resulting in enhanced Fe export from the cell. The cell senses a lack of Fe and DMT1 expression is upregulated in a compensatory manner and apical Fe uptake is subsequently increased (Han and Wessling-Resnick, 2002).

One possible point of interrelationship in the pathways of Fe and Cu absorption is DMT1. Human and rodent work has revealed that Cu has a dedicated transporter known as Ctr1; however, Arredondo et al. (2003) reported that at least some portion of Cu absorption is directly contributable to DMT1 function. These authors found that approximately 48% of Cu absorption was eliminated when Caco-2 cells were treated with a DMT1 antisense oligonucleotide, essentially knocking out DMT1 activity. These data suggest that DMT1 plays an important role in Cu import into cells. As shown in Figure 2, Arredondo et al. (2003) demonstrated reduced uptake of Cu^{64} by Caco-2 cells as the concentration of Fe in the

incubation media was increased from 0.5 to 20 μM. Collectively, these results suggest that high Fe may be antagonistic to Cu in two distinct manners, causing less DMT1 to be expressed by cells and also competing with Cu for uptake by DMT1 into the cell.

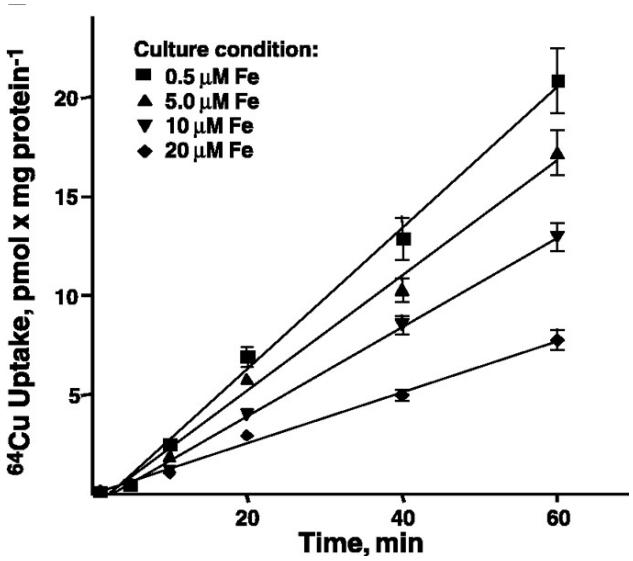

Figure 2. Competition between Fe and Cu uptake by Caco-2 cells.

Interactions between Iron and Manganese

The antagonism between dietary Fe and Mn has been demonstrated in multiple species (Standish et al., 1971, Rossander-Hulten et al., 1991; Davis et al., 1992). Hartman et al. (1955) examined the effect of increasing dietary Mn on hematological measurements in young lambs. Eight lambs (6-9 days of age) were maintained on cow's milk for a period of 2 months, and were bled occasionally during the second month to achieve hemoglobin concentrations of 4-6 g/dL. The lambs were gradually weaned onto a chopped soybean hay-based experimental diet (Mn content not provided) and were fed one of the following treatments for a period of 11 weeks. Treatment 1 received no supplemental Mn in addition to the basal diet, treatment 2 received 1000 mg Mn/kg DM and treatment 3 received 2000 mg

supplemental Mn/kg DM. Serum Fe and hemoglobin concentrations were determined during the experimental period of 11 weeks, as well as for an additional 3 weeks after dietary Mn treatments were suspended. Serum Fe concentrations over the 11 week period were lowest in lambs receiving supplemental Mn, but did not differ due to level of Mn supplementation. In addition, serum Fe concentrations in lambs supplemented with 1000 or 2000 mg Mn/kg DM rose markedly following discontinuation of the dietary treatments on week 11. Interestingly, serum Fe concentrations took longer to recover to normal levels in lambs receiving 2000 mg Mn/kg DM compared to those receiving 1000 mg Mn/kg DM. Hemoglobin concentrations during the 11 week period were also lower in lambs receiving supplemental Mn (maximizing at 7 g/dL) compared to those consuming the basal diet alone (maximizing at 11 g/dL). Following discontinuation of the treatments on week 11, hemoglobin concentrations began to rise in both supplemental Mn treatment groups. These data suggest that hemoglobin formation was compromised by excess dietary Mn, likely due to decreased Fe availability for heme synthesis because of competition for absorption between Fe and Mn (Hartman et al., 1955).

The effect of increased dietary Mn on absorption of Fe in humans has also been studied. Rossander-Hulten et al. (1991) utilized healthy human subjects with variable Fe status and ages (19-50 years) to determine the effect of increasing dietary Mn on the absorption of Fe. They found that the addition of 7.5 or 15 mg Mn to 3 mg radiolabeled Fe in solution decreased Fe absorption by 21% and 34%, respectively.

It has also been observed that high dietary Fe negatively affects Mn metabolism. Davis et al. (1992) reported that high Fe had a depressive effect on Mn absorption in male

weanling Sprague-Dawley rats. Rats were fed 0.9, 48, or 188 mg Mn/kg diet, respectively, and a marginal or high Fe level (19 or 276 mg Fe/kg diet). Rats fed the diet of 0.9 mg Mn/kg DM and 19 mg Fe/kg DM had much higher absorption than rats fed the other treatments, and increasing dietary Mn resulted in increasing endogenous Mn losses when Fe was marginal. However, when Fe was fed at the high level (276 mg/kg DM), endogenous Mn losses decreased across treatments and true absorption of Mn was significantly reduced (57.3% and 27.3%, true Mn absorption for low Mn/marginal Fe and low Mn/high Fe, respectively). Manganese concentrations were significantly lower in tibias, livers and mucosal cells of rats fed high Fe levels when compared to rats fed marginal levels of Fe, indicating that Fe was affecting the absorption of the dietary Mn (Davis et al., 1992).

In ruminants, Standish et al. (1971) examined the impact of varying levels of dietary Fe on tissue concentrations of several minerals, including Mn. These authors used 24 growing beef steers in a 2 x 2 factorial experiment containing 2 levels of Fe (100 or 1000 mg Fe/kg DM) and 2 levels of phosphorus (0.23 or 0.46%). Steers were provided with the ground corn-based diet for 77 days prior to harvest. Kidney Mn concentrations were lower (4.93 vs. 5.69 mg Mn/kg DM) in steers fed 1000 mg Fe/kg DM compared to 100 mg Fe/kg DM. Similarly, heart Mn concentrations were decreased (2.68 vs. 3.36 mg Mn/kg DM) by 1000 mg supplemental Fe/kg DM compared to 100 mg Fe/kg DM.

Two lines of rodents, the microcytic mouse and the Belgrade rat, are spontaneous mutants which suffer from Fe deficiency as a result of impaired Fe transport. These lines have been shown to have identical missense mutations in DMT1, resulting in Fe deficiency (Andrews, 1999). Metabolism of Mn has also been shown to be detrimentally affected in the

Belgrade rat, indicating that the missense of the DMT1 gene is causing disruption in absorption of Mn as well as Fe (Chua and Morgan, 1997). Subsequent research has shown that DMT1 is an important transporter of Mn into intestinal cells. As shown in Figure 3, Garrick et al. (2006a) used human embryonic kidney-293 fast growing variant cells (HEK-293) expressing doxycycline-inducible DMT1 to demonstrate that Mn^{54} uptake was increased in both –IRE (2/-dox) and +IRE (1A/+dox) isoforms of DMT1 (Figure 3). Endogenous expression (not treated with doxycycline) of the DMT1 isoforms in the cell lines are shown in bars 3 (2/-no) and 4 (1A/+no), while uptake of Mn^{54} by the tetres-responsive element and empty vector or tetres element alone (controls) are shown in the final two bars.

Further supporting evidence for DMT1 as a potential point of antagonism between these minerals is shown in Figure 4, as competition between Mn and Fe for uptake via DMT1 is clearly demonstrated. Uptake of Mn^{54}decreased as Fe sulfate in the media of HEK-293 cells was increased, with the authors reporting an IC_{50} of ~2.1 μM for Fe^{2+} (Garrick et al., 2006a). These data suggest that not only is Mn absorption likely to suffer if DMT1 is downregulated in response to high dietary Fe, but that high Fe may also out-compete what little dietary Mn is present for uptake into the absorptive enterocyte.

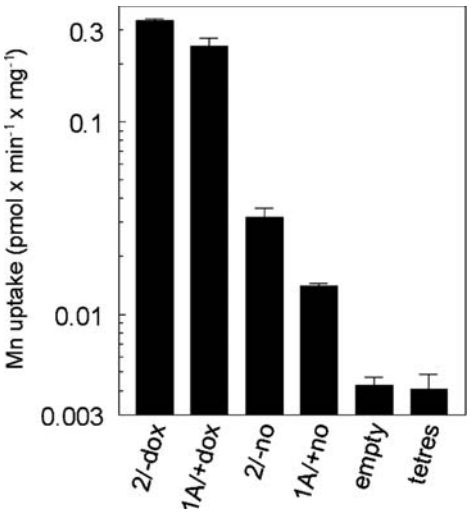

Figure 3. Mn uptake by HEK-293 cells

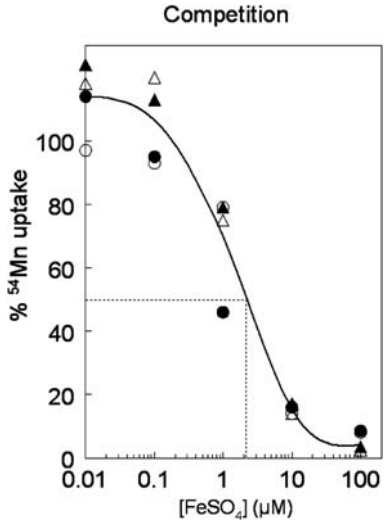

Figure 4. Competition between Fe and Mn for uptake by HEK-293 cells

Excessive Iron in Ruminant Diets and Impacts on Manganese and Copper

Excessive Fe in ruminant diets may come from a variety of sources, including feedstuffs that are naturally high in Fe such as alfalfa (300 mg Fe/kg DM) or soyhulls (500 mg Fe/kg DM; NRC, 1996; DePeters, 2000). Iron may also be in excess in some corn co-products, for example, distillers grains with solubles averages more than 600 mg Fe/kg DM (NRC, 1996). In addition to feedstuffs high in Fe, soil contamination of harvested feedstuffs may be an additional source of excessive Fe in ruminant diets. Using titanium concentrations of feces as an indicator of soil consumption in cattle, Mayland et al. (1975) suggested that grazing cattle ingested an average of 0.5 kg of soil per head per day. More recent work by Rafferty et al. (1994) reported that harvested feedstuffs such as hay and grass silage had a mean soil contamination level of 2% (w/w), with some contamination as high as 8%

reported. Soil Fe concentrations in the United States range from less than 0.5% in the southern Gulf States to greater than 5.5% in mountainous areas and many parts of the Western United States (United States Geological Survey, 2007).

Iron in soils is typically bound to clays or other chelating agents, and the majority of Fe is in an unsoluble form which should be unavailable to the animal for absorption (Whitehead, 2000). However, we have recently demonstrated that exposure to an acidic environment caused by fermenting silage resulted in significant increases in bioaccessible Fe concentrations following enzymatic in vitro ruminant digestion simulation (Hansen and Spears, 2008). Soil contamination of greenchop at harvest may represent an overlooked source of excessive Fe in ruminant diets. It is possible that high levels of available Fe in the silage may result in increased competition between minerals for common intestinal transporters.

Both Mn and non-transferrin bound Fe can be transported into cells via the action of DMT1, suggesting that high dietary Fe may result in increased competition for absorption of Mn by DMT1 (Garrick et al., 2006a). The role of Mn in cartilage formation makes it essential to the formation of the epiphyseal growth plate, which directly affects longitudinal bone growth, a deficiency of which could contribute to dwarfism (Leach and Muenster, 1962). Previously, we have observed skeletal defects in calves born to heifers fed diets low in Mn (16 mg Mn/kg DM) for a period of approximately 375 days (Hansen et al., 2006). The skeletal defects, which included dwarfism and shortened nasal maxillary bones, had not previously been reported in controlled studies examining the effects of Mn deficiency in beef cattle. A subsequent search of the literature revealed a series of field observations in Canada

which reported similar symptoms in newborn calves. Collectively, the symptoms included swollen joints, dwarfism and a similar nasal bone problem, which was referred to as superior brachygnathism. The condition is known as congenital joint laxity and dwarfism (CJLD). Observers noted that in many cases of CJLD births, pregnant cows had been wintered on grass silage or clover silage exclusively, and supplementation of cows with rolled barley or hay seemed to prevent any signs of CJLD (Ribble et al., 1989; Hidiroglou et al., 1990).

Hidiroglou et al. (1990) investigated the link between dietary Mn concentrations and appearance of CJLD in calves. The authors fed pregnant cows one of three feedstuffs (hay, red clover silage, or grass silage) and measured the frequency of CJLD in calves at birth for each group. While all three feedstuffs had similar levels of Mn (51, 64, and 63 mg Mn/kg DM for hay, red clover, and grass silage, respectively), 38% of calves born to cows fed red clover silage, and 28% of calves born to cows fed grass silage exhibited CJLD. None of the calves born to cows fed hay displayed any signs of the disorder. In addition, serum Mn concentrations were found to be lower in cows fed red clover or grass silage, compared to those cows wintered on hay. It is interesting to note that the cows in this study (Hidiroglou et al., 1990) were consuming silages containing well over the NRC (1996) recommended feeding level for Mn of 40 mg Mn/kg DM and yet some cows still gave birth to CJLD calves. One explanation for this may be the presence of a Mn antagonist in the silage. Previous research has suggested that high levels of Fe may negatively impact Mn metabolism in the body, possibly via competition for transporters in the intestine (Davis et al., 1992). However, little is known about the specific method(s) by which Mn absorption may be affected by these minerals. Iron, incorporated into the silage via soil contamination during

harvest may have been a contributing factor to the apparent decrease in Mn absorption in the incidences of CJLD in Canada.

Excessive levels of dietary Fe have been shown to negatively impact Cu metabolism in cattle. Standish et al. (1969) used growing beef steers to examine the effects of feeding 0, 400 or 1600 mg supplemental Fe/kg DM for a period of 84 days on plasma and tissue mineral concentrations. The basal diet was corn based and analyzed to contain 77 mg Fe/kg DM. Liver Cu concentrations were not different between steers supplemented with 0 or 400 mg Fe/kg DM; however, concentrations were lower in steers supplemented with 1600 mg Fe/kg DM compared to those provided 0 mg supplemental Fe/kg DM (44 and 260 mg Cu/kg DM, respectively). Several subsequent studies have found that Cu indices such as liver and plasma Cu concentrations or plasma ceruloplasmin activity are negatively affected by high dietary Fe in cattle (Humphries et al., 1983; Bremner et al., 1987; Phillipo et al., 1987; Gengelbach et al., 1994). In these studies, decreases in Cu status of calves fed high supplemental Fe were similar to those decreases observed in calves fed supplemental Mo. In addition, the negative impacts of Fe and Mo on plasma and liver Cu concentrations of calves appeared to be additive, suggesting differing mechanisms of Cu antagonism between the two minerals (Humphries et al., 1983; Bremner et al., 1987). Bremner et al. (1987) also noted that the severity of Fe-induced Cu deficiency did not differ between calves supplemented with 500 mg Fe/kg DM or 800 mg Fe/kg DM from Fe (II) carbonate, suggesting that both levels were at or above the threshold at which the body minimizes dietary Fe absorption, likely via regulation of proteins such as DMT1.

Bioavailability of Iron in Feedstuffs

There is an extremely limited body of work which has examined the bioavailability of Fe from various ruminant feedstuffs, and very little has actually been conducted with ruminants. Several workers have addressed ruminant bioavailability of Fe from supplemental sources such as dicalcium phosphate, ferrous sulfate and ferrous carbonate; however, little to no work has focused on availability from common feedstuffs such as soybeans, corn or forages. The distribution of minerals within forage has been examined to some extent however, and this information may prove useful when estimating the potential bioavailability of Fe in cattle. Kincaid and Cronrath (1983) reported that 77% of Fe in alfalfa hay was associated with the NDF fraction and 19% with the ADF fraction. Iron in grass silage was also predominately associated with the NDF fraction (45%) as opposed to the ADF fraction (17%). It is also interesting to note that 41 alfalfa hay samples and 39 grass silage samples analyzed in this study contained variable and excessive amounts of total Fe (382 ± 262 mg/kg DM and 505.2 ± 379.7 mg/kg DM for alfalfa hay and grass silage, respectively).

Thompson and Raven (1959) examined the bioavailability of Fe from perennial ryegrass, cocksfoot and timothy using hemoglobin regeneration in anemic weanling rats as their method of determining Fe bioavailability. In a subsequent publication, these same authors reported on the bioavailability of Fe from several legumes, including alsike clover, broad red clover, Kent wild white clover, trefoil (clover) and lucerne (alfalfa; Raven and Thompson, 1959). Using ferric chloride as the baseline (100% bioavailable Fe), these workers reported that only about 50% of the Fe in grasses was available to the anemic rats

based on hemoglobin regeneration. Specifically, perennial ryegrass was 50% bioavailable, cocksfoot was 48% bioavailable and timothy was 63% bioavailable.

In general, the bioavailability of Fe provided by legumes proved to be very similar to that of the grasses; however, all of the legumes analyzed in this study contain far greater concentrations of Fe than the grasses and as a result would contribute significantly more bioavailable Fe to the animal. Relative bioavailability (assuming 100% for ferric chloride) of Fe in the legumes was estimated to be between 46 and 57%. Based on this information, the red clover used in this study provided 413 mg bioavailable Fe/kg DM (analyzed to contain 756 mg Fe/kg DM total) and the alfalfa provided 436 mg bioavailable Fe/kg DM out of the 856 mg Fe/kg DM it was analyzed to contain. While the information provided by these rat studies is certainly interesting, it's usefulness in determining actual bioavailability of Fe from feedstuffs to cattle has yet to be determined.

Some work concerning the apparent absorption of Fe in ruminants has been conducted. Ivan et al. (1983) utilized cannulated sheep (rumen, duodenum and ileum) to determine concentrations of Fe in ruminal, duodenal and ileal digesta in order to estimate the apparent absorption of Fe from corn silage to be approximately 43.2%. Interestingly, the apparent absorption of Fe from alfalfa silage examined in this experiment was much lower (11.9%), which clearly demonstrates the wide range of potential bioavailability of Fe in different ruminant feedstuffs. Further studies on the bioavailability of Fe from common ruminant feedstuffs such as silage, alfalfa, grains, by-products and other forages are warranted.

Conclusion

While extensive research concerning Fe metabolism and its interactions with the essential trace minerals Cu and Mn has been conducted in monogastric species, only a small body of work has addressed this issue in ruminants. Because cattle are often exposed to high levels of Fe in their diet as a result of soil ingestion or consumption of feedstuffs naturally high in Fe it is especially important that the potentially negative effects of high dietary Fe are elucidated. Therefore, the subsequent studies were conducted to determine the in vitro bioavailability of Fe from soil contamination of corn silage, to identify proteins involved in Fe metabolism in pigs and cattle for the first time, and to determine the effects of varying concentrations of dietary Fe on gene expression and concentrations of these proteins in tissues important in trace mineral metabolism.

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CHAPTER 2

Silage Fermentation Increases Bioaccessibility of Iron from Soil Contamination in the Ruminant Digestive Tract

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ABSTRACT: Excessive dietary iron (Fe) can negatively impact absorption of other minerals and cause tissue damage through the production of free radicals. Ruminants are often exposed to high dietary Fe, frequently due to consumption of soil. Iron in soil is often found in the ferric form bound in insoluble complexes, however; exposure to an acidic environment similar to that occurring during silage fermentation may cause this Fe to be reduced to the more soluble ferrous form. To test this theory, 14 experimental silos (2 replicates of 7 treatments) were tightly packed with corn greenchop. Treatments included 3 types of soil (Cecil clay loam, 3.4% Fe; Georgeville silt loam, 4.3% Fe; and Dyke clay loam, 6.9% Fe) at 2 levels of soil inclusion (1 and 5% contamination, as-fed basis) added to greenchop prior to ensiling. In addition, one treatment consisting of greenchop with no soil added was ensiling to serve as a control. Greenchop was allowed to ferment for 90 days before silos were opened and silage was freeze-dried and ground. To simulate contamination after ensiling each soil type was added to control silage at the 2 levels of inclusion. Addition of soil to greenchop prior to ensiling resulted in greater amounts of water soluble Fe compared to soil addition after ensiling, suggesting that Fe-soil binding properties were altered due to ensiling. To test the potential bioaccessibility of Fe during ruminant digestion an enzymatic in vitro system was modified to simulate ruminal, abomasal and intestinal digestion. The presence of soil, regardless of time of addition, type or inclusion level, resulted in greater bioaccessible Fe concentrations after all 3 phases when compared to control silage. Ensiling further increased soluble Fe concentrations after each phase when compared to silage contaminated with soil after ensiling. In addition, dialyzable Fe concentration (15000 Dalton molecular weight cut off) following intestinal phase simulation was greater due to ensiling of soil. Iron

that becomes soluble during the intestinal phase may be available to the animal for absorption, and ensiling resulted in increased concentrations of this potentially bioavailable Fe. These results suggest that soil contamination of harvested feeds prior to ensiling may represent a major source of bioavailable Fe in the diets of cattle.

INTRODUCTION

Iron is often found in high levels in ruminant diets due to feedstuffs naturally high in Fe and/or soil contamination of feedstuffs (Standish et al., 1971). Some common feedstuffs that are often high in Fe include alfalfa, soyhulls and corn silage (NRC, 1996; DePeters et al., 2000). Humphries et al. (1983) noted that silages in the British Isles often contain greater than 2000 mg Fe/kg DM, which is clearly in excess of the NRC (1996) recommendation for cattle (50 mg Fe/kg DM). Soil contamination of silage is the most likely explanation for these high levels of Fe.

In the United States, concentrations of Fe in soils range from less than 0.5% in the southern Gulf States to greater than 5.5% in mountainous areas and many parts of the Western United States (United States Geological Survey, 2007). In general, Fe in soils is thought to be tightly bound to chelating agents and is therefore fairly unavailable for absorption by animals. However, in vitro work by Healy (1972) indicated that a considerable fraction of Fe from soil can become soluble in the ruminant digestive tract, suggesting that this Fe may be potentially available for absorption. It is also possible that exposure to an acidic environment, such as that found during fermentation of silage may result in increased bioavailability of soil Fe through reduction of ferric Fe to ferrous Fe (Whitehead, 2000).

High dietary Fe may adversely affect the bioavailability of Cu and Mn in ruminants. Numerous studies have demonstrated that excessive levels of dietary Fe reduce the Cu status of cattle (Standish et al., 1969; Humphries et al., 1983). It is well accepted that high Fe decreases Mn absorption in rats (Davis et al., 1992), however the exact relationship between Fe and Mn metabolism in ruminants is unclear. We recently reported skeletal defects in calves born to heifers fed a diet low in Mn (16 mg Mn/kg DM) for a period of approximately 375 days (Hansen et al., 2006). The skeletal defects, which included dwarfism and shortened nasal maxillary bones, had not previously been reported in clinical studies examining the effects of Mn deficiency in beef cattle. A search of the literature revealed a series of field observations in Canada which reported similar symptoms in newborn calves. Researchers noted that pregnant cows wintered on grass silage or clover silage gave birth to symptomatic calves while cows wintered on hay did not produce defective calves (Hidiroglou et al., 1990). In this study, both the grass silage and clover silage contained over 60 mg Mn/kg DM, suggesting that fetal demands for Mn should have been met. We hypothesized that Mn availability from the silages may have been affected by the presence of a Mn antagonist such as Fe in the diet, possibly due to soil contamination prior to ensiling.

Therefore, in the present study we endeavored to determine the effect of silage fermentation on the bioaccessibility of Fe from various levels and types of soil contamination using a ruminant simulated digestion system. For our purposes, bioaccessibility may be defined as the fraction of Fe that is soluble in the gastrointestinal environment and available for absorption or interaction with other minerals.

MATERIALS AND METHODS

Experimental Design and Silage Preparation

To determine the effects of silage fermentation on the potential bioaccessibility of Fe from soil contamination a 2 x 2 x 3 factorial design augmented with a control group (2 replicates per treatment) was utilized. Factors included time of soil addition (before or after ensiling of corn greenchop), level of soil addition (0 (control), 1 or 5% contamination on an as-fed basis), and type of soil (Cecil clay loam, Georgeville silt loam, or Dyke clay loam). Three soil types with differing soil properties and Fe content were examined: 1) Cecil clay loam (fine, Kaolinitic, thermic Type Kanhapludults; 3.4% Fe), 2) Georgeville silt loam (clayey, Kaolinitic, thermic Type Hapludults; 4.3% Fe), and 3) Dyke clay loam (clayey, mixed, mesic Type Rhodudults; 6.9% Fe). Soils were air dried, and crushed by hand to pass through a 1 mm screen before being mixed with greenchop.

To simulate contamination of corn greenchop prior to ensiling, 14 half-gallon, wide mouth plastic jars with screw top lids were utilized as experimental silos. Each silo was tightly packed with 1400 g (as-fed basis) of freshly harvested corn greenchop. Soil added before ensiling was thoroughly mixed with greenchop at the appropriate inclusion level (0, 1 or 5%) prior to packing. Greenchop was determined to contain 37% DM. Greenchop was allowed to ferment for 90 days, with occasional venting during the first few days to prevent gas buildup in the silos. On d 90 silos were opened and the top 5 cm of oxygen-exposed, spoiled silage was removed. The pH of silage from each silo was determined immediately after opening. Fifty grams of silage from each silo was added to 200 mL of deionized water

and stirred on a stir plate for 30 minutes. The slurry was then strained through a double layer of cheesecloth and pH of the supernatant was measured (Corning pH meter 340; Corning Inc. Life Sciences, Lowell, MA). The remaining silage was freeze-dried and ground to pass through a 1 mm sieve on a Wiley Mill (Model 4; Arthur A. Thomas Co., Philadelphia, PA). Silage samples from each silo were wet ashed for Fe analysis using microwave digestion (Mars 5; CEM Corp., Matthews, NC) as described by Gengelbach et al. (1994). Iron content of silage samples and all digestion solutions was determined by flame atomic absorption spectroscopy (AA-6701F; Shimadzu Scientific Instruments, Kyoto, Japan).

To simulate the effect of soil contamination of corn silage after ensiling the following procedure was used. Freeze dried and ground control silage was used in all analysis, to which was added the appropriate type (Cecil clay loam, Georgeville silt loam or Dyke clay loam) and level (1 or 5% as fed basis) of soil. Because control silage used was already dried, the level of soil added to each sample was adjusted to DM basis (approximately 3 and 15%, for 1 and 5% as fed, respectively). This procedure would most closely simulate consumption of soil by a grazing animal or consumption of recently contaminated feedstuffs. Each sample was freshly prepared, in duplicate, prior to in vitro simulation.

Water Solubility Procedures

The water solubility of Fe from soil added before or after ensiling was determined by adding 1 g of silage to 40 mL of deionized water in a 15 mL polypropylene tube and placing it in a 22˚C shaking water bath for 5 h. Samples were then filtered through ashless Whatman 541 paper and the filtrate was analyzed for soluble Fe concentrations.

In Vitro Digestion System

The simulated digestion system used was a modification of the procedure described by Ward and Spears (1993). A mixture of enzymes was used to simulate ruminal digestion, because rumen fluid is variable and may contain fairly high concentrations of trace minerals. In contrast, Ward and Spears (1993) used rumen fluid for the rumen digestion stage. Preliminary experiments were conducted to determine the necessary pH and enzyme inclusion levels for optimal DM digestion in the rumen stage (data not shown). These experiments were based on a target rumen dry matter disappearance rate of ~70% for corn silage (Jones et al., 1980). Digestion of silage in the rumen was simulated by adding 0.5 g silage to a 50 mL Erlenmeyer flask. Thirty milliliter of an acetate buffer was then added to each flask. To make this buffer 2.95 mL glacial acetic acid was brought up to 500 mL with deionized water (Part A) and 13.6 g sodium acetate was brought up to 1 L with deionized water (Part B; De Boever et al., 1986). Parts A and B were added together to reach a pH of ~5.1 for optimum enzyme activity and ruminal digestion of silage. All enzymes were purchased from Sigma-Aldrich (St. Louis, MO). Cellulase (EC 3.2.1.4) from *Aspergillus niger* was included at a level of 10 units/mL buffer (De Boever et al., 1986), hemicellulase (EC 232-799-9) from *Aspergillus niger* at a level of 1.67 units/mL buffer (Nocek and Hall, 1984), and 50 μL of heat stable amylase (Sigma product number A 3306; EC 3.2.1.1) was added to each flask. Flask openings were covered with parafilm, and agitated in a 39°C water bath for 24 h. After 24 h of digestion flasks were swirled and a 3 mL aliquot of the fluid was removed and centrifuged briefly to remove any solid contents prior to analysis for Fe concentrations. Dry matter disappearance for control silage was determined following the

24 h ruminal stage. In addition, in vitro true DM disappearance was determined for control silage using fermentation vessels (Ankom Technology Corp., Fairport, NY) as reported by Huntington and Burns (2007).

Abomasal digestion followed the rumen stage, and included the addition of 5% pepsin (388 units/mg; EC 3.4.23.1) solution in 1 M HCl. Approximately 3 mL of this solution was required to lower the pH to ~2.5. Flasks were then agitated in a 37°C water bath for 1 h. Following the abomasal stage a 3mL aliquot was taken as previously described following the ruminal stage. To simulate intestinal digestion the pH of the remaining solution was lowered through the drop wise addition of 1 M NaOH to a pH of approximately 6.8 (approximately 2 mL NaOH). Once the pH was stabilized, 0.4 mL of 10% pancreatin (Sigma product number P 1500; EC 232-468-9) solution in deionized water was added to each flask and flasks were again agitated in a 37°C water bath for 2 h. To more stringently test the amount of potentially bioaccessible Fe following intestinal digestion, dialyzable Fe concentrations were determined. This procedure utilized dialysis tubing with a molecular weight cutoff of 15000 Daltons (Spectra/Por 7 Dialysis Membrane; Spectrum Laboratories, Rancho Dominguez, CA). Following the intestinal stage of digestion, flasks were swirled, poured off into 50 mL polypropylene tubes, and centrifuged at 580 x *g* for 10 min to pellet any remaining silage. Eight mL of supernatant was pipetted into a segment of dialysis tubing approximately 8 cm in length and the ends clamped off with clips. Tubing was suspended in 500 mL of deionized water in a 600 mL beaker, covered with parafilm and placed in a gently oscillating 37°C water bath for 2 h to simulate approximate retention time of digesta in the intestine. Soluble Fe concentrations of the remaining intestinal supernatant, dialysis tube

contents and the dialysate were analyzed. Dialyzable Fe concentrations were determined as the amount of soluble Fe that disappeared from the dialysis tubing during the 2 h time period (initial supernatant Fe content – dialysis tubing Fe content).

Statistical Analysis

Statistical analysis of all data was performed by ANOVA using the MIXED procedure of SAS (SAS Institute Inc, Cary, NC, USA). The model included the fixed effects of time of soil addition (before or after ensiling), level of soil inclusion (1 or 5%) and type of soil utilized (Cecil clay loam, Georgeville silt loam or Dyke clay loam) nested within control treatment (factor) and all appropriate interactions. Interactions that were not significant ($P > 0.05$) for the measurement of interest were removed from the model. P-values ≤ 0.05 were considered to be statistically significant. Least square means are presented and represent the mean of duplicate analysis of each replicate (2 replicates per treatment for ensiled treatments and 1 replicate per treatment for nonensiled treatments).

RESULTS

Silage Characteristics

The wide-mouth half gallon plastic jars used as experimental silos in the present study appeared to work quite well. A small amount of silage at the mouth of each jar was spoiled due to oxygen exposure, but beneath this layer the silage looked and smelled like normal silage. Silage pH did not differ due to level or type of soil inclusion and averaged

4.0, suggesting that silage fermentation was normal. Chemical composition of the control silage is presented in Table 1.

Water Soluble Iron Concentrations

Water soluble concentrations of Fe were affected by a time by level of soil addition interaction ($P = 0.007$; Table 2). Soluble Fe concentrations were greater ($P = 0.001$), regardless of level of soil contamination (1 or 5%) when soil was added to greenchop prior to ensiling. However, level of soil contamination did not affect ($P > 0.05$) water soluble concentrations of Fe if soil was added after ensiling (Table 2). The percentage of total Fe that was water soluble decreased ($P = 0.004$) as total Fe concentrations increased, and was greater ($P = 0.01$) if soil was added prior to ensiling compared to if soil was added after ensiling.

There was a time of soil addition by soil type interaction ($P = 0.003$) observed with water soluble Fe concentrations (Table 3). No differences between soil types was observed if soil was added after ensiling, however, concentrations of water soluble Fe were greater ($P < 0.05$) with the Dyke clay loam compared to the Georgeville silt loam and Cecil clay loam soils when soil was added prior to ensiling. The percentage of total Fe soluble in water was increased ($P = 0.01$) across soil types when soil was exposed to the acidic environment of silage fermentation for 90 days.

In Vitro Bioaccessible Iron Concentrations

Dry matter disappearance of the control silage following the 24 h simulated ruminal digestion averaged 70% (n = 4). To confirm that enzymatic ruminal digestion was similar to that of digestion estimates using the Tilley and Terry (1963) method, in vitro true dry matter disappearance was determined. In vitro true DM disappearance averaged 76% (n = 4) for the control silage, suggesting that the enzymatic technique provided a good approximation of ruminal dry matter disappearance. For all stages of simulated digestion two or three way interactions between time, level and type of soil addition were observed. These interactions are described in Figures 1-4, but main effects as well as control silage Fe concentrations at all stages are summarized in Table 4.

Bioaccessible Fe fractions following simulated ruminal digestion were affected by a time by level by type of soil addition interaction ($P = 0.01$; Figure 1). Across soil inclusion levels and soil types, Fe concentrations were increased ($P = 0.001$) when soil was added prior to ensiling compared to when soil was added after ensiling. Concentrations of soluble Fe did not differ between 1 or 5% soil addition when soil was added after ensiling, suggesting that level of soil contamination was not as important when soil was not exposed to the acidic environment of silage fermentation. However, when soil was added prior to ensiling soluble Fe concentrations were increased ($P = 0.001$) due to the addition of 5% soil compared to 1%. Soluble Fe concentrations did not differ among soil types at 1% inclusion level if soil was added prior to ensiling, but at the 5% inclusion level Dyke clay loam had higher ($P < 0.05$) soluble Fe concentrations compared to the other two soils.

Regardless of soil level or type, bioaccessible Fe concentrations were much higher (P = 0.01) following ruminal and abomasal digestion when soil was added prior to ensiling compared to soil addition after ensiling (Table 4). Following simulated ruminal and abomasal digestion, bioaccessible Fe fractions were affected by time of soil addition by type of soil addition (P = 0.001; Figure 2a) and time of soil addition by level of soil addition (P = 0.001; Figure 2b) interactions. No differences in bioaccessible Fe were observed between soil types when soil was added to silage after ensiling (Figure 2a). However, when soil was added prior to ensiling, greater amounts of soluble Fe from the Dyke clay loam were observed at this stage compared to the other two soils. This may be partially explained by the greater Fe content (6.9%) of the Dyke clay loam soil. Conversely, when soil was added prior to ensiling more soluble Fe (P = 0.02) was observed when the contaminating soil was Cecil compared to the Georgeville soil, suggesting that chemical properties of the soils may be affecting availability of Fe, as the Georgeville soil analyzed 4.3% Fe compared to 3.4% Fe for the Cecil soil. Increasing the level of soil contamination from 1 to 5% increased (P = 0.001) bioaccessible Fe concentrations when soil was added prior to ensiling but not when soil was added after ensiling (P = 0.72; Figure 2b).

Bioaccessible Fe concentrations following simulated ruminal, abomasal and intestinal digestion are shown in Figure 3. Soluble Fe concentrations were affected by a time by level by type of soil addition to corn silage interaction (P = 0.001). For both 1 and 5% soil contamination levels, the addition of soil prior to ensiling resulted in increased ($P < 0.05$) concentrations of soluble Fe compared to the addition of soil after ensiling. Soluble Fe concentrations at the 1% inclusion level did not differ between soil types if soil was added

after ensiling; however, when soil was added prior to ensiling, both Cecil and Georgeville soils resulted in lower ($P < 0.05$) soluble Fe concentrations compared to the Dyke soil. At the 5% inclusion level an increase ($P = 0.01$) in soluble Fe concentrations was observed as soil Fe concentrations increased (Cecil < Georgeville < Dyke) when soils were added prior to ensiling. When soils were added after ensiling at the 5% inclusion level, Fe concentrations were greatest from Cecil clay loam ($P = 0.04$ compared to Georgeville), though minimal differences were observed between the 3 soil types at this inclusion level.

To more stringently test the amount of Fe from soil contamination of corn silage which might truly be of an available size for absorption by the animal, dialyzable Fe concentrations following ruminal, abomasal and intestinal digestion were determined (Figure 4). Using a dialysis membrane with a molecular weight cut off of 15000 Daltons, dialyzable Fe concentrations were affected by a time by level by type of soil addition interaction ($P = 0.02$). No differences due to soil inclusion level or soil type were observed if soil was added to corn silage after ensiling. When soil was added prior to ensiling, the addition of 1% Cecil clay loam resulted in the lowest concentration of dialyzable Fe, and Dyke clay loam the highest concentration at this inclusion level, with the Georgeville soil being intermediate to the Dyke soil. Dialyzable Fe concentrations were the greatest ($P < 0.05$) when 5% Dyke clay loam was added to corn silage prior to ensiling, while Cecil and Georgeville soils at the 5% inclusion level did not differ from the Georgeville and Dyke soils at the 1% inclusion level.

DISCUSSION

The results of the present study suggest that bioaccessibility of Fe from soil contamination of harvested corn greenchop is increased following ensiling. Soil contamination of feedstuffs or consumption by animals can be estimated using x-ray fluorescence analysis of titanium (Ti), a metal which is abundant in soils, but is present in only very low concentrations in plants (< 1 mg Ti/kg). Fries and Marrow (1981) estimated soil contamination of various ruminant feedstuffs, including corn silage and greenchop using the Ti method. They reported that apparent soil concentrations in corn silage ranged from < 0.01-0.20% DM, while greenchop soil contamination appeared slightly higher, ranging from 0.19-0.73% DM. More recently, Rafferty et al. (1994) examined rates of soil contamination of both freshly harvested and stored feedstuffs in Ireland. These authors also used Ti levels in feedstuffs as an indicator of soil presence on the surface of feeds. Soil contamination of hay and grass silage was measured at four time points, directly from the field, and 4, 8 and 12 months after initial harvest and storage. Soil contamination was found to average less than 2% (w/w) for both hay and grass silage, though contamination rates as high as 8% were observed in some samples taken after 4 months in storage. This sharp increase in soil contamination compared to samples taken directly from the field suggests that contamination probably occurred during harvest of the feedstuffs.

Several potential sources of excessive dietary Fe may be found in ruminant diets, including drinking water, soil ingestion and feedstuffs naturally high in Fe (NRC, 1996; DePeters, 2000). Little is known about the bioavailability of Fe from feedstuffs such as

alfalfa and soyhulls which are often high in Fe. It has long been assumed that soil contamination would not represent a highly bioavailable form of dietary Fe (Whitehead, 2000). The results of the present study appear to support this assumption. In general, the bioaccessibility of Fe from soil contamination was very low if soil was not first exposed to the acidic environment of silage fermentation. These results suggest that consumption of soil by grazing ruminants may not have a dramatic effect on mineral status of the animal. However, exposure of soil to the low pH associated with silage fermentation appears to alter the chemistry of Fe bound in the soil. It remains unclear as to how quickly this change may occur following exposure to an acidic environment, it may take days or weeks.

Approximately 14.7% of Fe in the control silage was soluble in water following the 5 h incubation time (Table 2). Ivan and Veira (1981) reported similar findings, with approximately 17.6 (± 1.46%) of Fe in a sheep diet consisting of 89.5% corn silage, 9.2% cornstarch and 1.3% vitamin-mineral mix becoming soluble during extraction with deionized water over a 24 h period. The slightly higher percentage Fe solubility in water reported by Ivan and Veira (1981) may be due to a 24 h water extraction procedure as compared to the 5 h used in our study.

In the present study, soluble concentrations of Fe in water were dramatically increased when soil was added to corn greenchop prior to ensiling compared to after ensiling (16 and 29 fold greater for the 1% and 5% soil inclusion levels, respectively; Table 2). This suggests that significant changes in the chemical composition of Fe in the soil-silage mix occurred during the process of silage fermentation. These changes may have included

reduction of soil Fe from the ferric to ferrous forms, as well as dissociation of some Fe from insoluble complexes such as hydrous oxides in the soil (Whitehead, 2000). In addition, bioaccessiblity of Fe from all soil types was increased following all 3 stages of digestion when soil was added prior to ensiling of corn greenchop compared to after ensiling (Figures 1-3). These results support the higher water soluble Fe concentrations observed when soil was added prior to ensiling. It appears that chemical properties of Fe bound in soils may be altered when exposed to a reducing environment such as that found with fermenting corn silage. During the process of silage fermentation, lactic acid produced by anaerobic bacteria decreases the pH to approximately 4. At this pH, silage is considered stable and until re-exposed to oxygen, can be safely stored for a considerable length of time (Schroeder, 2004). It should also be noted that any ruminant feedstuff with a low pH that may come into contact with soil during harvest, processing or storage should also be considered as potential sources of bioaccessible Fe. Both Rooke et al. (1983) and Ibrahim et al. (1990) observed that the solubility of several minerals were elevated in grass silage and corn silage compared to unfermented feedstuffs, suggesting that the acidic environment of ensiling also promoted increased mineral release of elements naturally present in the silages.

Dialyzable Fe concentrations following the 3 stage simulated digestion procedure were greatest with 5% Dyke clay loam soil added prior to ensiling (Figure 4). It is unclear why such a large fraction of the soluble Fe from this soil was of such small size. Differences in chemical properties between the soils likely affected both the potential solubility of Fe as well as the size of Fe released. A variety of factors can affect the mineral content of soils, including organic matter content, binding of minerals as insoluble complexes, weathering

and leaching of minerals and pH of the soil (Whitehead, 2000). Based on our results, it appears that if soil contamination of a feedstuff occurs before exposure to an acidic environment not only is Fe bioaccessibility increased, but at least some fraction of that Fe is of a small enough size to be absorbed by the animal. As a result, increased dietary concentrations of Fe resulting from soil contamination may have deleterious effects on the absorption of other essential trace metals.

Elevated levels of dietary Fe have been shown to negatively impact Cu status in cattle. Standish et al. (1969) reported that addition of 1600 mg supplemental Fe/kg DM to a basal diet containing 77 mg Fe/kg DM reduced liver Cu concentrations in growing beef steers from 260 to 44 mg Cu/kg DM compared to steers receiving the basal diet alone. Several subsequent studies have reported that high levels of dietary Fe result in a reduction in indices of Cu status in growing cattle similar to decreases observed by the potent Cu antagonist Mo (Humphries et al., 1983; Bremner et al., 1987; Phillipo et al., 1987). In addition to negative effects on Cu metabolism, limited research suggests that Mn metabolism in cattle may be negatively affected by high dietary Fe. Standish et al. (1971) reported that feeding a beef calves a diet containing 1000 mg Fe/kg DM for 77 days reduced both heart and kidney Mn concentrations compared to calves receiving a diet containing 100 mg Fe/kg DM.

Recent advances in molecular biology have increased our knowledge about pathways of absorption and metabolism of trace minerals such as Fe, Cu and Mn (Sharp, 2004; Mackenzie and Garrick, 2005). These advances have shed light on the molecular

mechanisms which result in antagonisms between these trace minerals. For example, the transport of ferrous Fe into the absorptive enterocyte of the small intestine has been shown to require the action of divalent metal protein 1 (DMT1) in rodents and in vitro models. Interestingly, Mn is also transported into the enterocyte by DMT1, and some evidence suggests that Cu may also utilize this route of absorption to some extent (Arredondo et al., 2003, Garrick et al., 2006). Concentrations of intestinal DMT1 are regulated by Fe status in the body as well as dietary Fe concentrations. As a result, high dietary Fe may lead to impaired absorption of Mn or Cu. We have recently demonstrated the presence of DMT1 in the duodenum of beef cattle (Hansen et al., 2008), suggesting that the molecular machinery potentially responsible for the antagonism between Fe, Mn and possibly Cu, is present in ruminants.

In summary, in vitro bioaccessibility of Fe from soil contamination of corn silage was increased when soil was exposed to the acidic environment of silage fermentation. Iron is a well known antagonist of Cu in ruminants, and appears to share a similar route of intestinal absorption with Mn. Therefore, situations of high dietary Fe may result in increased competition for intestinal absorption with Mn and Cu, negatively affecting the status of these essential trace minerals in ruminants. While there are many known sources of elevated Fe in the diets of cattle, our findings suggest that soil contamination of fermented feedstuffs may represent an overlooked source of bioavailable Fe in ruminant diets.

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Table 1. Chemical composition of control silage after a 90 day ensiling period.

Component	DM basis
Crude protein, %	8.3
Acid detergent fiber, %	24.4
Neutral detergent fiber, %	43.9
TDN, %	69
Calcium, %	0.23
Phosphorus, %	0.23
Magnesium, %	0.23
Potassium, %	1.00
Sodium, %	0.004
Iron, mg/kg	52
Zinc, mg/kg	25
Copper, mg/kg	5
Manganese, mg/kg	38
Molybdenum, mg/kg	0.5
Sulfur, %	0.10

Table 2. The effect of time and level of soil addition to corn silage on total and water soluble concentrations of Fe and percent water soluble Fe[1].

Soil Added, %	Time[2]	Total Fe, µg/g DM	Water Soluble Fe, µg/g DM	Water Soluble Fe, %
Control (0)	-------	54[d]	8[c]	14.70[a]
1	Before	1591[c]	193[b]	12.88[a]
1	After	1586[c]	12[c]	0.87[c]
5	Before	5498[b]	435[a]	7.64[b]
5	After	6830[a]	15[c]	0.24[c]
SEM	-------	235	35	1.13

[1] Time x level interaction for total Fe and water soluble Fe ($P = 0.01$); percent water soluble Fe ($P = 0.06$).

[2]Time of soil addition to corn silage, before or after ensiling for 90 days.

[a-c] Values with different superscripts in the same column differ ($P < 0.05$).

Table 3. The effect of time and type of soil addition to corn silage on total and water soluble Fe concentrations and percent water soluble Fe[1].

Soil Type	Time[2]	Total Fe, µg/g DM	Water Soluble Fe, µg/g DM	Water Soluble Fe, %
Cecil clay	Before	2518 [c]	243 [b]	11.00 [a]
Cecil clay	After	2633 [c]	13 [c]	0.78 [b]
Georgeville silt	Before	2502 [c]	169 [b]	9.33 [a]
Georgeville silt	After	3800 [b]	14 [c]	0.54 [b]
Dyke clay	Before	5614 [a]	529 [a]	10.44 [a]
Dyke clay	After	6191 [a]	14 [c]	0.35 [b]
SEM	-------	288	43	1.38

[1] Time x type interaction for total Fe ($P = 0.16$), water soluble Fe ($P = 0.003$); percent water soluble Fe ($P = 0.85$).

[2]Time of soil addition to corn silage, before or after ensiling for 90 days.

[a-c] Values with different superscripts in the same column differ ($P < 0.05$).

Table 4. Summarized least square means for the main effects of time, level and type of soil addition on bioaccessible Fe fractions following simulated ruminant digestion.

	Bioaccessible Fe Fraction, μg/g silage DM			
Main effect[1]	Ruminal	Abomasal	Intestinal	Dialyzable
Control	4	10	9	0
Before[2]	72	204	592	40
After[2]	8	20	88	6
1%[3]	28	98	150	14
5%[3]	52	126	530	32
SEM[4]	1.6	2.7	9.5	3.3
Cecil clay loam[5]	38	104	260	12
Georgeville silt loam[5]	38	98	302	16
Dyke clay loam[5]	44	134	458	42
SEM[6]	2.0	3.3	11.8	4.0

[1]For all stages of digestion: time effect ($P = 0.001$); level effect ($P = 0.001$); type effect ($P \leq 0.06$).

[2]Time of soil addition to corn silage, before or after ensiling for 90 days.

[3]Level of soil addition to corn silage.

[4]SEM presented for time and level of soil addition to corn silage across columns.

[5]Type of soil added to corn silage.

[6]SEM presented for type of soil added to corn silage across columns.

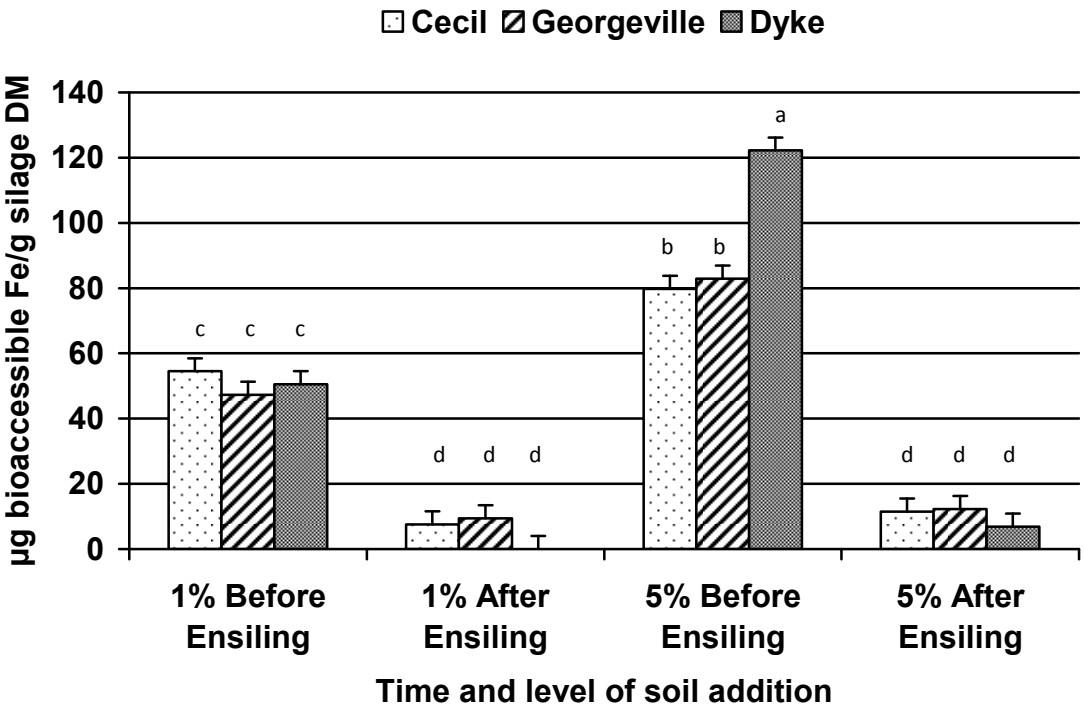

Figure 1. The effect of time, level and type of soil addition to corn silage on bioaccessible Fe concentrations following simulated ruminal digestion. Time x level x type interaction (P = 0.01). Bars with differing superscripts are different ($P < 0.05$).

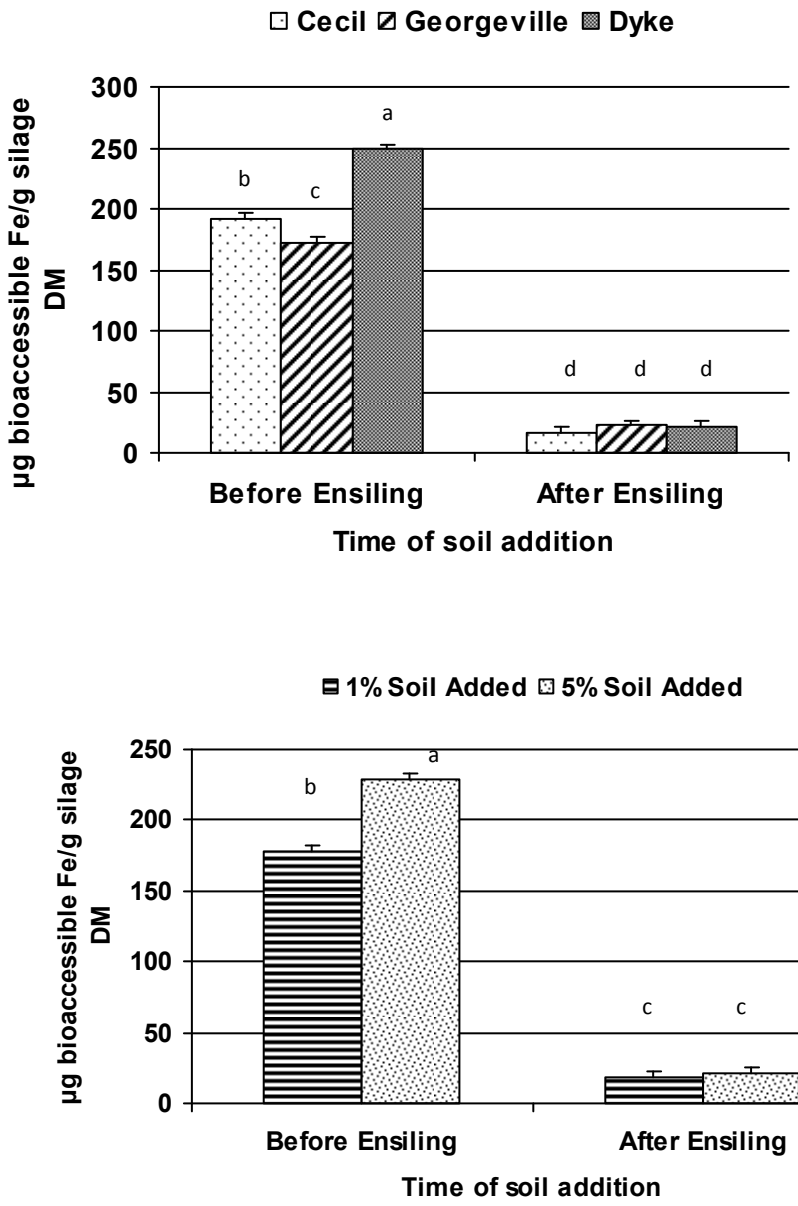

Figure 2. The effect of time and type of soil addition (2a; $P = 0.001$) and time and level of soil addition to corn silage (2b; $P = 0.001$) on bioaccessible Fe concentrations following simulated ruminal and abomasal digestion.

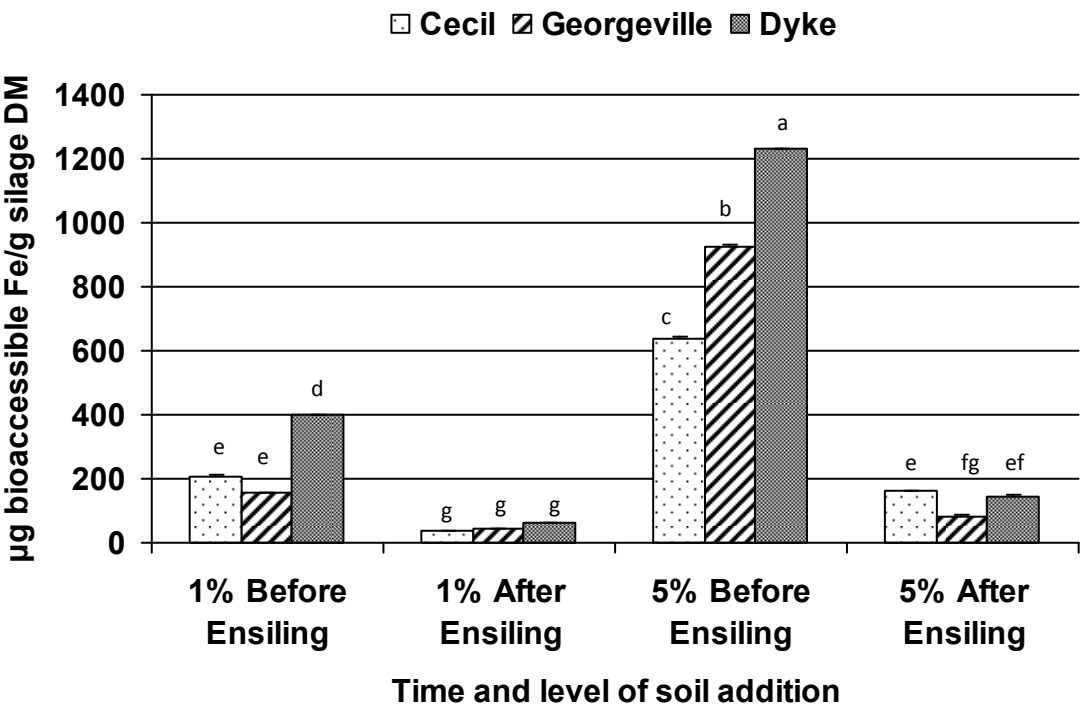

Figure 3. The effect of time, level and type of soil addition to corn silage on bioaccessible Fe concentrations following simulated ruminal, abomasal, and intestinal digestion ($P = 0.001$).

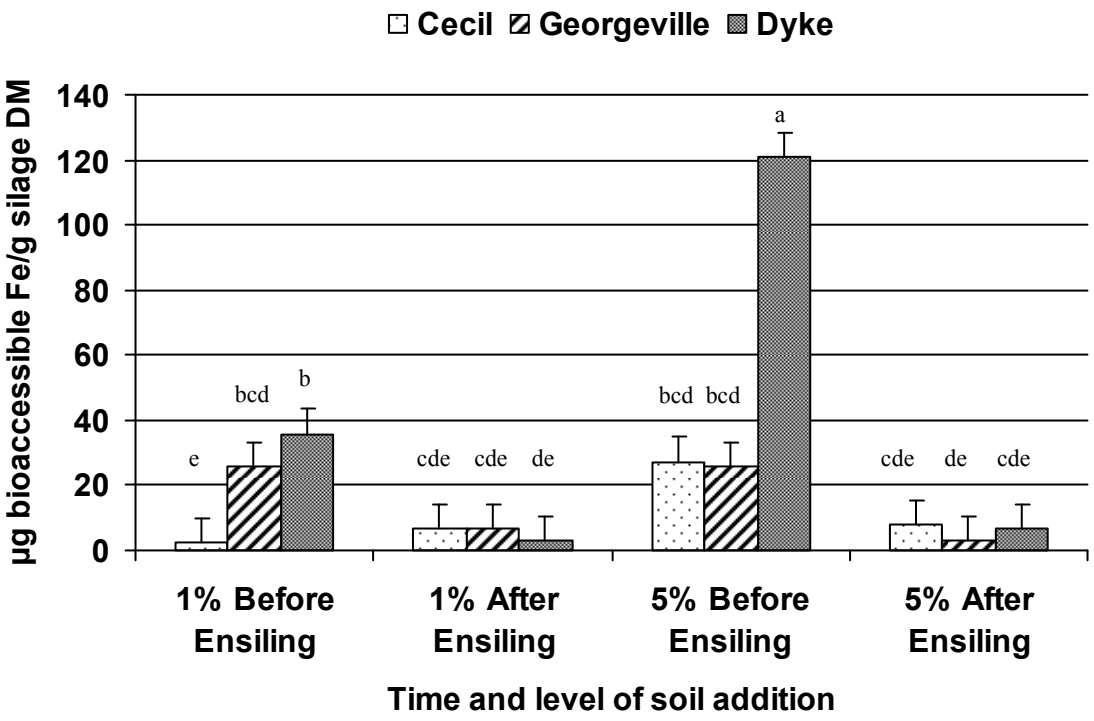

Figure 4. The effect of time, level and type of soil addition to corn silage on dialyzable Fe concentrations (< 15000 Daltons in molecular weight) following simulated ruminal, abomasal and intestinal digestion ($P = 0.02$).

CHAPTER 3

Proteins involved in iron metabolism in beef cattle are affected by copper deficiency in combination with excessive dietary manganese, but not by copper deficiency alone

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ABSTRACT: A 493-d study was conducted to determine the impact of a severe, long-term Cu deficiency on Fe metabolism in beef cattle. Twenty-one Angus calves were born to cows receiving one of the following treatments: 1) adequate Cu (+Cu), 2) Cu deficient (-Cu), and 3) Cu deficient plus high Mn (-Cu+Mn). Following weaning, calves remained on the same treatment as their dam through growing (basal diet analyzed 7 mg Cu/kg DM) and finishing (analyzed 4 mg Cu/kg DM) phases. Plasma Fe concentrations were positively correlated ($P < 0.01$; $R^2 = 0.49$) with plasma Cu concentrations. Liver Fe concentrations were increased ($P = 0.05$) in -Cu vs. +Cu calves and further increased ($P = 0.07$) in -Cu+Mn vs. -Cu calves. There was a negative relationship ($P < 0.01$; $R^2 = -0.31$) between liver Cu and Fe concentrations. This relationship is likely explained by lower ($P < 0.01$) ceruloplasmin activity in -Cu. vs. +Cu calves. In liver, relative gene expressions of both *hepcidin* and *ferroportin* were down regulated ($P < 0.10$) in Cu-deficient compared to Cu-adequate calves. In duodenum, relative gene expression of *ferritin* was upregulated ($P = 0.06$) in -Cu vs. +Cu calves. Based on Western blotting of duodenal tissue protein, concentrations of hephaestin were greater ($P = 0.02$) in -Cu+Mn compared to -Cu calves. Similarly, concentrations of ferroportin tended ($P = 0.10$) to be higher in -Cu+Mn vs. -Cu calves. However, duodenal concentrations of DMT1 were lower ($P = 0.04$) in -Cu+Mn calves vs. -Cu calves. Concentrations of hephaestin, ferroportin and DMT1 did not differ ($P > 0.1$) between +Cu and -Cu calves. In summary, while Fe and Cu status of calves were correlated, Cu deficiency alone had no impact on duodenal concentrations of proteins important in Fe metabolism. However, the addition of 500 mg Mn/kg DM to a diet low in Cu did affect levels of duodenal proteins involved in Fe metabolism.

INTRODUCTION

Copper (Cu) deficiency in cattle is a problem in many parts of the world. Based on a nationwide survey of more than 3000 serum samples from the 23 leading cow-calf states in the United States, the USDA reported that 41.6% of cattle were either moderately or severely Cu deficient (< 0.65 mg Cu/L serum; USDA, 2000). In the bovine, Cu deficiency may result in reduced growth and anemia, with the latter being due to the relationship between Cu and Fe. Copper deficiency results in reduced ferroxidase activity of two important cuproenzymes, hephaestin and ceruloplasmin, enzymes which are essential for mobilization of Fe from tissues (Sharp, 2004). Reduced ferroxidase activity causes a buildup of tissue Fe, and circulating levels of Fe are reduced, resulting in the characteristic anemia (Sharp, 2004).

We have previously reported that excessive dietary Mn further depresses both liver and plasma Cu concentrations in cattle already experiencing severe Cu deficiency (Legleiter et al., 2007; Hansen et al., 2008a). It is not uncommon to find Mn concentrations greater than 100 mg/kg DM in many types of forage (Grace et al., 1973). Increased dietary Mn has been demonstrated to negatively impact the absorption of Fe in humans (Rossander-Hulten et al., 1991). In ruminants, Hartman et al. (1955) found that hemoglobin formation and serum Fe concentrations were adversely affected in young lambs fed 1000 or 2000 mg Mn/kg DM compared to those receiving no supplemental Mn.

While there is a demonstrable interaction which exists between Cu, Mn and Fe, essentially no information exists concerning the molecular mechanisms behind this interrelationship in ruminants. Therefore, the objectives of the present study were twofold: 1) to determine if proteins known to be involved in Fe metabolism in rodents and humans are

present in the bovine and 2) to determine if gene expression and concentrations of these proteins are altered by a long-term, severe Cu deficiency in the presence or absence of excessive dietary Mn.

MATERIALS AND METHODS

Animals and Experimental Design

Care, handling and sampling procedures were approved by the North Carolina State University Animal Care and Use Committee prior to initiation of the trial. Twenty-one Angus steers (n = 12) and heifers (n = 9) averaging 38.9 ± 2.4 kg at birth were used in this study. Calves were born to cows that had been on treatments for at least 410 d by calving. Birth of the calves was considered d 0 of the study, and all days mentioned are based on average calf age on that day. Birth and management of calves through weaning has been described elsewhere (Hansen et al., 2008a). Briefly, calves were born over a 36 d period and were weaned at 183 d of age. After weaning, calves were bunk fed by treatment for a period of 43 d. On d 226 calves were moved to pens with electronic Calan gate feeders (American Calan, Northwood, NH) and were fed individually through a 136-d growing phase and a 139-d finishing phase. Calves were fed once daily, with feed amounts based on what they would consume in a 24-h period. Diets were formulated to meet or exceed all NRC recommendations (1996) with the exception of Cu. Ingredient and chemical compositions of the basal diets are shown in Table 1.

Dietary treatments included the following: 1) 10 mg supplemental Cu/kg DM (Cu adequate, +Cu; n = 6), 2) no supplemental Cu, 2 mg Mo/kg DM (Cu deficient, -Cu; n = 8), and 3) no supplemental Cu, 2 mg Mo/kg DM, and 500 mg Mn/kg DM (Cu deficient plus Mn,

-Cu+Mn; n = 7). Experimental induction of Cu deficiency in treatments -Cu and -Cu+Mn was achieved through the addition of sodium molybdate and lack of Cu supplementation. Supplemental Mo results in the formation of ruminal thiomolybdates, which bind dietary Cu in the rumen and reduce Cu absorption (Suttle, 1991). Supplemental minerals were provided as: Cu from tribasic Cu chloride ($Cu_2(OH)_3Cl$; Micronutrients, Indianapolis, IN), Mn from $MnSO_4 \cdot H_2O$ (Sulfamex, Veracruz, Mexico) and Mo from $NaMoO_4$ (Eastern Minerals, Inc., Henderson, NC).

Jugular blood samples were collected at birth and on d 114, 183, 241, 297, 422, and 459 for analysis of plasma Fe. Blood samples for hematocrit and ceruloplasmin activity were collected on d 422 and 490, respectively. Blood was collected in heparinized vacuum tubes designed for trace mineral analysis (Becton Dickenson, Rutherford, NJ), transferred on ice to the laboratory and centrifuged at 1,200 × *g* for 20 min at 20°C. Plasma was removed and stored at -20°C until analysis. Liver biopsy samples were obtained as described by Engle and Spears (2000) on d 114, 183, 297, and 422 for liver Fe determination.

Tissue Collection and Analytical Procedures

On d 493 of the study calves were harvested at a commercial abattoir. Calves were stunned via captive bolt, exsanguinated, and a sample of liver and mucosal scrapings of the proximal duodenum were collected (Hansen et al., 2008a). Plasma preparation for mineral analysis and the ceruloplasmin assay were performed as previously described (Hansen et al., 2008b). Feed, liver and duodenal scrapings were prepared for mineral analysis by wet ashing using microwave digestion (Mars 5; CEM Corp., Matthews, NC) as described by Gengelbach

et al. (1994). Mineral content of feed, plasma, liver and mucosal samples was determined by flame atomic absorption spectroscopy (Shimadzu Scientific Instruments, Kyoto, Japan).

DNA Analysis

To validate sample identity from all tissues collected at the slaughterhouse DNA from tissues was compared to DNA isolated from jugular blood samples previously collected during the trial. DNA was isolated from 30 mg tissue using the Gentra Puregene DNA Purification kit (Qiagen). The DNA was PCR amplified with 7 fluorescently labeled microsatellite markers and unique polymorphic markers were selected for animal differentiation. Fragment analysis for 5 markers was performed on an ABI 3100 Genetic Analyzer (Applied Biosystems) and alleles were sized using Genemapper v3.7 software (Applied Biosystems).

Protein Extraction and Immunoblotting Procedures

Isolation of proteins from duodenal scrapings and subsequent gel electrophoresis and transfer to a polyvinylidene diflouride membrane was performed as previously described (Hansen et al., 2008a). Membranes were probed with one of the following antibodies overnight at 4°C: monoclonal mouse anti-divalent metal transporter 1 (DMT1; Novus Biologicals, Littleton, CO; 2 μg/mL), or affinity purified polyclonal rabbit anti-ferroportin (Alpha Diagnostics, San Antonio, TX; 1 μg/mL). For determination of hephaestin concentrations membranes were probed for 1 h at room temperature using a polyclonal mouse anti-hephaestin (Abcam, Inc, Cambridge, MA; 1:1500 dilution). A partial recombinant mouse divalent metal transporter 1 protein (Novus Biologicals, Littleton, CO)

served as the positive control for DMT1 analysis and water served as the negative control for all proteins.

After incubation with the primary antibody, membranes were washed several times in PBS containing 0.05% Tween 20, incubated for 30 min with the appropriate alkaline phosphatase-linked secondary antibody, and then washed several times with the PBS-Tween 20 wash. Membranes were rinsed 2 times with water and then visualized using Lumi-Phos WB (Pierce, Rockford, IL). Images were captured on autoradiography film (CL-XPosure Film; Pierce) and band densities were semi-quantified using Image Quant TL software (Amersham Biosciences, Piscataway, NJ). Membranes were stripped with Restore Western Blot Stripping Buffer (Pierce, Rockford, IL) and reprobed with β-actin (Abcam; 1:5000 dilution for 1 h at room temperature) as a loading control. Results are reported as β-actin adjusted relative optical intensities (ROI) in arbitrary units.

RNA isolation and Real-time RT-PCR

Total RNA was isolated using an RNeasy Mini kit (Qiagen, Valencia, CA). Briefly, 30-40 mg of frozen tissue sample was homogenized in 800 µl buffer RLT using tissue homogenizer model VDI 25 and dispersion tool S100NK-7 (VWR International, West Chester, PA) and processed with RNeasy mini column following the manufacturer's protocol. On-column DNase digestion was performed with RNase-Free DNase set (Qiagen, Valencia, CA) to remove a trace of carried over genomic DNA. In addition, DNA contamination was also evaluated by an observation of "no" amplification signal (SYBR Green) of PCR reaction containing RNA sample and gene specific (RPL4) primers. The quantity of total RNA was determined using a NanoDrop spectrophotometer (Wilmington, DE) and RNA integrity was

examined on 1.2% agarose gel electrophoresis. First-strand cDNA was reverse transcribed from 4 μg of total RNA using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen Carisbad, CA) following the manufacture's recommended procedure. A total volume of 20 μl reaction of the first-strand cDNA was further diluted with 80 μl of nuclease free water to a final concentration of 40 ng/μl cDNA from total RNA. Quantitative RT-PCR was performed in triplicate on an iQ5 Real Time PCR detection System (Bio-Rad Hercules, CA) with a total volume of 20 μl reaction containing cDNA from 80 ng of total RNA, 500 nM each of forward and reverse primers and 1X SYBR Green Master Mix (Qiagen). Real-time PCR of 40 cycles (95 °C for 10 sec and 56 °C for 30 sec) was performed with an initial denaturation step (95 °C for 10 min) and a final melting curve analysis for all primers used (Table 2). Gene specific primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) except for *rps9* which was reported by Janovick-Guretzky et al. (2007). Amplification efficiency of each primer pair was evaluated using a 10-fold serial dilution of cDNA and specificity of amplified product was evaluated by melting curve analysis (iQ5 optical system software, Bio-Rad) and gel electrophoresis. The Ct values were analyzed by a PCR base line subtracted curve fit, a default setting of the iQ5 software. The Ct data was then transformed to quantities using the $2^{-\Delta Ct}$ method described by Livak and Schmittgen (2001) by which the highest quantity of each gene and tissue type was set to 1. The relative transcript abundance was then normalized by an appropriate housekeeping gene based on its stable expression between treatments.

Statistical Analysis

Statistical analysis of all data was performed by ANOVA for a completely randomized design using the MIXED procedure of SAS (SAS Institute Inc, Cary, NC). Individual animal served as the experimental unit in all analysis. For analysis of protein data and duodenal mineral concentrations the model included the fixed effect of treatment and sex, the treatment by sex interaction was not found to be significant ($P > 0.10$) for any variable. When treatment was significant ($P \leq 0.10$), differences among means were separated using single df orthogonal contrasts. The comparisons made included: +Cu vs. -Cu and -Cu vs. -Cu+Mn. Plasma and liver Fe data were analyzed as repeated measures and the model included the fixed effects of treatment, sex, time and the appropriate interactions. When a treatment by time interaction was observed, data were further analyzed by sampling day. Interactions that were not significant ($P > 0.10$) for the measurement of interest were removed from the model. When treatment was significant, the previously stated contrasts were also used for analysis of these data. Simple correlations were determined using the CORR procedure of SAS. Relative gene expression data were analyzed using the MIXED Procedure of SAS using the previously stated contrasts.

RESULTS

Plasma and blood measurements

Plasma and liver Cu concentrations have been published elsewhere (Hansen et al., 2008a). Plasma Cu concentrations across all sampling d averaged 1.07 mg/L for +Cu, 0.22 mg/L for -Cu and 0.12 mg/L for -Cu+Mn. Plasma Fe concentrations, analyzed as repeated measures over the course of the study were affected by calf sex ($P = 0.05$), treatment ($P =$

0.01), time ($P = 0.01$) and treatment by time ($P = 0.02$; Table 3). Iron concentrations in the plasma were greater in heifers (1.60 mg Fe/L) compared to steers (1.46 mg Fe/L). Plasma Fe concentrations at weaning (d 183) were lower ($P = 0.01$) in -Cu calves compared to +Cu calves, and did not differ between -Cu and -Cu+Mn calves. During the growing phase (d 241 and 297) plasma Fe was lower ($P < 0.05$) in calves receiving the -Cu diet compared to calves receiving the +Cu diet, while the addition of high Mn to the low Cu diet (-Cu+Mn) tended ($P \leq 0.10$) to further reduce plasma Fe concentrations compared to -Cu. During the finishing phase (d 422) plasma Fe concentrations increased in calves receiving the -Cu diet, and were not different from +Cu calves at this time point, while plasma Fe in calves receiving the -Cu+Mn diet remained lower ($P = 0.04$) than -Cu calves. Interestingly, no differences in plasma Fe concentrations between treatments were observed on d 459 of the study. As shown in Table 5, plasma Cu and Fe concentrations were positively correlated ($R^2 = 0.49$; $P = 0.001$). Hematocrit was measured on d 422 and did not differ between +Cu (31.8%) and -Cu (32%) calves, but was lower ($P = 0.04$) in -Cu+Mn (28.1%) compared to –Cu calves.

Tissue mineral concentrations

Based on repeated measures analysis of liver biopsies taken during the trial liver Fe concentrations were greater ($P = 0.05$) in -Cu calves vs. +Cu calves and tended to be greater ($P = 0.07$) in -Cu+Mn calves vs. -Cu calves (Table 4). Liver Fe concentrations were affected by time ($P = 0.001$) and tended ($P = 0.12$) to be affected by a time by treatment interaction. When analyzed by sampling d, Fe concentrations in the liver did not differ due to treatment on d 114 and 183, but tended to be lower ($P = 0.07$) in +Cu calves compared to -Cu calves, and higher ($P = 0.07$) in -Cu+Mn calves compared to -Cu calves on d 297. Liver Fe

concentrations did not differ due to dietary treatment on d 422, which may be related to the much lower Fe concentrations in the finishing diet (52 mg Fe/kg DM) relative to the growing diet. Liver Cu and Fe concentrations were negatively correlated (Table 5; R^2 = -0.31; *P* = 0.002). Duodenal Cu concentrations were lower (*P* = 0.01) in -Cu calves compared to +Cu calves, and did not differ between the Cu-deficient treatments (Table 6). Mucosal Mn concentrations did not differ between +Cu and -Cu calves, but were greater (*P* = 0.01) in -Cu+Mn calves compared to -Cu calves. Concentrations of Fe in the mucosal scrapings did not differ between treatments.

Liver and duodenal gene expression

Expression of genes important in Fe metabolism in liver and duodenum were determined. Because of uneven numbers of heifers and steers in each experimental treatment, only tissues collected from steers were utilized in this analysis. This resulted in an overall n of 12, with the following breakdown by treatment: n = 3 (+Cu); n = 4 (-Cu); and n = 5 (-Cu+Mn). As shown in Table 7, *ferroportin* expression in liver tended (*P* = 0.09) to be down regulated in -Cu steers compared to +Cu steers. Hepatic *hepcidin* expression was downregulated (*P* = 0.03) in –Cu steers compared to +Cu steers. *Dmt1 non-ire, dmt1 ire* and *ferritin* expression did not differ between -Cu and +Cu steers. Expression of *dmt1 non-ire*, *dmt1 ire*, *ferroportin*, *ferritin* and *hepcidin* did not differ between -Cu and -Cu+Mn steers. In duodenal scrapings (Table 8), relative expression of *ferritin* was upregulated (*P* = 0.06) in -Cu steers vs. +Cu steers. Relative expression of *dmt1 non-ire, dmt1 ire and ferroportin* did not differ between -Cu and +Cu steers and expressions of both *dmt1* isoforms, *ferroportin* and *ferritin* did not differ between -Cu and -Cu+Mn steers.

Duodenal protein levels

Duodenal levels of hephaestin were increased ($P = 0.01$) in -Cu+Mn calves compared to -Cu calves and did not differ ($P = 0.53$) between +Cu and -Cu calves (Figure 1). Hephaestin migrated to an apparent molecular weight of approximately 150 kDa. Intestinal levels of ferroportin were also not different between +Cu and -Cu calves; however, ferroportin levels tended to be greater ($P = 0.10$) in -Cu+Mn calves compared to -Cu calves (Figure 2). Bovine intestinal ferroportin had an apparent molecular weight of approximately 60 kDa. Levels of DMT1 were lower ($P = 0.04$) in calves receiving the -Cu+Mn diet compared to -Cu calves (Figure 3). No differences in DMT1 concentrations were observed between +Cu and -Cu calves, and DMT1 had an apparent molecular weight of approximately 55 kDa.

DISCUSSION

In this study we demonstrated the presence of DMT1, ferroportin and hephaestin, proteins important in Fe metabolism, in bovine duodenum for the first time. Additionally, this study is the first to report on the expression of both isoforms of *dmt1, ferroportin,* and *hepcidin* in the bovine.

It is well accepted that the metabolism of Fe and Cu are closely related (Sharp, 2004). Two multi-copper oxidases, ceruloplasmin and hephaestin, are necessary for Fe to be mobilized out of the liver and intestine, respectively (Osaki, 1971; Chen et al., 2006). In the present study, both -Cu and -Cu+Mn calves exhibited severe Cu deficiency, as evidenced by extremely low plasma Cu concentrations and decreased liver Cu concentrations (approximately 6 mg/kg DM; Hansen et al., 2008a). Ceruloplasmin activity was also greatly

reduced in both Cu deficient treatments by d 490 (34.94 mg/dL in +Cu; 4.72 mg/dL in -Cu and 2.96 mg/dL in -Cu+Mn). Limited ceruloplasmin activity probably prevented Fe from being mobilized out of the liver, causing Fe to accumulate in the liver and limiting Fe availability for extrahepatic tissues. There was a strong negative correlation between liver Cu and liver Fe concentrations, with low liver Cu concentrations leading to increased Fe concentrations. It appears that the greatest impact of Cu deficiency on liver Fe accumulation was during the growing phase when the diet contained a rather excessive amount of Fe (765 mg/kg DM), perhaps allowing for a more rapid buildup of hepatic Fe.

Because of reduced export of Fe from the liver, plasma Fe concentrations of –Cu and –Cu+Mn calves were decreased. It has been suggested that plasma Fe concentrations of less than 1.32 mg/L may be indicative of Fe deficiency in beef cattle (Puls, 1994). Based on this suggestion, -Cu+Mn calves were at least marginally Fe-deficient on four out of the five sampling dates, and were only slightly over this threshold on d 422. Calves receiving the –Cu diet averaged approximately 1.47 mg Fe/L plasma over the course of the study, but had plasma Fe concentrations less than 1.32 mg/L on d 183 and 241. Plasma Fe concentrations were normal for calves receiving the Cu-adequate diet.

Calves receiving the –Cu+Mn diet had lower plasma Cu concentrations compared to –Cu calves and appeared to suffer from a more extensive Cu deficiency (Hansen et al., 2008a). The increased severity of Cu deficiency may have more negatively affected the Fe status of –Cu+Mn calves compared to calves receiving the Cu-deficient diet alone, as evidenced by lower plasma Fe concentrations and reduced hematocrit on d 422. Excessive dietary Mn may have antagonized intestinal Cu absorption or interacted with Cu metabolism

in some other manner to further exacerbate the effects of a low Cu, molybdenum-supplemented diet on Cu status of these calves.

Alternately, supplementation with high dietary Mn may have directly interfered with Fe metabolism. Hartman et al. (1955) found that hemoglobin concentrations in weaned lambs were lower in lambs supplemented with 1000 or 2000 mg Mn/kg DM compared to those fed a diet with no supplemental Mn. Additionally, following discontinuation of the treatments hemoglobin concentrations began to rise in both supplemental Mn groups. The authors speculated that excessive dietary Mn compromised hemoglobin formation, possibly due to decreased Fe availability for heme synthesis because of competition for absorption between Fe and Mn (Hartman et al., 1955).

Recently, in vitro work has suggested that both Mn and Fe can be transported by DMT1, and excessive concentrations of either mineral have been demonstrated to negatively affect cellular uptake of the other (Gunshin et al., 1997; Garrick et al., 2006). Additionally, the Belgrade rat, which suffers from a spontaneous mutation of DMT1 that renders the protein inactive, has provided supporting evidence for the role of DMT1 in cellular Mn uptake. Belgrade rats are not only highly anemic because of limited absorption of dietary Fe, they also have lower tissue Mn concentrations than their wild type counterparts, suggesting that DMT1 is important in Mn absorption as well (Chua and Morgan, 1997).

Iron metabolism is a closely regulated process which requires the coordinated efforts of several different proteins. At the intestinal level, the Fe importer DMT1 is essential for adequate Fe uptake by the enterocyte, while another Fe transporter, ferroportin, works in concert with the ferroxidase hephaestin to mobilize Fe out of the enterocyte and into the

bloodstream (De Domenico et al., 2008). In the present study intestinal expressions of both isoforms of *dmt1* and *ferroportin* were not affected by Cu deficiency alone or in the presence of excessive dietary Mn. However, *ferritin* expression was increased in Cu-deficient calves, suggesting that Cu deficiency was causing accumulation of Fe in the duodenum, probably due to reduced Fe export because of reduced activity of the Cu oxidase hephaestin. This reduced Fe export results in low plasma Fe levels, causing release of iron regulatory proteins into circulation which act to stabilize mRNA of proteins important in Fe acquisition such as DMT1 and ferroportin, and to increase degradation of mRNA of proteins involved in Fe storage such as ferritin (De Domenico et al., 2008).

Hepatic expression of *ferroportin* tended to be reduced in Cu-deficient calves compared to Cu-adequate calves. This observed downregulation in the cellular Fe exporter may be a result of reduced mobilization of Fe out of the liver because of reduced ceruloplasmin activity attributable to the severe Cu deficiency. Liver expression of *hepcidin* was down regulated in Cu-deficient calves compared to Cu-adequate calves. Hepcidin is a small liver-derived peptide that is expressed when body Fe status is high. Active hepcidin binds to the cellular Fe exporter ferroportin, causing it to be internalized and degraded, resulting in a drop in cellular Fe export, and thus plasma Fe concentrations (De Domenico et al., 2008). In the present study the down regulation of *hepcidin* is probably due to the moderate deficiency of Fe that both Cu-deficient treatments were experiencing. Similarly, Chen et al. (2006) reported that hepcidin expression was down regulated in Cu-deficient mice and attributed the down regulation to a secondary Fe deficiency induced by the primary deficiency of Cu. In the present study a combination of increased liver Fe concentrations and

reduced plasma Fe concentrations in both Cu-deficient treatments suggests that *hepcidin* expression is regulated by systemic signals of Fe status rather than local signals of Fe stores.

In the present study, increased concentrations of ferroportin and hephaestin protein were only observed in -Cu+Mn calves. Chen et al. (2006) also noted that expression and protein levels of ferroportin were increased in Cu-deficient mice in response to a secondary deficiency of Fe. In the present study it appears that -Cu+Mn calves were experiencing a more extensive Fe deficiency than -Cu calves, and thus higher protein levels of ferroportin and hephaestin in these calves may have been an attempt to increase absorption of Fe. Although hephaestin protein was increased in -Cu+Mn calves, hephaestin activity was not measured in the present study and because Cu is required for the enzyme to function, it is unlikely that much activity was present due to the extreme state of Cu deficiency these cattle were experiencing.

The results of the current study are intriguing, because based on the observed increases in ferroportin and hephaestin protein in the duodenum of -Cu+Mn calves, we expected to observe a similar increase in DMT1 protein levels. It is not entirely clear why DMT1 protein was lower in -Cu+Mn calves. The decrease in DMT1 levels may suggest indirect regulation of this protein by excessive levels of dietary Mn in combination with a severe deficiency of Cu. Low dietary Cu may have limited hephaestin activity, causing Fe to buildup in the enterocyte. This local signal of increased Fe stores may have provided negative feedback to the DMT1 protein, resulting in degradation of the active protein to reduce Fe import into the enterocyte. Because relative expression of both isoforms of *dmt1* was not different due to dietary treatment it appears that lower protein levels may be a result

of degradation of DMT1 protein or transcript. Limited in vitro work has suggested that Cu may also be transported via DMT1, in addition to Fe and Mn (Arredondo et al., 2003).

In summary, it appears that while severe Cu deficiency resulted in decreased Fe status and alterations in expression of several genes involved in Fe metabolism, the Fe status of these calves was not lowered enough to induced changes in intestinal proteins involved in Fe metabolism. However, the addition of high dietary Mn to a diet low in Cu (-Cu+Mn) resulted in a more severe Cu deficiency, and appeared to induce a more robust deficiency of Fe. As a result, increases in intestinal hephaestin and ferroportin protein levels were observed in -Cu+Mn calves.

In conclusion, we were able to detect for the first time proteins important in Fe metabolism in bovine intestinal tissues. These proteins included hephaestin, ferroportin and DMT1. In addition, we report that gene expressions of *hepcidin* and *ferroportin* in the bovine liver and intestinal *ferritin* were affected by Cu status of the animal. In rodents, the role of DMT1 in transport of Fe, Mn and Cu may at least partially explain the interactions often observed between these trace elements. The presence of DMT1 in the bovine duodenum provides an intriguing possibility that this protein may be the common link in the pathways of absorption of Fe, Mn and Cu in the ruminant. Further research is necessary to characterize the interaction among these trace minerals and determine how these interactions affect the trace mineral requirements of cattle.

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Table 1. Ingredient composition of growing and finishing diet.

Ingredient	Growing Phase[1]	Finishing Phase[2]
	% DM	
Corn silage	86.88	---
Ground corn	---	83.80
Soybean meal (48%)	9.00	7.00
Cottonseed hulls	---	5.00
Urea	1.00	0.80
$CaSO_4$	0.80	0.80
Limestone	0.10	0.40
NaCl	0.20	0.20
Vitamin premix[3]	0.01	0.01
Trace mineral premix[4]	0.01	0.01
Monensin[5]	---	+
Treatment supplement[6]	2.00	2.00

[1] Analyzed 7 mg Cu, 59 mg Cu and 765 mg Fe per kg DM.

[2] Analyzed 4 mg Cu, 32 mg Mn and 52 mg Fe per kg DM.

[3] Provided per kilogram of premix: 1.98 g all-trans retinol; 38 mg cholecalciferol, and 4.2 g DL-α-tocopheryl acetate.

[4]Provided per kilogram of diet: 30 mg of Zn as $ZnSO_4$; 0.5 mg of I as $Ca(IO_3)_2(H_2O)$; 0.2 mg of Se as Na_2SeO_3; and 0.1 mg of Co as $CoCO_3$.

[5]Provided 33 mg of monensin/kg DM.

[6]A ground corn 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); -Cu+Mn (500 mg Mn/kg DM, 2 mg Mo/kg DM).

Table 2. Real-time RT-PCR Primers.

Primer[1]	GenBank GI Number	Primer Sequence Forward (5'-3')	Primer Sequence Reverse (5'-3')	Product Length (bp)
dmt1non-ire	114051719	TGGTTTCAAAGCCATTGTGC	TGGTACCAGTGCCCCAGTC	113
dmt1 ire	154426003	AGTTGCTCTGGGTTCTTCTGT	CTGGGATACTGGCGGTGACAC	121
ferroportin	118151031	CAGTTGCTGCAAGAAAATGTT	CCATGATGAAGTGCAGAAGGT	100
ferritin	54262148	AAGCCATCTCAAGATGAGTG	TTCTCCAGGAAGTCACAGAT	146
hepcidin	167583521	CACGACAGCTCACAGACCTC	CTTTACGACAGCAGCCACAG	133
rps9[2]	155372028	CCTCGACCAAGAGCTGAAG	CCTCCAGACCTCACGTTTGTTC	63
rpl4	62460479	CGTTTCTGCATTTGGACTGA	GAGCATCTTGTGCATAGGGA	114

[1] Primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/).
[2]Rps9 primers were reported by Janovick-Guretzky et al. (2007).

Table 3. Effect of dietary Cu and Mn on plasma Fe concentrations in growing calves.

	Treatment[1]				Contrasts	
Calf age	+Cu	-Cu	-Cu+Mn	SEM	+Cu vs. -Cu *P*-value	-Cu vs. -Cu+Mn *P*-value
Overall[2,3,4,5]	1.86	1.47	1.27	0.06	0.01	0.04
Day 183	2.14	1.16	1.10	0.14	0.01	0.79
Day 241	1.56	1.27	1.06	0.08	0.03	0.10
Day 297	2.00	1.45	1.17	0.09	0.01	0.07
Day 422	1.99	2.07	1.42	0.22	0.80	0.04
Day 459	1.63	1.39	1.29	0.12	0.18	0.59

[1] +Cu, Cu-adequate; -Cu, Cu-deficient; -Cu+Mn, Cu-deficient plus high Mn.

[2] Means across all sampling times expressed as mg/L plasma.

[3] Time effect ($P < 0.001$).

[4] Time x treatment effect ($P = 0.02$).

[5] Sex effect ($P = 0.05$).

Table 4. Effect of dietary Cu and Mn on liver Fe concentration in growing calves.

	Treatment[1]				Contrasts	
Calf age	+Cu	-Cu	-Cu+Mn	SEM	+Cu vs. -Cu *P*-value	-Cu vs. -Cu+Mn *P*-value
Overall[2,3,4]	321.2	390.9	454.8	23.93	0.05	0.07
Day 114	250.0	265.4	233.1	33.67	0.73	0.51
Day 183	329.4	427.4	532.8	54.54	0.21	0.18
Day 297	393.3	533.6	666.1	50.71	0.07	0.07
Day 422	312.0	337.2	392.7	46.48	0.70	0.40

[1] +Cu, Cu-adequate; -Cu, Cu-deficient; -Cu+Mn, Cu-deficient plus high Mn.

[2] Means across all sampling times expressed as mg/kg DM.

[3] Time effect ($P < 0.001$).

[4] Time x treatment effect ($P = 0.12$).

Table 5. Simple correlation of plasma Cu or liver Cu concentrations to plasma Fe or liver Fe concentrations of calves fed varying amounts of dietary Cu and Mn.

Copper Indices	Iron, R^2	*P*-value
Plasma	0.49	0.001
Liver	-0.31	0.002

Table 6. Effect of dietary Cu and Mn on mineral concentration of duodenal scrapings.

	Treatment[1]				Contrasts	
Mineral[2]	+Cu	-Cu	-Cu+Mn	SEM	+Cu vs. -Cu *P*-value	-Cu vs. -Cu+Mn *P*-value
Copper	22.0	6.5	6.4	3.5	0.01	0.98
Manganese	36.2	30.0	207.4	42.9	0.93	0.01
Iron	290.4	265.0	345.6	72.0	0.82	0.39

[1] +Cu, Cu-adequate; -Cu, Cu-deficient; -Cu+Mn, Cu-deficient plus high Mn.

[2] Values expressed as mg/kg DM.

Table 7. Gene expression profiles in liver.

Gene[2]	Treatment[1]			SEM	Contrasts	
	+Cu	-Cu	-Cu+Mn		+Cu vs. -Cu *P*-value	-Cu vs. -Cu+Mn *P*-value
dmt1 non-ire	0.85	1.33	0.94	0.29	0.32	0.30
dmt1 ire	1.42	1.21	1.22	0.14	0.35	0.96
ferroportin	1.52	1.09	1.18	0.14	0.09	0.60
ferritin	1.23	0.94	0.66	0.29	0.52	0.49
hepcidin	1.76	1.08	0.72	0.17	0.03	0.15

[1] +Cu, Cu-adequate; -Cu, Cu-deficient; -Cu+Mn, Cu-deficient plus high Mn.

[2]Cycle threshold (CT) ratios are shown as the ratio of the target gene/*rpl4*.

Table 8. Gene expression profiles in duodenum.

Gene[2]	Treatment[1]			SEM	Contrasts	
	+Cu	-Cu	-Cu+Mn		+Cu vs. -Cu *P*-value	-Cu vs. -Cu+Mn *P*-value
dmt1 non-ire	0.35	0.58	0.49	0.18	0.41	0.72
dmt1 ire	0.54	0.59	0.69	0.14	0.83	0.59
ferroportin	0.70	0.65	0.67	0.19	0.88	0.96
ferritin	0.59	0.94	0.91	0.11	0.06	0.89

[1] +Cu, Cu-adequate; -Cu, Cu-deficient; -Cu+Mn, Cu-deficient plus high Mn.

[2]Cycle threshold (CT) ratios are shown as the ratio of the target gene/*rps9*.

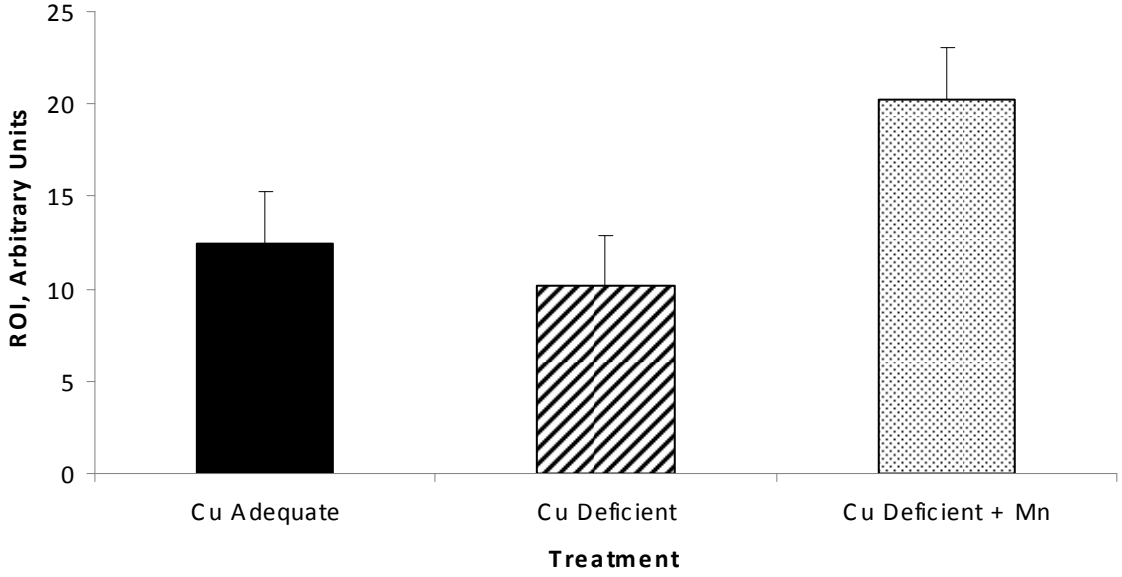

Figure 1. Actin-adjusted relative optical intensities of hephaestin concentrations in duodenal mucosal scrapings based on Western blot analysis. Contrast: -Cu vs. –Cu+Mn ($P = 0.02$).

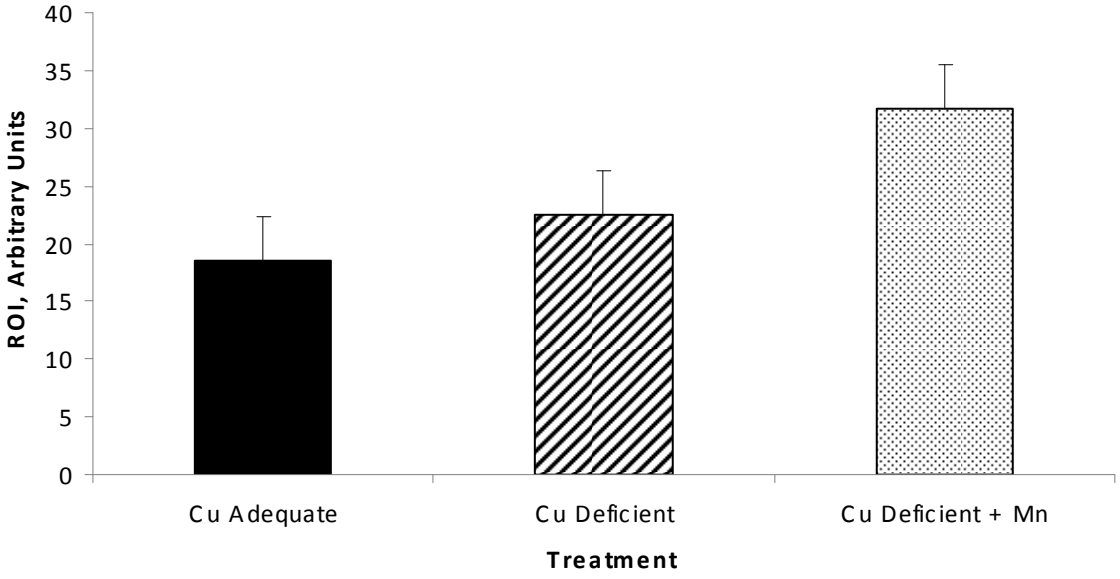

Figure 2. Actin-adjusted relative optical intensities of ferroportin concentrations in duodenal mucosal scrapings based on Western blot analysis. Contrast -Cu vs. –Cu+Mn: ($P = 0.10$).

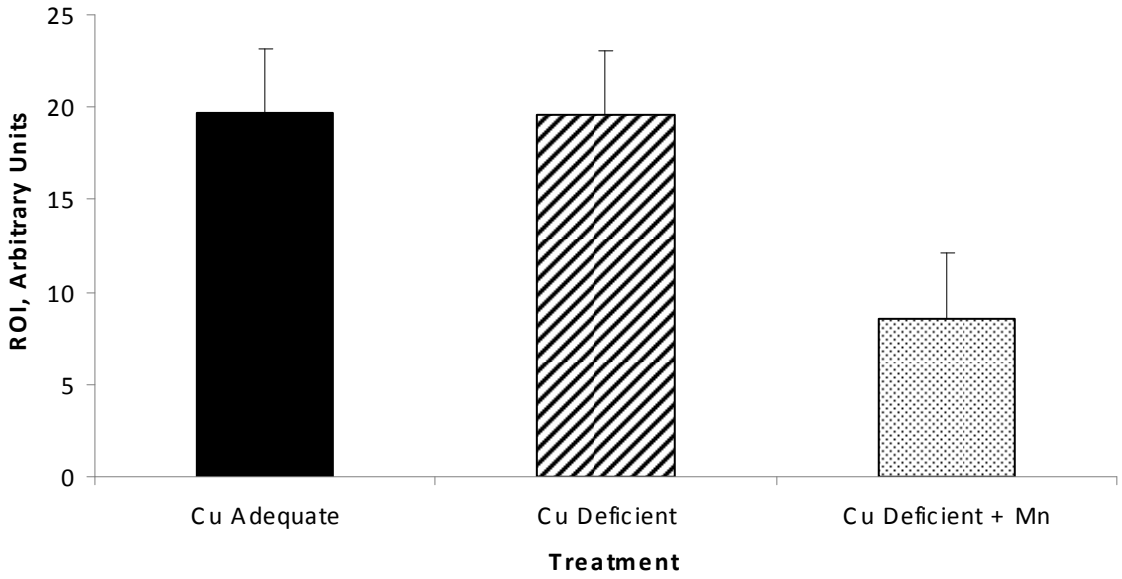

Figure 3. Actin-adjusted relative optical intensities of divalent metal transporter 1 concentrations in duodenal mucosal scrapings based on Western blot analysis. Contrast –Cu vs. –Cu+Mn: ($P = 0.04$).

CHAPTER 4

The effect of dietary iron on iron status and intestinal proteins important in iron metabolism in nursery pigs

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ABSTRACT: Twenty-four weanling male pigs, averaging 21 d of age, were blocked by litter and weight, and randomly assigned to 1 of 3 dietary treatments: 1) no supplemental Fe (low Fe; L-FE); 2) 100 mg supplemental Fe/kg DM (adequate Fe; A-FE); and 3) 500 mg supplemental Fe/kg DM (high Fe; H-FE). The basal diet contained 20 mg Fe/kg DM and supplemental Fe was provided as $FeSO_4$. Pigs were harvested for tissue collection and Ussing chamber experiments after 32 days on study. Average daily gain for the 32-d period was greater ($P < 0.01$) in A-FE pigs (328 g/d) compared to L-FE pigs (224 g/d), and did not differ between A-FE pigs and H-FE pigs (290 g/d). Liver Fe increased ($P < 0.01$) with increasing dietary Fe. Hemoglobin concentrations on d 32 were lower ($P < 0.05$) in L-FE pigs (6.2 g/dL) compared to A-FE (12.8 g/dL), but did not differ between A-FE and H-FE (13.3 g/dL). Duodenal concentrations of divalent metal transporter 1 (DMT1) tended ($P = 0.18$) to be lower in H-FE pigs compared to L-FE pigs. Intestinal levels of ferroportin also tended ($P = 0.17$) to be lower in H-FE pigs compared to L-FE pigs. Liver Mn concentrations were lower ($P < 0.05$) in H-FE pigs (8.1 mg/kg DM) than in A-FE (12.7 mg/kg DM) or L-FE pigs (11.7 mg/kg DM). Duodenal Mn concentrations were greater ($P < 0.05$) in L-FE pigs compared to A-FE or H-FE pigs. In conclusion, supplementation of 500 mg Fe/kg DM to weanling pigs tended to decrease levels of DMT1 and ferroportin, which may have resulted in reduced absorption of Mn.

INTRODUCTION

It is well known that the rapidly growing nursery pig has a high Fe requirement (80 mg/kg DM; NRC, 1998); however, many commercial swine diets contain levels of Fe in

considerable excess of this requirement. Though bioavailability of Fe from most swine feedstuffs is relatively unknown, many are very high in Fe, including blood meal (3,000 mg/kg DM), dicalcium phosphate (10,000 mg Fe/kg DM), and limestone (3500 mg Fe/kg DM; NRC, 1998). High dietary Fe may increase tissue oxidative stress through production of free radicals, as well as interfere with the absorption of other essential trace minerals such as Mn and Cu through competition for common intestinal transporters (Arredondo et al., 2003; Garrick et al., 2006, Ganz and Nemeth, 2006). In the past decade, much has been learned about Fe absorption and the many proteins involved in this pathway. Included in this pathway are two well characterized proteins, the cellular Fe importer divalent metal transporter 1 (DMT1) and the cellular Fe exporter ferroportin (Fpn). A limited body of work has demonstrated the presence of DMT1 and Fpn in the digestive tract of pigs (Blachier et al., 2007; Öhrvik et al., 2007; Tako et al., 2007). However, little work exists which shows the potential regulation of these proteins by dietary concentrations of Fe in the weaned pig. Because in vitro work has suggested that both Mn and Cu may also be transported by DMT1 (Arredondo et al., 2003), it is also of interest to determine the impact of excessive dietary Fe on tissue concentrations of these two trace elements.

Therefore, our objective was to examine proteins important in Fe metabolism in the weaned pig as well as the regulation of these proteins by dietary Fe. In addition, we examined the potential impact of varying levels of dietary Fe on the metabolism of Mn and Cu.

MATERIALS AND METHODS

Animals and experimental design

Care, handling, and sampling procedures were approved by the North Carolina State University Animal Care and Use Committee prior to initiation of the trial. Twenty-four weanling male pigs averaging 21 d of age and 5.53 ± 0.44 kg BW were used. All piglets received an injection of only 100 mg Fe from Fe dextran at birth to minimize body Fe stores prior to initiation of the trial. Pigs were weaned and blocked by litter and weight, and randomly assigned to one of 12 pens. Each pen contained a self feeder and a nipple waterer to allow for ad libitum access to food and water throughout the 32-d trial. Treatments included: 1) no supplemental Fe (low Fe; L-FE), 2) 100 mg supplemental Fe/kg DM (adequate Fe; A-FE) and 3) 500 mg supplemental Fe/kg DM (high Fe; H-FE). In order to formulate a diet which was well below the young pigs nutritional requirement for Fe, casein and dried skim milk were substituted for soybean meal which can contain more than 100 mg Fe/kg DM (NRC, 1998). The basal diet was formulated based on NRC (1998) recommendations, to meet or exceed dietary requirements of the pigs for all nutrients with the exception of Fe. The ingredient and chemical composition of the basal diet is shown in Table 1. The basal diet analyzed to contain 20 mg Fe/kg DM (approximately 25% of the young pig's requirement for Fe) and supplemental Fe was provided as $FeSO_4$.

Pigs were housed in pens of 2 (four replicate pens per treatment) in an environmentally controlled nursery. Room temperature in the nursery was 28°C for the first week and was lowered by 1°C each subsequent week.

Sample collection and Ussing chamber experiments

Piglet weights were collected on d 0 and 32 of the study. Jugular blood samples were collected on d 0, 14 and 32 of the trial for analysis of serum Fe and hemoglobin. Blood was collected in trace mineral-free heparinized vacuum tubes designed for trace mineral analysis (Becton Dickenson, Rutherford, NJ, USA) and transferred on ice to the laboratory. Blood for serum analysis was collected in vacuum tubes and allowed to clot at room temperature for 1 h prior to centrifugation.

Due to access to a limited number of Ussing chambers, 2 pigs per treatment were harvested over 3 consecutive d (33-35). A total of 18 pigs (n = 6 per treatment) were utilized in the Ussing chamber experiments. On d 36 the remaining 6 pigs were euthanized and tissues were collected in an identical manner (described below), for protein analysis. In order to minimize intestinal stress, pigs were sedated prior to euthanasia. Pigs were sedated using a combination of Telazol, Ketamine and Xylazine at a dose of 0.03 mL/kg BW. Once completely sedated, pigs were euthanized using Fatal Plus (pentobarbital sodium) at a dose of 2.2 mL/kg BW given intravenously via a catheterized ear vein. Immediately following euthanasia a segment of the proximal duodenum extending approximately 10 cm distal to the pyloric sphincter was harvested from the pig and placed in cold oxygenated (95% O_2-5% CO_2) Ringer solution (Moeser et al., 2007). The next 25 cm of duodenum was removed from the pig, cut open longitudinally and rinsed generously with 0.87% saline to remove any digesta prior to sample collection. Duodenal scrapings of the exposed mucosa were performed as previously described (Hansen et al., 2008b) for trace mineral and protein analysis, and immediately flash frozen in liquid N_2 to prevent protein degradation. Liver

samples collected from each pig were rinsed thoroughly with PBS to remove hepatic blood contamination of tissue, wrapped in aluminum foil and flash frozen in liquid N_2.

For Ussing chamber experiments, the mucosa was stripped from the seromuscular layer and mounted in 1.13 cm^2 apperature Ussing chambers as previously described (Moeser et al., 2007). Spontaneous potential difference and short-circuit current measurements were taken every 15 minutes over the course of 2 h. Radiolabeled Mn^{54} (PerkinElmer, Waltham, MA) was added to the mucosal chamber after a 30 minute equilibration period at a concentration of 0.2μ/mL. Fifteen minutes after Mn^{54} was added duplicate samples (200 μL aliquots) were taken from both the mucosal and serosal chambers to represent the initiation of the Mn^{54} flux. Aliquots were taken from the serosal chamber at 1 h and samples were taken from both sides at 2 h to represent the end of the flux period. At the conclusion of the Ussing chamber experiments tissues were carefully removed from the chambers and rinsed briefly in a 5 μM EDTA solution to remove any adhering Mn^{54} prior to counting. Samples were counted using an ORTEC High Purity Germanium Detector coupled to Canberra GENIE 2000 MCA software. Manganese content of tissues is expressed on a dry tissue basis and was calculated as the product of dpm, specific activity of the radiolabeled Mn, and the cold Mn:hot Mn ratio (398:1), divided by the mg dry tissue weight. Manganese content of the mucosal buffers was determined to be the product of dpm, activity of Mn^{54}, cold Mn:hot Mn ratio and volume of the buffer (10 mL). Mucosal Mn disappearance was calculated as the initial Mn content – the end (2 h) Mn content.

Analytical procedures

Blood was analyzed for hemoglobin content using Drabkin's Reagent (Sigma, 1990). The hemoglobin standard was purchased from Pointe Scientific, Canton, MI. Blood for serum analysis was centrifuged at 1,200 × *g* for 20 min at 20°C and serum was prepared for analysis as previously described (Hansen et al., 2008a) Feed, liver and intestinal scrapings were prepared for analysis by wet ashing using microwave digestion (Mars 5; CEM Corp., Matthews, NC, USA) as described by Gengelbach et al. (1994). The mineral content of serum, feed, liver and intestine was determined by flame atomic absorption spectroscopy (Model AA-6701F; Shimadzu Scientific Instruments, Kyoto, Japan). A bovine liver standard (1577b Bovine Liver; National Institute of Standards and Technology, Gaithersburg, MD), was included in all runs to verify instrument accuracy.

Protein extraction and immunoblotting procedures

Isolation of proteins from duodenal scrapings and gel electrophoresis was performed as previously described (Hansen et al., 2008b), and 65 μg of protein was loaded into each well. Following transfer and blocking, membranes were probed with one of the following antibodies overnight at 4°C: monoclonal mouse anti-divalent metal transporter 1 (DMT1; Novus Biologicals, Littleton, CO; 2 μg/mL), or affinity purified polyclonal rabbit anti-Fpn (Dr. Mitchell Knutson, University of Florida; 1 μg/mL). For determination of hephaestin concentrations membranes were probed for 1 h at room temperature using a polyclonal mouse anti-hephaestin (Novus Biologicals, Littleton, CO; 1:1000 dilution). A partial recombinant mouse divalent metal transporter 1 protein (Novus Biologicals, Littleton, CO)

served as the positive control for DMT1 analysis, and bovine spleen isolate served as the positive control for Fpn analysis.

After incubation with the primary antibody, membranes were washed several times in PBS containing 0.05% Tween 20, incubated for 30 minutes with the appropriate alkaline phosphatase-linked secondary antibody and then washed several times with the PBS-Tween 20 wash. Membranes were rinsed 2 times with water and then visualized using Lumi-Phos WB (Pierce, Rockford, IL). Images were captured on autoradiography film (CL-XPosure Film; Pierce) and band densities were semi-quantified using Image Quant TL software (Amersham Biosciences, Piscataway, NJ). Membranes were stripped with Restore Western Blot Stripping Buffer (Pierce, Rockford, IL) and reprobed with β-actin (Abcam; 1:5000 dilution for 1 h at room temperature) as a loading control. Data are presented as a proportion of a pooled sample (standard) run on each gel to normalize for between gel variations.

Statistical analysis

Statistical analysis of all data was performed by ANOVA for an unbalanced incomplete block design using the MIXED procedure of SAS (SAS Institute Inc, Cary, NC, USA). The model for performance and blood measurements included the fixed effects of treatment and pen served as the experimental unit (n = 4 per treatment). For tissue mineral, protein and Ussing chamber experiment data analysis the model included the fixed effect of treatment. Block and the treatment by block interaction term were random and individual animal served as the experimental unit (n = 8 per treatment for mineral and protein analysis and n = 6 per treatment for Ussing chamber experiments). All data are presented as LSM values with pooled SEM. Correlations were determined using the Corr procedure of SAS.

RESULTS

Performance

Final body weights of pigs were lower in L-FE ($P = 0.05$) pigs compared to A-FE pigs and did not differ between L-FE and H-FE or H-FE and A-FE pigs (Table 2). Daily body weight gain was lower in L-FE pigs compared to A-FE pigs ($P = 0.02$) and tended ($P = 0.11$) to be lower in L-FE pigs compared to H-FE pigs. Daily gain did not differ between A-FE and H-FE pigs ($P = 0.37$). Daily dry matter intake was lower ($P = 0.01$) in L-FE pigs compared to A-FE and tended ($P = 0.08$) to be lower in L-FE pigs compared to H-FE pigs. Intake did not differ between A-FE and H-FE pigs and feed efficiency did not differ due to dietary treatment (Table 2).

Hemoglobin and serum Fe measurements

Hemoglobin concentrations did not differ at the onset of the experiment (Table 3). By d 14, hemoglobin concentrations were lower ($P = 0.01$) in L-FE pigs compared to H-FE pigs and tended ($P = 0.08$) to be lower in L-FE pigs compared to A-FE pigs. Hemoglobin concentrations were greater ($P = 0.001$) in A-FE and H-FE pigs on d 32 compared to L-FE pigs. Hemoglobin did not differ between A-FE and H-FE pigs at any sampling time point. Initial serum Fe concentrations did not differ between experimental treatment groups (Table 3). Day 14 serum Fe concentrations tended ($P = 0.06$) to be greater in H-FE pigs relative to L-FE pigs. Serum Fe concentrations on d 32 were greater ($P = 0.001$) in A-FE and H-FE pigs compared to L-FE pigs and tended ($P = 0.10$) to be greater in H-FE pigs compared to A-FE pigs.

Tissue mineral concentrations

Liver mineral concentrations are shown in Table 4. Liver Fe concentrations were lower ($P = 0.002$) in L-FE pigs than in A-FE pigs and H-FE pigs, and greater in H-FE pigs ($P = 0.001$) compared to A-FE pigs. Liver Mn concentrations did not differ between L-FE and A-FE pigs, but were lower ($P = 0.02$) in H-FE pigs compared to either L-FE or A-FE pigs. Liver Cu concentrations were greater ($P = 0.02$) in L-FE compared to A-FE pigs, and did not differ between L-FE and H-FE pigs. Liver Cu concentrations were also increased ($P = 0.01$) in H-FE pigs relative to A-FE pigs. Mineral concentrations in the duodenum, based on mucosal scrapings collected at harvest are presented in Table 4. Iron content of the duodenum was lower ($P = 0.03$) in L-FE pigs compared to A-FE and H-FE pigs and greater ($P = 0.01$) in H-FE pigs compared to A-FE pigs. Manganese content of the duodenal scrapings was greater ($P = 0.02$) in L-FE pigs compared to A-FE and H-FE pigs. Additionally, intestinal Mn tended ($P = 0.15$) to be lower in H-FE pigs compared to A-FE pigs. Intestinal Mn and intestinal Fe concentrations were negatively correlated (R^2: -0.49 $P = 0.02$; data not shown). Intestinal concentrations of Cu did not differ due to dietary Fe concentration.

Proteins Involved in Iron Metabolism

Duodenal levels of DMT1 tended ($P = 0.18$) to be lower in H-FE pigs relative to L-FE pigs and did not differ between L-FE and A-FE or A-FE and H-FE pigs (Figure 1). The apparent molecular mass of DMT1, as estimated by SDS-PAGE, was approximately 50 kDa. Intestinal DMT1 levels and liver Fe concentrations in H-FE pigs were highly correlated ($R^2 = -0.72$; $P = 0.04$).

Ferroportin levels tended ($P = 0.17$) to be lower in H-FE pigs compared to L-FE pigs (Figure 2). Relative Fpn concentrations also tended ($P = 0.18$) to be lower in H-FE pigs compared to A-FE pigs. The apparent molecular mass of Fpn was approximately 60 kDa, which is similar to the predicted molecular weight based on the amino acid sequence of the protein (62 kDa; Blachier et al., 2007). Intestinal concentrations of hephaestin were greater ($P = 0.03$) in A-FE pigs compared to H-FE pigs and tended ($P = 0.06$) to be greater in A-FE pigs compared to L-FE pigs (Figure 3). Hephaestin levels did not differ between L-FE and H-FE pigs. Hephaestin protein migrated to an apparent molecular weight of approximately 145 kDa.

Ussing chamber experiments

Manganese accumulation in mounted duodenal tissues did not differ due to dietary treatment (Figure 4). Manganese-54 export into the serosal buffer was below minimal detectable limits of the gamma counter. Disappearance of Mn from the mucosal buffer did not differ between treatments (Figure 5).

DISCUSSION

In the present study feeding a diet containing 20 mg Fe/kg DM to newly weaned, rapidly growing nursery pigs resulted in development of a moderate Fe deficiency by d 32. Hemoglobin concentrations of 10 g/dL or greater are considered adequate in the pig, while levels of 7.0 g/dL or lower are indicative of anemia (NRC, 1998). Reduced hemoglobin and serum Fe content of L-FE pigs suggests this treatment group was anemic by d 32. Underwood and Suttle (1999) outline several phases of Fe deficiency in the young pig, with the most severe phase being characterized by labored breathing and reduced growth. No

labored breathing or "thumps" were observed in the present study; however, L-FE pigs did exhibit a significant reduction in growth over the 32-d period. Reduced growth of pigs fed diets low or high in Fe may be at least partially explained by the observed reductions in feed intake by these treatment groups.

Within the last two years several groups have reported the presence of the Fe import protein DMT1 and the Fe export protein Fpn in the small intestine and colon of the pig (Blachier et al., 2007; Öhrvik et al., 2007; Tako et al., 2007). The results of the present study indicate that proteins important in Fe metabolism such as DMT1 and Fpn are not only present in swine intestine, but appear to be regulated by dietary Fe concentration.

Because the body has a limited capacity for physiological excretion of Fe, a very complex system of check and balances has evolved to adjust intestinal absorption of Fe (DeDomenico et al., 2008). Many of the proteins involved in Fe metabolism are regulated by body Fe status through the concerted actions of iron regulatory proteins and iron responsive elements, including DMT1 (Fe import), transferrin receptor (Fe uptake) and ferritin (Fe storage; Muckenthaler et al., 2008). Iron regulatory proteins, released into the cytosol in response to low Fe stores, bind to iron responsive elements located on transcribed mRNA, resulting in improved translation of mRNA to protein (Muckenthaler et al., 2008). Subsequently, synthesis of proteins such as DMT1 is increased, improving the absorptive capacity of the intestine for Fe and raising plasma Fe levels (Garrick et al., 2003). Rincker et al. (2005) recently demonstrated the presence of an active iron regulatory protein/iron responsive element system in young pigs that is affected by dietary Fe concentration. These

authors reported that binding activity of iron regulatory proteins in the liver of pigs receiving no supplemental Fe was greater than that of pigs supplemented with 150 mg Fe/kg DM.

In the present study, we found that intestinal levels of DMT1 and Fpn tended to be regulated by dietary Fe concentration. It is possible that anemic L-FE pigs had increased activity of iron regulatory proteins and thus increased synthesis of proteins such as DMT1 in order to meet the Fe demands of the body. Therefore, we postulate that in the pig, regulation of Fe metabolism occurs via a mechanism similar to that present in rodents. Certainly, the strong negative relationship between liver Fe concentration and DMT1 protein levels in the intestine of H-FE pigs supports the regulation of DMT1 by body Fe status.

Intestinal Fpn concentrations are regulated through the actions of a small liver-derived peptide called hepcidin which is released into circulation when body Fe status is high, causing removal of Fpn from the plasma membrane of the enterocyte rendering it unable to effectively export Fe into the plasma (Nemeth et al., 2004). Vokurka et al. (2006) reported that hepcidin expression in mice decreased when erythropoiesis was stimulated, and increased when erythropoiesis was inhibited, suggesting that Fe availability for erythropoiesis is a sensitive marker for hepcidin production by the liver. Decreased hepcidin production would lead to greater levels of Fpn in the intestinal mucosa to improve Fe export into plasma. Pigs in the current study were on feed for 32 days; however, the average lifespan of a pig red blood cell is about 72 days (Withrow and Bell, 1969). Therefore, it is possible that because complete red blood cell turnover did not occur in these pigs erythropoietic demand for Fe may not have been great enough to affect the expression and production of hepcidin and thus Fpn.

Hephaestin is a multi-copper oxidase which works in concert with Fpn to export Fe from through the basolateral portion of the enterocyte into the bloodstream (Han and Kim, 2007). In the present study, hephaestin concentrations were decreased in H-FE pigs compared to A-FE pigs. In mice, it has been reported that supplementation with high amounts of Fe results in decreased levels of hephaestin transcript and protein (Chen et al., 2003). Decreased levels of hephaestin may serve a protective function against excessive Fe absorption. It is unclear why hephaestin levels in L-FE pigs were decreased relative to A-FE pigs when the opposite would have been expected.

Our data suggest that the concentration of Fe in the diet directly impacts the metabolism of Mn in swine. Increasing dietary Fe concentration negatively impacted intestinal and liver Mn concentrations. Liver Mn was reduced when pigs were fed the H-FE diet, while intestinal Mn concentrations were reduced in A-FE pigs relative to L-FE pigs and tended to be further reduced in H-FE pigs relative to controls. Davis et al. (1992) reported that feeding rats a diet of 275 mg Fe versus 19 mg Fe/kg DM reduced tibia and liver Mn concentrations regardless of dietary Mn concentration. In vitro work has suggested that both Fe and Mn are potential substrates for DMT1 (Gunshin et al., 1997). This is supported by in vivo documentation in the Belgrade rat, a model which has a DMT1 mutation that renders the protein ineffective. Belgrade rats are highly anemic and also demonstrate reduced tissue Mn concentrations (Chua and Morgan, 1997). In the present study, reductions in tissue Mn concentrations may have resulted from a combination of reduced intestinal DMT1 concentrations in H-FE pigs and increased competition between Fe and Mn for absorption. These results support the role of DMT1 in uptake of both Fe and Mn into the absorptive

enterocyte of the small intestine in the pig. Because commercial swine diets may contain 500 mg Fe/kg DM or higher, the level of dietary Fe which H-FE pigs received in the present study, our findings with high dietary Fe could certainly be applicable to the swine industry.

It is unclear why Mn transport was unaffected by dietary Fe treatment in the Ussing chamber experiments. Based on tissue Mn and intestinal DMT1 and Fpn changes observed in the present study it was expected that Mn transport would differ. Activity of DMT1 has been shown to be both pH and temperature dependent, with optimum activity at a pH of between 5.5 and 6 and a temperature of 37˚C (Mackenzie et al. 2007). Because the pH of the mucosal buffer used in the chamber system is 7.2-7.4, less than optimal activity of DMT1 may have contributed to the lack of differences observed due to dietary Fe treatment. Intestinal barrier function of the duodenum was also determined in these pigs and found to be significantly impaired in H-FE pigs (Moeser et al., unpublished data); therefore it is possible that some leakage of Mn^{54} may have occurred in these pigs.

Though liver Cu concentrations for L-FE, A-FE and H-FE pigs were all within physiologically acceptable levels, changes in liver Cu concentrations due to dietary Fe concentration were observed. Increased liver Cu content in –Fe pigs may have resulted from the anemia these pigs were experiencing, as Hahn and Fairman (1936) reported a tendency for liver Cu to increase in anemic dogs. Yu et al. (1994) also observed increased hepatic Cu concentrations in rats fed low Fe diets compared to those fed normal or high Fe diets. These authors also reported that biliary excretion of Cu decreased in rats as dietary Fe increased, which may offer an explanation as to why increased liver Cu concentrations were observed in H-FE pigs in the present study.

In conclusion, proteins important in intestinal Fe absorption, DMT1 and Fpn, tended to be regulated by dietary Fe concentration in pigs. Feeding high levels of dietary Fe to nursery pigs also affected Mn metabolism, as both liver and duodenal Mn concentrations were lower in pigs receiving 520 mg Fe/kg DM (H-FE). In addition, dietary Fe concentration affected liver Cu concentrations, although the implications of these data are unclear at this time. These results support the role of DMT1 in uptake of both Fe and Mn into the absorptive enterocyte of the small intestine in the pig, but do not support an essential role for DMT1 in Cu absorption in the pig. Because Mn plays an important role in skeletal development, which clearly is important in the rapidly growing nursery pig, the effect of high dietary Fe on Mn absorption through impacts on proteins such as DMT1 warrants further investigation. Because commercial swine diets may contain 500 mg Fe/kg DM or higher, the level of dietary Fe which H-FE pigs received in the present study, our findings with high dietary Fe may be applicable to the swine industry.

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Table 1. Ingredient composition of the diet.[1]

Ingredient	% of diet
Dried skim milk	37.30
Ground corn	21.72
Corn starch	14.92
Casein	8.45
Sucrose	12.43
Poultry fat	2.98
Antibiotic[2]	1.00
Limestone	0.55
Salt	0.25
Phosphoric acid[3]	0.25
Vitamin premix[4]	0.12
Trace mineral mix[5]	0.03

[1] Diet analyzed to contain 20 mg Fe/kg DM.
[2] Carbadox.
[3] Provided as food grade phosphoric acid, a gift from Potash Corporation (Aurora, NC).
[4] Provided per kilogram of diet: 3409 IU vitamin A, 341 IU vitamin D_3, 17 IU vitamin E, 0.01 mg vitamin B12, 7.7 mg niacin, 6.4 mg pantothenic acid, and 1.8 mg riboflavin.
[5] Provided per kilogram of diet: 80 mg Zn as $ZnSO_4$, 0.25 mg Se as Na_2SeO_3, 3 mg Mn as $MnSO_4$, 0.14 mg I as $Ca(IO_3)_2(H_2O)$, 5 mg Cu as $CuSO_4$, 0·2 mg of Se as Na_2SeO_3; and 0·1 mg of Co as $CoCO_3$.

Table 2. Effect of dietary iron on performance of nursery pigs.[1]

Item	Dietary Fe[2]			
	L-FE	A-FE	H-FE	SEM
Initial body weight, kg	5.8	5.5	5.3	0.30
Final body weight, kg	13.0[a]	16.0[b]	14.8[a,b]	0.93
Weight gain, g/d	224.0[a]	328.3[b]	291.7[a,b]	27.2
Dry matter intake, g/d	303.6[a]	404.8[b]	368.4[a,b]	23.05
Gain:feed	0.74	0.81	0.79	0.04

[1] Pooled standard errors presented (n = 4 per treatment). Means in a row without a common superscript differ ($P < 0.05$).
[2] Dietary treatments are: L-FE (20 mg Fe/kg DM); A-FE (120 mg Fe/kg DM); and H-FE (520 mg Fe/kg DM).

Table 3. Effect of dietary iron on hemoglobin and serum Fe concentrations in nursery pigs.[1]

	Dietary Fe[2]			
Item	L-FE	A-FE	H-FE	SEM
Hemoglobin, g/dL				
Day 0	7.0	7.9	7.5	0.51
Day 14	7.2[a]	8.8[ab]	9.7[b]	0.59
Day 32	6.2 [a]	12.8[b]	13.3[b]	0.79
Serum Fe, mg/L				
Day 0	0.40	0.38	0.25	0.11
Day 14	0.44	1.40	1.63	0.37
Day 32	0.34[a]	2.47[b]	2.78[b]	0.11

[1] Pooled standard errors presented (n = 4 per treatment). Means in a row without a common superscript differ ($P < 0.05$).
[2] Dietary treatments are: L-FE (20 mg Fe/kg DM); A-FE (120 mg Fe/kg DM); and H-FE (520 mg Fe/kg DM).

Table 4. Effect of dietary iron on tissue mineral concentrations in nursery pigs.[1]

	Dietary Fe[2]			
Item	L-FE	A-FE	H-FE	SEM
Liver mineral, mg/kg DM				
Iron	71.6[a]	227.8[b]	411.3[c]	23.96
Manganese	11.2[a]	12.4[a]	8.1[b]	1.00
Copper	35.3[a]	25.4[b]	36.7[a]	2.64
Duodenal mineral, mg/kg DM				
Iron	107.8[a]	624.2[b]	1239.7[c]	140.98
Manganese	7.7[a]	5.1[b]	3.7[b]	0.64
Copper	12.5	12.4	13.5	0.49

[1] Pooled standard errors presented (n = 8 per treatment). Means in a row without a common superscript differ ($P < 0.05$).

[2] Dietary treatments are: L-FE (20 mg Fe/kg DM); A-FE (120 mg Fe/kg DM); and H-FE (520 mg Fe/kg DM).

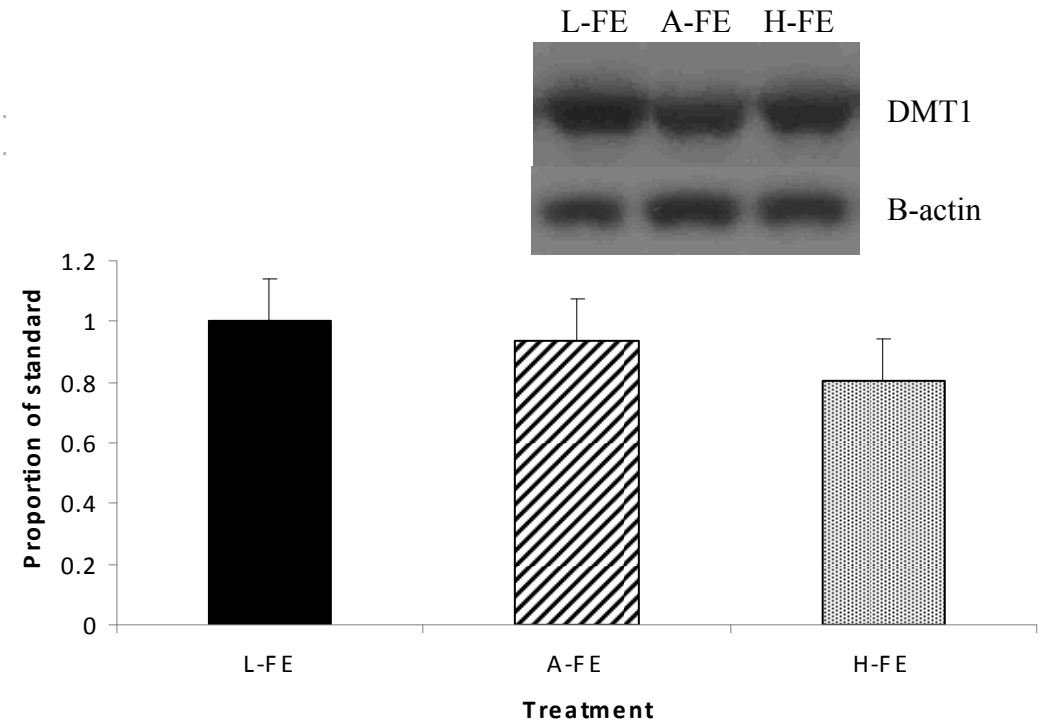

Figure 1. Effect of dietary iron on intestinal divalent metal transporter 1 concentrations. L-FE vs. H-FE ($P = 0.18$). A representative immunoblot is shown.

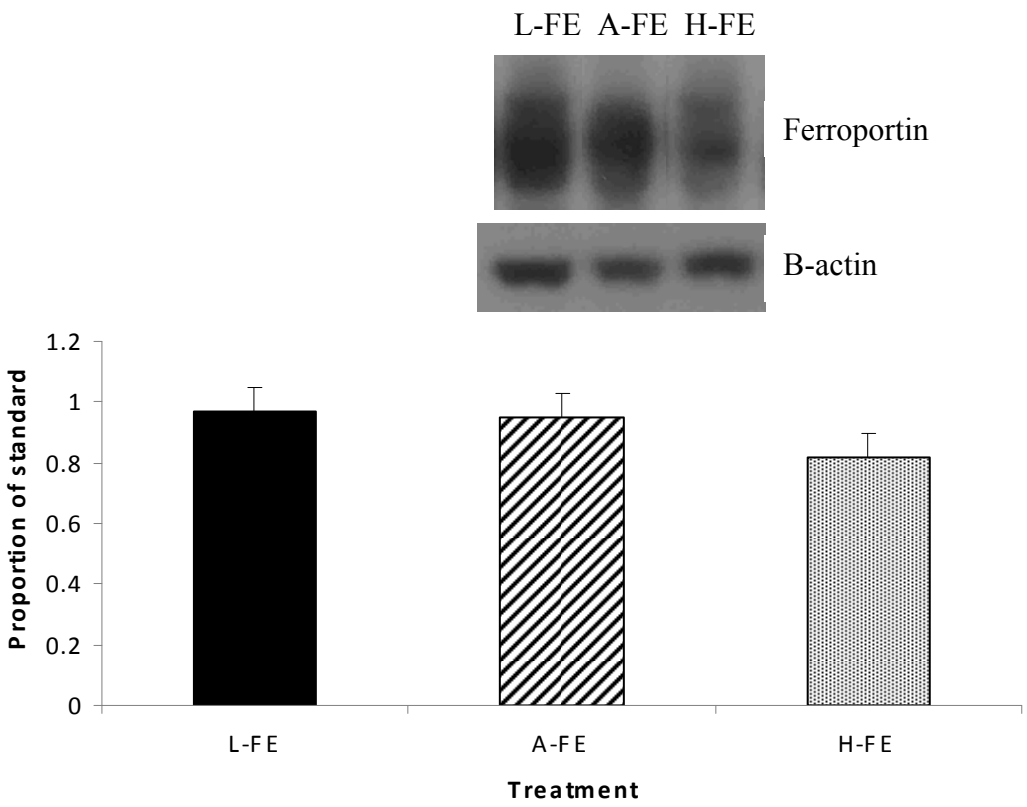

Figure 2. Effect of dietary iron on intestinal ferroportin concentrations. L-FE vs. H-FE (P = 0.17), A-FE vs. H-FE (P = 0.18). A representative immunoblot is shown.

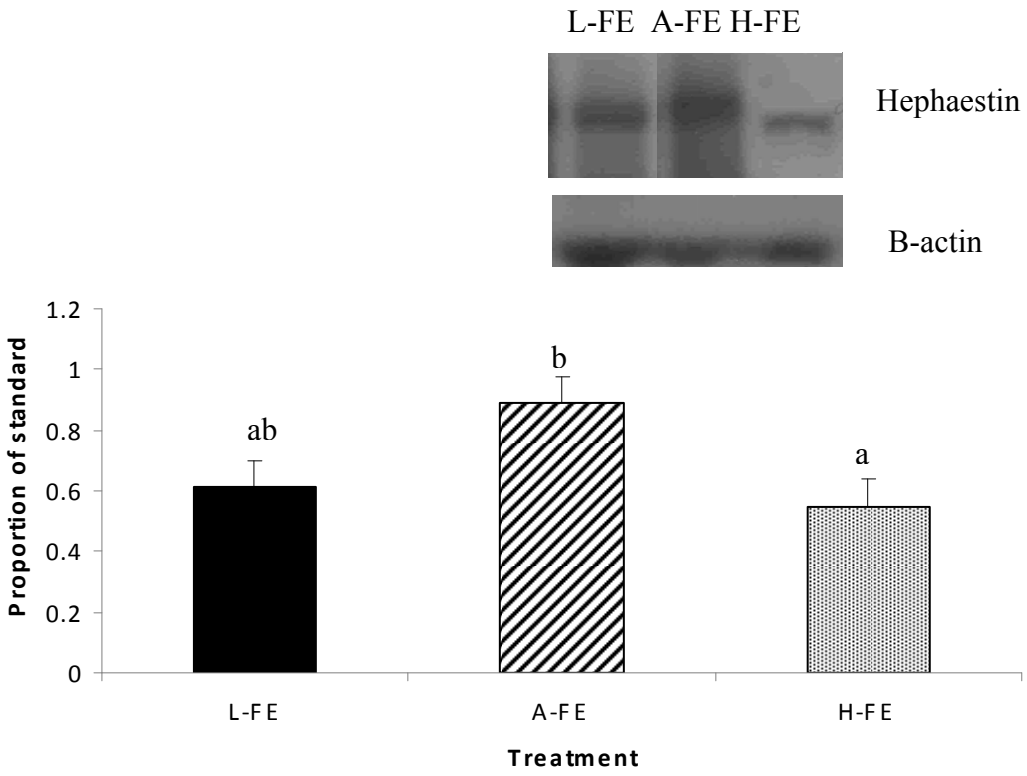

Figure 3. Effect of dietary iron on intestinal concentrations of hephaestin. Bars without common superscripts differ ($P < 0.05$). A representative immunoblot is shown.

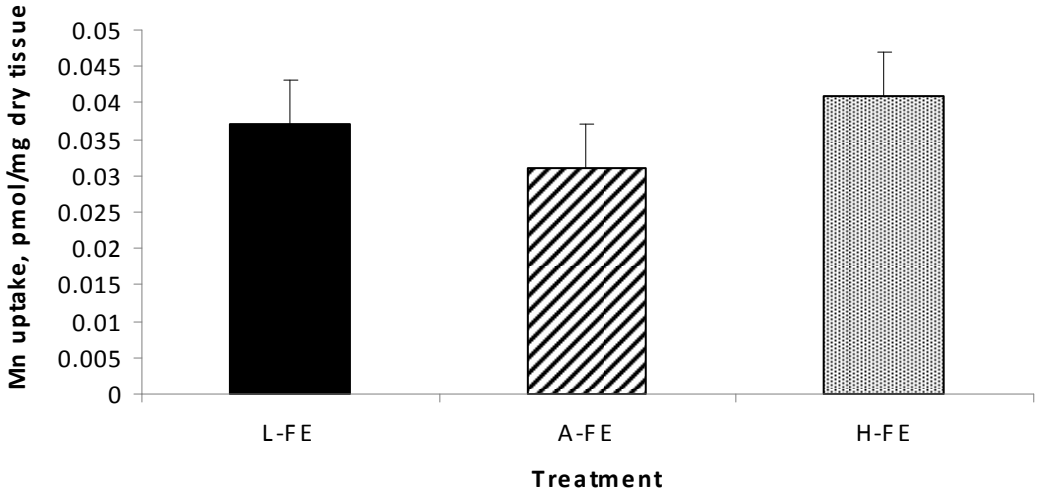

Figure 4. Effect of dietary iron on uptake of manganese by duodenal epithelium.

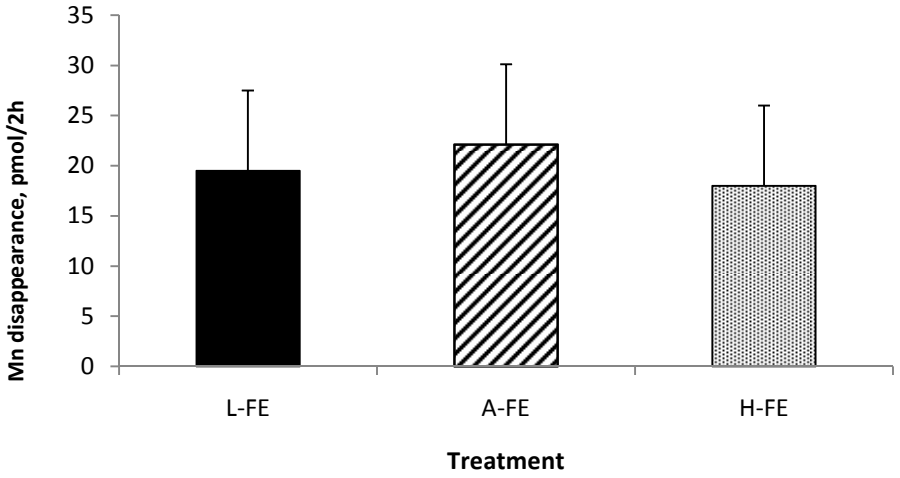

Figure 5. Effect of dietary iron on rate of Mn disappearance from the mucosal buffer.

CHAPTER 5

High dietary iron reduces levels of proteins important in iron and manganese metabolism in young calves

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Keywords: cattle, iron, metal transporters

ABSTRACT: Fourteen weaned Holstein calves were used in a 56-d experiment examining the impacts of high dietary iron (Fe) on proteins involved in Fe metabolism. Calves were randomly assigned to one of two diets: 1) no supplemental Fe (normal Fe) or 2) 750 mg supplemental Fe/kg DM (high Fe). Supplemental Fe was provided as $FeSO_4$. At the end of the trial 6 calves per treatment were harvested, and tissues collected for mineral and protein analysis. Feeding a diet high in Fe decreased ($P < 0.05$) average daily gain, dry matter intake and feed efficiency. Hemoglobin, hematocrit and serum Fe concentrations did not differ due to dietary treatment at any time in the study. Feeding a diet high in Fe increased concentrations of Fe in the liver ($P = 0.03$), but did not affect heart or duodenal Fe concentrations. Duodenal Mn was lowered ($P = 0.05$) by feeding a high Fe diet, but liver and heart Mn concentrations did not differ due to dietary treatment. Feeding a high Fe diet to calves tended ($P = 0.13$) to reduce duodenal levels of divalent metal transporter 1, the Fe import protein and decreased ($P = 0.03$) levels of ferroportin, the cellular Fe export protein. Duodenal hephaestin levels did not differ between treatments. In summary, feeding calves a diet high in Fe reduced duodenal levels of proteins involved in Fe metabolism and appeared to interfere with duodenal Mn uptake.

INTRODUCTION

Oftentimes, cattle diets are very high in iron (Fe). High dietary Fe may result from feedstuffs which are high in Fe or from contamination by soil. Some common ruminant feedstuffs which may be high in Fe are: alfalfa (300 mg/kg DM), corn gluten feed (400

mg/kg DM), dried distillers grains with solubles (600 mg/kg DM) and soyhulls (600 mg/kg DM; NRC, 1996; Kerr et al., 2008).

Iron is an essential trace element and is required for several important biological processes; however, excessive Fe can lead to the production of free radicals and expose sensitive tissues to oxidative stress (Ganz and Nemeth, 2006). As a result of this delicate balance, nature has evolved a very complex system by which the body is able to regulate the absorption of dietary Fe. Two proteins in particular play an important role in modulation of Fe absorption, the cellular Fe importer divalent metal transporter 1 (DMT1) and the cellular Fe exporter ferroportin. We recently demonstrated the presence of these proteins in the duodenum of growing beef cattle (Hansen et al., 2008b). In addition to Fe, DMT1 can transport a number of other divalent metals, including manganese (Mn; Garrick et al., 2006). High dietary Fe decreased tissue Mn concentrations and tended to decrease intestinal levels of DMT1 and ferroportin in pigs, suggesting a possible role for DMT1 in the antagonism between Fe and Mn in the pig (Hansen et al., 2008c). The role of DMT1 in this relationship is supported through work using cell culture and rodent models (Chua and Morgan, 1997; Gunshin et al., 1997); however, the molecular mechanisms behind the interactions between Fe and Mn have yet to be elucidated in ruminants. The objective of the present study was to determine if duodenal levels of proteins involved in Fe absorption in the young calf are affected by dietary Fe concentration. In addition, we examined the effects of excessive dietary Fe concentration on tissue concentrations of Fe and Mn.

MATERIALS AND METHODS

Animals and experimental design

Care, handling and sampling procedures were approved by the North Carolina State University Animal Care and Use Committee prior to initiation of the trial. Fourteen Holstein bull calves aged 1 to 7 d were purchased from a local commercial dairy farm. Calves were housed in individual hutches and fed whole milk until 8 weeks of age, during which time they were allowed ad libitum access to a starter diet (Table 1). Approximately 1 week prior to weaning calves were moved from the hutches to individual pens, which provided approximately 3 m^2 pen space.

At weaning, calves were stratified by body weight (average body weight 73.8 ± 3.7 kg) and randomly assigned to 1 of 2 dietary treatments: 1) no supplemental Fe and 2) 750 mg supplemental Fe/kg DM (provided as $FeSO_4$). Calves received the Grower 1 diet from d 0 through d 38 and the Grower 2 diet from d 39 through the end of the study (Table 1). The Grower 2 diet was formulated without casein and contained slightly higher levels of soybean meal and urea. Calves were fed once daily, with feed amounts based on what they would consume in a 24-h period. Ad libitum access to water was provided throughout the experiment. Diets were formulated to meet or exceed all NRC recommendations (2001). Supplemental Mn and Cu were provided to exactly meet the NRC recommendation when combined with Mn and Cu provided by dietary ingredients. The control Grower 1 and 2 diets analyzed 67 and 62 mg Fe/kg DM, respectively.

Sample collection

Body weights of calves were obtained on d 0, 35, and 56. Jugular blood samples were collected on d 0, 35, and 56 of the trial for analysis of serum Fe, iron binding capacity, transferrin saturation, and hemoglobin. Blood was collected in heparinized vacuum tubes (Becton Dickenson, Rutherford, NJ, USA) and transferred on ice to the laboratory. Blood for serum analysis was collected in vacuum tubes, and allowed to clot at room temperature for 1 h prior to centrifugation at 1,200 × *g* for 20 min at 20°C.

Six calves from each treatment group were randomly selected to be harvested at the termination of the study for tissue collection. Three calves per treatment were harvested on two consecutive days, the lightest group on d 57 and the heaviest group on d 58. Calves were transported approximately 11 km to the North Carolina State University College of Veterinary Medicine for harvest. To avoid intestinal stress prior to tissue collection calves were sedated via an i.v. dose of xylazine (100 mg) and euthanized with an i.v. overdose of pentobarbital sodium.

Immediately following euthanasia a midline incision was made and a 25 cm section of duodenum, approximately 12 cm distal to the pyloric sphincter, was removed and gently squeezed to remove digesta. Segments were cut open longitudinally, rinsed briefly in water to remove any remaining digesta and thoroughly rinsed with 0.87% saline before mucosal scrapings were collected. Scrapings were performed as previously described (Hansen et al., 2008a). A section of liver and piece of heart (apex) were also collected. All

tissue samples for protein determination were snap frozen in liquid N_2 to prevent protein and RNA degradation.

Analytical procedures

Whole blood samples were analyzed for hemoglobin content using Drabkin's Reagent (Sigma, 1990). The hemoglobin standard was purchased from Pointe Scientific, Canton, MI. Serum Fe, unsaturated iron binding capacity (UIBC), total iron binding capacity (TIBC) and transferrin saturation were determined using a colorimetric assay (Iron/TIBC Reagent Set; Pointe Scientific, Canton, MI). The assay was modified for use in a microwell plate (50 µl serum) and serial dilutions of the provided Fe standard were tested to validate the procedure. Absorbance was determined at 560 nm on a plate reader (Synergy HT, Bio-Tek Instruments, Inc., Winooski, VT). Total iron binding capacity was calculated as the sum of serum Fe and UIBC, and transferrin saturation percentage as serum Fe/TIBC x 100.

Feed, liver and intestinal scrapings were prepared for mineral analysis by wet ashing using microwave digestion (Mars 5; CEM Corp., Matthews, NC, USA) as described by Gengelbach et al. (1994). Iron and Mn content of feed, liver and intestinal scrapings was determined by flame atomic absorption spectroscopy (Model AA-6701F; Shimadzu Scientific Instruments, Kyoto, Japan). A bovine liver standard (1577b Bovine Liver; National Institute of Standards and Technology, Gaithersburg, MD), was included in all runs to verify instrument accuracy.

Protein extraction and immunoblotting procedures

Isolation of proteins from duodenal scraping and SDS-PAGE was performed as previously described (Hansen et al., 2008a). One hundred and thirty micrograms of protein isolate were loaded into each well prior to electrophoresis. Following transfer and blocking, membranes were probed with one of the following antibodies overnight at 4°C: monoclonal mouse anti-DMT1 (Novus Biologicals, Littleton, CO; 2 μg/mL), or affinity purified polyclonal rabbit anti-ferroportin (Dr. Mitchell Knutson, University of Florida; 1 μg/mL). For determination of hephaestin concentrations membranes were probed for 1 h at room temperature with polyclonal mouse anti-hephaestin (Novus Biologicals, Littleton, CO; 1:1,000 dilution). A partial recombinant mouse DMT1 protein (Novus Biologicals, Littleton, CO) served as the positive control for DMT1 analysis, and bovine spleen isolate served as the positive control for Ferroportin analysis.

After incubation with the primary antibody, membranes were washed several times in tris-buffered saline (TBS) containing 0.1% Tween 20 (TTBS), incubated for 30 minutes with the appropriate alkaline phosphatase-linked secondary antibody and then washed several times with the TTBS. Membranes were rinsed 2 times with deionized H_2O and then visualized using Lumi-Phos WB (Pierce, Rockford, IL). Images were captured on autoradiography film (Blue Devil Autoradiography Film; Genesee Scientific, San Diego, CA) and band densities were semi-quantified using Image Quant TL software (Amersham Biosciences, Piscataway, NJ). Membranes were stripped with Restore Western Blot

Stripping Buffer Plus (Pierce, Rockford, IL) and reprobed with β-actin (Abcam, Cambridge, MA; 1:5,000 dilution for 1 h at room temperature) as a loading control.

Statistical analysis

Statistical analysis of all data was performed by ANOVA for a completely randomized design using the MIXED procedure of SAS (SAS Institute Inc, Cary, NC, USA). The model included the fixed effect of treatment. Individual animal served as the experimental unit for all data. Day 0 BW was used in a covariate analysis for d 56 BW and daily dry matter intake data. Significance was declared at $P \leq 0.05$. Least square means and pooled standard errors are presented, where n = 7 calves per treatment for performance and blood measurements and n = 6 calves per treatment for tissue protein and mineral data.

RESULTS

Performance

Weight gain, dry matter intake and feed efficiency for the 56-d period are shown in Table 2. Feeding calves a diet high in Fe reduced average daily weight gain ($P = 0.01$), daily dry matter intake ($P = 0.02$), and efficiency of feed conversion ($P = 0.03$).

Blood and serum iron measurements

Hemoglobin and hematocrit did not differ between treatments at any time point during the experiment (Table 3). Day 0 serum Fe concentrations, UIBC, TIBC, and transferrin saturation were similar among treatments (Table 4). No differences in serum Fe

concentrations and TIBC were observed due to dietary Fe concentration on d 35 or 56. Feeding a diet high in Fe tended ($P = 0.13$) to increase UIBC on d 35 and did increase UIBC ($P = 0.02$) on d 56. Transferrin saturation percentages were lower in calves receiving high dietary Fe on d 35 ($P = 0.04$) and 56 ($P = 0.02$).

Tissue mineral concentrations

Iron and Mn concentrations in liver, duodenal and heart tissue are presented in Table 5. High dietary Fe increased Fe concentrations in liver ($P = 0.03$), but not in heart or duodenum. Manganese concentrations were reduced ($P = 0.05$) in duodenal tissue in calves receiving high dietary Fe; however, liver and heart Mn concentrations were not affected by treatment.

Intestinal protein levels

Intestinal levels of DMT1 tended ($P = 0.13$) to be reduced by high dietary Fe (Figure 1). Ferroportin levels in the duodenal mucosal scrapings were lower ($P = 0.03$) in calves fed high Fe (Figure 2). Levels of hephaestin protein did not differ due to dietary treatment (Figure 3; $P = 0.67$).

DISCUSSION

The dietary requirement of cattle for Fe is suggested to be 50 mg/kg DM (NRC 1996; 2001); however, dietary Fe concentrations are often much greater. In the present study, feeding a diet high in Fe (approximately 800 mg/kg DM) to calves for 56 d decreased intestinal ferroportin protein levels and tended to decrease DMT1 protein levels. Superfluous

dietary Fe may come from a variety of sources. Soil ingestion by grazing ruminants is nearly unavoidable, and has been suggested by some authors to make up as much as 10-14% of the daily DM intake of ruminants when pasture vegetation is limited (Suttle et al., 1975). In the United States, soil Fe concentrations range from as low as 0.5% to greater than 5.5% (United States Geological Survey, 2007). Using a simulated in vitro digestion system, we have demonstrated that only a very small fraction of the Fe bound in soils is soluble in the ruminant gastrointestinal tract (Hansen et al., 2008d). However, a considerable amount of Fe from soil was found to be soluble if soil was first exposed to a low pH environment such as that found with fermenting corn silage. In this situation, soil contamination of silage could easily provide amounts of dietary Fe well beyond an animal's requirement. Several common ruminant feedstuffs are high in Fe as well: dicalcium and monocalcium phosphate contain approximately 1% Fe and soyhulls contain approximately 600 mg Fe/kg DM (Kerr et al., 2008). In addition, many grasses contain greater than 100 mg Fe/kg DM, and legumes such as alfalfa and white clover have been reported to contain greater than 200 mg Fe/kg DM (NRC, 1996; Greene, 1999).

In the present study, feeding calves a diet high in Fe decreased daily weight gain, daily dry matter intake and feed efficiency. Standish et al. (1969) also reported a negative relationship between increasing levels of supplemental Fe (0, 400 and 1,600 mg/kg DM) and DM intake in growing beef steers. These authors observed a linear decrease in average daily gain over the 77-d study as supplemental Fe levels increased. Other studies have also shown a depressive effect of high dietary Fe on feed intake and gain (Koong et al., 1970; Standish et al., 1971). It is possible that the large amount of $FeSO_4$ in the high Fe diet in the current

study reduced the palatability of the diet, resulting in lower dry matter intake and gain. It is also possible that high dietary Fe reduced ruminal fermentation. High Fe has been shown in vitro to reduce ruminal dry matter digestibility (Harrison et al., 1992).

It is unclear why transferrin saturation was decreased in calves receiving high dietary Fe in the present study. It was expected that transferrin saturation would increase as dietary Fe increased, as other studies have reported (Bremner and Dalgarno, 1973; Prabowo et al., 1988). In the present study, feeding calves a diet high in Fe for 56 d did not affect serum Fe concentrations, and only minor tissue Fe increases were observed, suggesting that Fe absorption was reduced in these calves. Our laboratory recently demonstrated the presence of several proteins important in Fe absorption in bovine duodenum (Hansen et al., 2008b). Divalent metal transporter 1 is a membrane-bound H^+/divalent metal co-transporter located on the apical surface of enterocytes which serves as an importer of non-transferrin bound Fe into cells (Hubert and Hentze, 2002). Ferroportin is the only known exporter of Fe from the cell, and is a membrane-bound protein localized to the basolateral portion of the enterocyte (Wessling-Resnick, 2006). Hephaestin is a cuproenzyme which works in concert with ferroportin by oxidizing ferrous Fe to ferric Fe so that ferric Fe may bind to transferrin for transport in the bloodstream.

It is unclear why feeding a diet high in Fe to calves did not reduce hephaestin levels in the present study, although it is possible that hephaestin requires a more dramatic change in Fe status to induce protein changes. Anderson et al. (2002) indicated that minimal changes in hephaestin transcript and protein levels occurred in response to Fe deficiency or

excess in rodents, and suggested that hephaestin may not play a major role in the regulation of Fe absorption. It should also be pointed out that while dietary treatment did not affect hephaestin protein levels in the present study, ferroxidase activity of hephaestin was not determined and it is unknown if this activity was affected by dietary Fe concentration.

Duodenal ferroportin concentrations were reduced in calve fed high dietary Fe, probably in an attempt to reduce Fe export into the plasma. Reduced Fe export from the cell may cause Fe to build up within the enterocyte which would then be lost in the feces when the cell is sloughed off. Additionally, intracellular buildup of Fe may eventually create negative feedback on DMT1, causing levels of the protein to be reduced either through restricted synthesis or increased degradation of the protein (De Domenico et al., 2008). The addition of high Fe to the control diet tended to reduce duodenal DMT1 protein levels in the current study, suggesting that DMT1 may be regulated by Fe status in cattle.

Two putative *Bos taurus* DMT1 isoforms are reported in the NCBI database, one which appears to contain an iron responsive element (IRE) consensus sequence and one which does not. In rodents, the presence of an IRE in the 3' untranslated region of DMT1 mRNA allows for regulation by intracellular Fe concentrations. Iron regulatory proteins (IRP) become active when Fe status is low and bind to the IRE, increasing the half-life of DMT1 mRNA (Garrick et al., 2006). Reduced IRP activity in calves receiving high dietary Fe may have limited the half-life of DMT1 mRNA, resulting in the observed tendency for DMT1 to be decreased compared to calves receiving the normal Fe diet in the present study. It is unclear why protein levels of DMT1 were not as dramatically affected by dietary Fe as

ferroportin protein levels were; however, this discrepancy may be explained by the method of Fe-dependent regulation of ferroportin compared to DMT1. Ferroportin levels are mediated by the release of hepcidin, a small peptide released from the liver in situations of high Fe, which binds to ferroportin causing it to be internalized and degraded to decrease export of Fe into plasma (Nemeth et al., 2004). Regulation of DMT1, however, appears to occur via a different mechanism. Johnson et al. (2005) reported that in Caco-2 cells, incubation with 100 μM Fe for just 4 h caused DMT1 to be removed from the plasma membrane and relocated to intracellular compartments. These authors also reported that total cellular DMT1 protein levels, determined by Western blotting, did not differ due to Fe exposure. This suggests that Fe absorption via DMT1 is reduced through intracellular redistribution of the protein, in addition to transcriptional regulation by the IRP/IRE system. In the present study, total cellular concentrations of DMT1 were determined, so it is possible that DMT1 protein in calves receiving the high Fe diet may have actually been located inside the cell, and therefore unable to transport Fe.

Additionally, the anti-DMT1 antibody used in the present study was not specific to the IRE-containing isoform of bovine DMT1 and may also detect the non-IRE bovine DMT1 isoform. Numerous studies have reported that, in rodents, the non-IRE isoform of DMT1 is not affected by dietary Fe concentration (Frazer et al., 2003; Johnson et al., 2005). Because essentially nothing is known about the distribution of DMT1 isoforms in the bovine duodenum, we are unable to ascertain whether DMT1 protein changes in the present study represent translational or post-translational modifications to the protein. At the molecular level mineral metabolism in ruminants may be quite different from monogastrics, and

sensitivity of DMT1 to dietary and cellular Fe changes, method of regulation of DMT1 by Fe, and intestinal abundance of the transporter have yet to be determined. .

In vitro work has indicated that DMT1 is capable of transporting Mn in addition to Fe (Gunshin et al., 1997), suggesting that Mn absorption may be impaired if DMT1 expression is decreased for any reason. Two lines of rodents, the microcytic mouse and the Belgrade rat, are spontaneous mutants which suffer from Fe deficiency as a result of impaired Fe transport. These lines have been shown to have identical missense mutations in DMT1, resulting in Fe deficiency (Andrews, 1999). Metabolism of Mn has also been shown to be detrimentally affected in the Belgrade rat, indicating that the DMT1 gene mutation is causing disruption in absorption of Mn as well as Fe (Chua and Morgan, 1997). In addition, Conrad et al. (2000) reported that Mn uptake in a normal cell line (HEK-293) was reduced in a linear fashion when DMT1 activity was blocked by increasing amounts of DMT1 antibody.

Numerous studies have demonstrated the negative effects of excessive dietary Fe on Cu status of cattle (Humphries et al., 1983; Phillippo et al., 1987; Mullis et al., 2003); however, few studies have evaluated the effects of high Fe on Mn metabolism in cattle. Standish et al. (1971) reported that beef steers consuming diets of 1,000 mg Fe/kg DM had lower heart Mn concentrations and a numerical decrease in the apparent absorption of Mn compared to steers fed diets containing 100 mg Fe/kg DM, supporting a role for bovine DMT1 in uptake of Mn. In the present study feeding a diet high in Fe to calves reduced duodenal concentrations of Mn but did not affect liver Mn concentrations. It is possible that feeding this diet for 56 d did not allow sufficient time for initial Mn stores to be depleted.

Previously in our laboratory we have observed a negative relationship between dietary Fe concentrations and tissue Mn concentrations in weaned pigs (Hansen et al., 2008c). Liver Mn concentrations were lower in pigs fed diets containing 520 mg Fe/kg DM compared to those fed diets containing 20 or 120 mg Fe/kg DM. We also observed a tendency for intestinal DMT1 and ferroportin concentrations to be decreased in pigs receiving high Fe compared to low Fe diets (Hansen et al., 2008c). These data, in combination with the present study, suggest that DMT1 may play an essential role in both Fe and Mn absorption in pigs and cattle.

In conclusion, proteins involved in Fe absorption in the weaned dairy calf appear to be regulated by body Fe status. Excessive dietary Fe negatively impacted duodenal ferroportin level and tended to reduce intestinal DMT1 protein expression. In addition, intestinal concentrations of Mn were decreased by high dietary Fe, suggesting that excessive dietary Fe may result in competitive inhibition of Mn absorption. Because ruminant diets are often high in Fe, whether from soil ingestion or consumption of feedstuffs naturally high in Fe, further research on the impacts of high dietary Fe on metabolism of other trace minerals is warranted.

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Table 1. Ingredient composition of diets

Ingredient	Starter[a]	Grower 1[b]	Grower 2[c]
	% DM		
Cottonseed hulls	15.0	30.0	30.0
Ground corn	57.1	49.0	47.8
Soybean meal	24.0	13.0	15.8
Casein	--	1.75	--
Limestone	1.5	1.3	1.3
Rumensin	1.25	1.25	1.25
White salt	0.5	0.5	0.5
Urea	--	0.45	0.6
Phosphoric acid[d]	0.3	0.5	0.5
Trace mineral premix	0.2[e]	0.1[f]	0.1[f]
Vitamin premix[g]	0.1	0.1	0.1
Sucrame[h]	0.04	0.05	0.05
Corn supplement[i]	--	2.0	2.0

[a] Starter diet was provided in addition to milk through weaning and analyzed 60 mg Fe/kg DM.
[b] Grower 1 diet fed from day 0 through day 38 and analyzed 67 mg Fe/kg DM.
[c] Grower 2 diet fed from day 38 through day 56 and analyzed 62 mg Fe/kg DM.
[d] Provided as food grade phosphoric acid, a gift from Potash Corporation (Aurora, NC).
[e] Provided per kg of diet: 40 mg of Zn as $ZnSO_4$; 40 mg Mn as $MnSO_4$; 0.25 mg of I as $Ca(IO_3)_2(H_2O)$; 0.2 mg of Se as Na_2SeO_3; and 0.1 mg of Co as $CoCO_3$.
[f] Formulated to provide per kg of diet: 40 mg Zn as $ZnSO_4$; 0.25 mg of I as $Ca(IO_3)_2(H_2O)$; 0.2 mg of Se as Na_2SeO_3; 0.1 mg of Co as $CoCO_3$ and 28 mg Mn as $MnSO_4$ and 4 mg Cu as $CuSO_4$.
[g] Provided per kg of diet: 9912 IU Vitamin A; 2203 IU Vitamin D_3; and 4.4 IU Vitamin E.
[h] Flavoring agent.
[i] Provided dietary Fe treatment.

Table 2. Effect of dietary iron on gain and feed efficiency of calves.

Item	Treatment		SEM[a]	*P* Value
	Control	High Fe		
Initial body weight, kg	73.3	74.4	3.66	0.75
Final body weight, kg[b]	118.8	105.5	4.38	0.005
Weight gain, g/d	1.01	0.79	0.05	0.01
Gain:feed	0.28	0.23	0.013	0.03
DMI, kg/d[b]	3.26	2.92	0.165	0.02

[a] Pooled standard error of the mean (n = 7).
[b] Day 0 body weights used as covariate.

Table 3. Effect of dietary iron on hematological measures in calves.

Item	Treatment		SEM[a]	*P* Value
	Control	High Fe		
Hemoglobin, g/dL				
Day 0	11.9	11.9	0.79	0.97
Day 35	12.2	13.4	0.58	0.19
Day 56	10.3	10.8	0.48	0.50
Hematocrit, %				
Day 35	23.2	25.4	1.45	0.32
Day 56	24.0	25.6	1.13	0.35

[a] Pooled standard error of the mean (n = 7).

Table 4. Effect of dietary iron on serum iron measurements of calves.

Item	Treatment		SEM[a]	*P* Value
	Control	High Fe		
Serum Fe, ug/dL				
Day 0	70.3	92.4	14.9	0.32
Day 35	172.8	148.3	17.8	0.35
Day 56	203.4	182.9	12.6	0.28
UIBC, ug/dL[b]				
Day 0	224.0	244.1	25.3	0.42
Day 35	237.3	280.2	24.7	0.13
Day 56	277.1	328.9	19.7	0.02
TIBC, ug/dL[c]				
Day 0	301.4	345.2	40.9	0.30
Day 35	395.9	410.9	33.5	0.76
Day 56	482.8	514.8	31.4	0.34
Transferrin Saturation, %				
Day 0	25.3	29.0	2.8	0.36
Day 35	42.0	35.5	2.3	0.04
Day 56	44.3	38.1	1.5	0.02

[a] Pooled standard error of the mean (n = 7).
[b] Serum unsaturated iron binding capacity.
[c] Serum total iron binding capacity.

Table 5. Effect of dietary iron on tissue mineral concentrations in calves.

Item	Treatment		SEM[a]	*P* Value
	Control	High Fe		
Liver, mg/kg DM				
Fe	116.8	152.9	10.98	0.03
Mn	11.1	11.4	0.64	0.67
Duodenum, mg/kg DM				
Fe	224.8	297.5	57.13	0.39
Mn	10.7	7.4	1.05	0.05
Heart, mg/kg DM				
Fe	179.7	194.3	6.68	0.16
Mn	4.7	4.2	0.71	0.60

[a] Pooled standard error of the mean (n = 6).

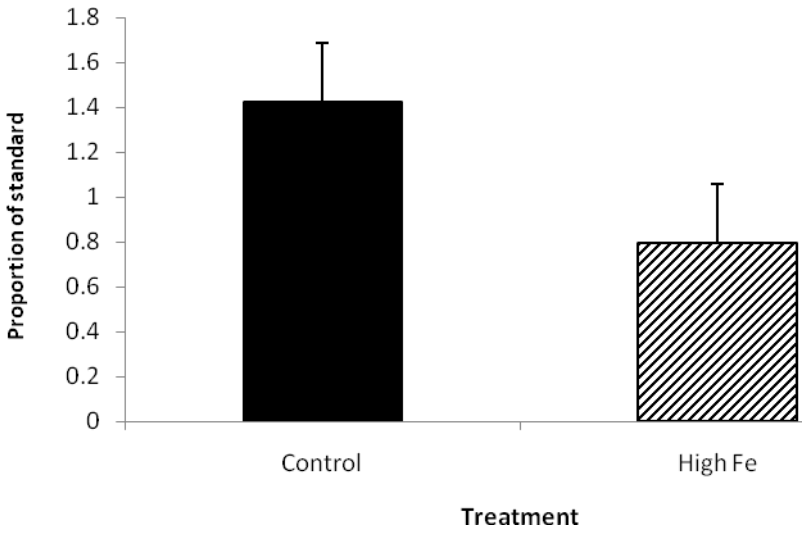

Figure 1. Effect of dietary Fe concentration on intestinal levels of divalent metal transporter 1 protein in young dairy calves (P = 0.13).

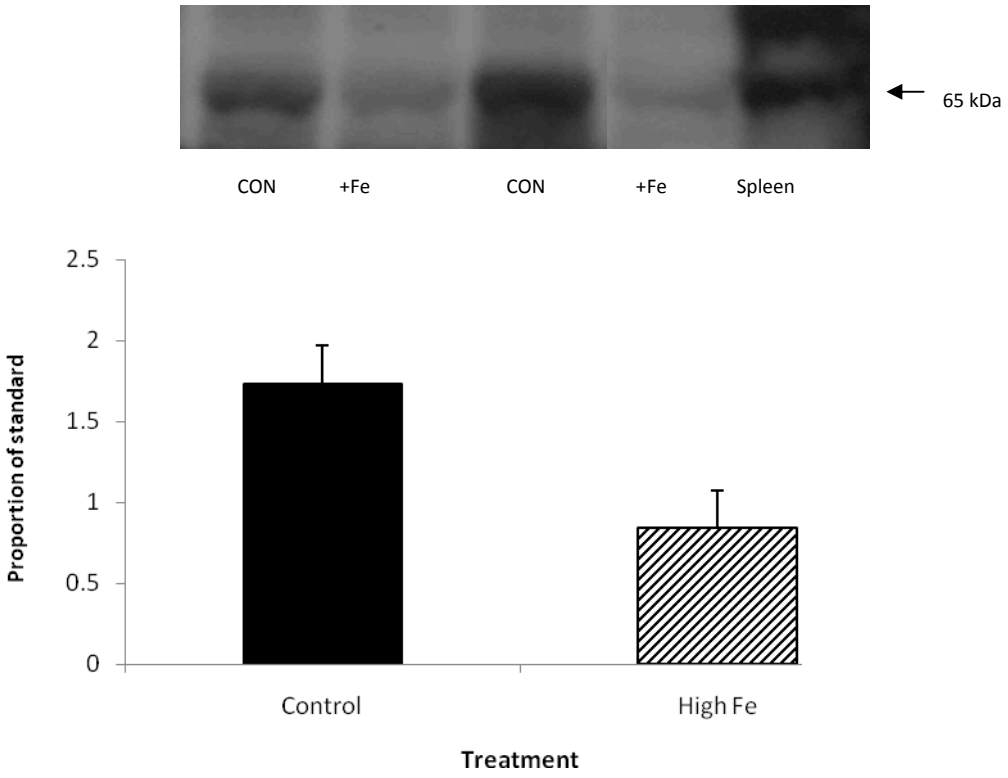

Figure 2. Effect of dietary Fe concentration on intestinal levels of ferroportin protein in young dairy calves (P = 0.03)

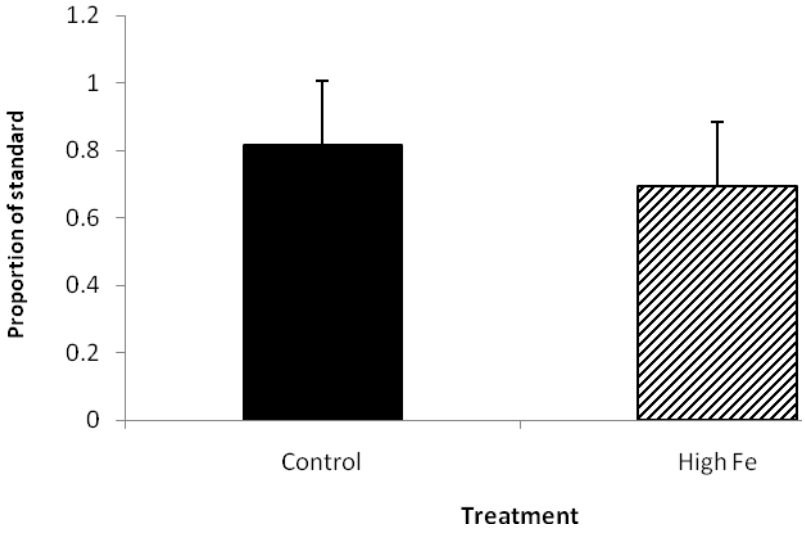

Figure 3. Effect of dietary Fe concentration on intestinal levels of hephaestin protein in young dairy calves (P = 0.67).