INVESTIGATION OF IRON CONCENTRATION, VALENCE, AND SOURCE EFFECTS ON SHORT-TERM IRON (Fe) STATUS AND DRINKING WATER PREFERENCE OF LACTATING DAIRY COWS

By

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ABSTRACT

INVESTIGATION OF IRON CONCENTRATION, VALENCE, AND SOURCE ON SHORT-TERM IRON (Fe) STATUS AND DRINKING WATER PREFERENCE OF LACTATING DAIRY COWS

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Drinking water can contain high concentrations of ferrous iron (Fe^{2+}), which is more absorbable than ferric iron (Fe^{3+}) found in feed and may negatively impact cow health and productivity. Our objective was to characterize Fe status, oxidative stress, and drinking water preference of lactating dairy cows in response to different concentrations, valences and sources (salts) of Fe. In two dosing experiments, cows were abomasally administered 0, 0.75, or 1.5 mg Fe/kg bodyweight from ferrous lactate; or, 0 or 1.5 mg Fe/kg bodyweight from ferrous or ferric sulfate. Blood was sampled hourly for 12 h, and liver biopsies were taken at 0, 18 and 36 h post-dosing. The Fe dosed had minimal effects on Fe status or oxidative stress, suggesting that amounts of Fe administered do not affect short-term Fe metabolism. A protocol was developed to evaluate water preference and drinking behavior of lactating cows for subsequent experiments. In four preference experiments, cows were offered pairs of drinking water treatments. Water intake was measured to determine preference for water with Fe of different concentrations, valences and sources. Water intake was not different between 0 and 4 mg Fe/L from ferrous lactate, but intake with 8 mg Fe/L from ferrous lactate was reduced. Water intakes of treatments with 8 mg Fe/L from ferrous lactate, sulfate or chloride were less compared with control water, but not different among Fe sources. Direct metal analysis commonly used to determine Fe content of drinking water for livestock greatly underestimates total recoverable Fe (acid-digested water sample). Total recoverable Fe values as used in our research are listed above.

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ABBREVIATIONS

Ctr1 = copper transporter 1

DMT-1 = divalent metal transporter 1

Fe^{2+} = ferrous iron

Fe^{3+} = ferric iron

GSH-Px = glutathione peroxidase

Hb = hemoglobin

SEM = standard error of the mean

TDS = total dissolved solids

TIBC = total iron binding capacity

UIBC = unsaturated iron binding capacity

INTRODUCTION

Excess Fe in drinking water may have a negative impact on production and health of lactating dairy cows through decreased water intake, trace mineral interactions, and/or oxidative stress. Iron supplied by drinking water typically is less than the amount supplied by feed. However, Fe in water may be more available for absorption than Fe in feed, because ferrous ($\mathbf{Fe^{2+}}$) Fe, the main form of Fe in water (Colter and Mahler, 2006) is more available than ferric ($\mathbf{Fe^{3+}}$) Fe in feed (NRC, 2001). However, there is little research relating Fe concentration of water with Fe status, health or productivity if lactating dairy cows. In chapter one, literature related to Fe absorption, metabolism, trace mineral interactions, oxidative stress, and current information about utilization of Fe in feed and drinking water is reviewed.

High Fe consumption can increase measurements of Fe status; have a potential negative impact on Cu and Zn status and cause oxidative damage. Iron in water may be more easily absorbed than Fe in feed, and thus has the potential to cause health problems, though no research has been completed in dairy cows to prove this theory. Chapter two describes experiments completed to investigate the effects of abomasally dosing with Fe sources providing differing concentrations and valences on short-term (12 h) measurements of Fe and antioxidant status and oxidative stress in lactating dairy cows.

High concentration of Fe in drinking water is a palatability concern for humans (EPA, 2004), and although this has not been shown in lactating dairy cows. The current maximum tolerable concentration is 0.3 mg Fe/L (EPA, 2004). The third chapter describes four experiments to evaluate the effects of Fe concentration, valence, and chemical sources (salt form) on drinking water preference among lactating dairy cows.

Chapter 4 provides an overall summary, with conclusions and implications of the completed research.

We expect our research will provide a foundation for additional research to better understand the effects of Fe in drinking water on Fe status, health and productivity of lactating dairy cows.

CHAPTER 1

LITERATURE REVIEW

Introduction

There is little information on the effects of Fe concentration and valence in drinking water on lactating dairy cows (Coup and Campbell, 1964). Most research has been completed in humans, rats and other non-ruminants. There also is no established upper limit for the concentration of Fe in drinking water for cattle. The current maximum tolerable concentration (0.3 mg/L) is based on the human limit for palatability (EPA, 2004). However, it has been suggested that cattle can tolerate greater concentrations without negative consequences on water intake or productivity (NRC, 2005).

Anecdotal information suggests that water Fe concentration of 2 mg/L or greater may negatively impact milk production and cow health (Beede, Michigan State University, East Lansing, MI, personal communication). It is hypothesized that this effect could be mediated either through Fe overload and oxidative stress or through drinking water palatability. Iron in water is presumed to be in the soluble ferrous form ($\mathbf{Fe^{2+}}$), which is the form likely absorbed in the small intestine (Sharp and Srai, 2007). In contrast, Fe from feed (specifically forages) is present mainly in the ferric form ($\mathbf{Fe^{3+}}$) and poorly absorbed (NRC, 2001). Therefore, Fe from water may have a greater potential to impact milk production and Fe status of lactating cows more than feed Fe.

Additionally, there is no information regarding the palatability of drinking water with Fe^{2+} or Fe^{3+} for lactating dairy cows. Some studies suggest that greater Fe concentration in feed

affects the palatability of feed (Hansen et al. 2010; Standish and Ammerman, 1971), though no conclusions about a relationship between valence and palatability were made definitively.

Biology of Iron Utilization

Iron is an important trace element required by the body for many different aspects of metabolism. The main function of Fe is as a component of heme-containing proteins in oxygen transport. Iron is needed in cytochromes and Fe-sulfur proteins for electron transport, and it also is found in many metalloenzymes and oxygenases used in other metabolic processes (Jacobs, 1980).

Because Fe is vital to oxygen transport, about two-thirds of the total Fe content of the body is in hemoglobin. A much smaller amount (about 3%) is found in myoglobin, the oxygen storage molecule in the muscle. There is a small pool of Fe within the cells, stored in vacuoles, that is used to form Fe-containing proteins (Halliwell and Gutteridge, 1985). The majority of the remainder is found as ferritin or hemosiderin the storage forms of Fe in various organs, or as transferrin, the protein used for Fe transport throughout the body (Underwood, 1977).

Absorption. The absorption of Fe in cattle is dependent upon several factors including age, Fe status and health, secretions of the gastrointestinal tract (acids), and the nutrient composition of the diet (Underwood, 1977). Pre-ruminants require more absorbed Fe than adult cattle as a function of body weight; so, by necessity their absorption of Fe is much more efficient (NRC, 2001). When an animal begins ruminating, its efficiency of absorption decreases (NRC, 2001). Adult cattle do not require additional Fe for growth or increasing blood volume like calves, so Fe required for normal Fe metabolism is lower. Forages supplied to adult ruminants

also contain more than the amount of Fe required, frequently due to soil contamination, although the availability of this Fe for absorption is unknown. To avoid Fe toxicity, the mechanisms involved in Fe absorption are down-regulated in adulthood, decreasing the efficiency of absorption. The NRC (2001) set the absorption coefficient of Fe from common feeds at 0.10. Absorption efficiency can range from approximately 2 to 20% depending on Fe status, age and health of the animal.

The mechanisms of Fe absorption are summarized in Figure 1.1. Iron from the diet is absorbed in the duodenum and the upper portion of the jejunum. Although this information comes primarily from the human literature, there was no information found that suggests that the site of absorption is different in ruminants. Iron is absorbed as Fe^{2+}, and some of the Fe^{3+} that is consumed can be reduced and solubilized by hydrochloric acid secretions in the stomach. Additionally, Fe^{3+} can be reduced by ferrireductases present on the brush border of the duodenum, likely duodenal cytochrome b (DCYTB; McKie et al., 2001). Ferrous Fe is co-transported into cells with protons by divalent metal transporter 1 (DMT-1; Mackenzie et al., 2007; Sharp, 2004). Mechanisms of Fe transport within the enterocyte are not well understood, but depending on Fe status, Fe can either be bound to mucosal ferritin, or brought to the basolateral surface of the enterocyte for export (Sharp and Srai, 2007). Mucosal ferritin is a storage compound that remains in the enterocyte until the cell dies and is sloughed off, removing both the Fe and the cell from the body via fecal excretion (Sharp and Srai, 2007).

When the animal has a need for Fe, instead of being incorporated into mucosal ferritin the Fe is transported out of the enterocyte by the Fe transporter ferroportin in the basolateral surface of the cell (Muckenhaler et al., 2008). Once it has reached the blood stream, Fe^{2+} is oxidized by

the ferroxidase hephaestin, and then bound to transferrin for transport to other areas of the body (Sharp, 2004). In the case of adequate or excessive Fe, export of Fe from the enterocyte is another site for regulation. When excess Fe is absorbed, the peptide hormone hepcidin is released which causes ferroportin to be removed from the membrane and degraded (Nemeth and Ganz, 2006).

The process of absorption and production and localization of binding proteins involved in Fe metabolism is regulated through post-transcriptional control by the iron-responsive element/iron-binding protein (IRE/IRP) system (Muckenthaler et al. 2008). Iron regulatory proteins (IRP1 and IRP2) bind to IREs on proteins involved in metabolism to allow for Fe absorption and utilization. When Fe is needed, IRP binds to IRE and stabilizes both DMT1 and Tfr1 to prevent degradation (Núñez, 2010). Additionally, IRP binding to the IRE region of ferritin inhibits translation and reduces Fe stored (Núñez, 2010). When a system is overloaded with Fe, DMT-1 is localized in the cytoplasm, decreasing Fe transport (Trinder et al., 2000). It was also found that in cells exposed to Fe, approximately 30% of DMT1 on the surface of the cell is internalized within 10 minutes of Fe exposure (Ma et al., 2006). This suggests that the internalization of DMT1 operates through a mechanism that is faster than the transcriptional and translational control of the IRE/IBP system.

Post-absorptive transport and metabolism. The mechanisms of post-absorptive Fe transport and metabolism are summarized in Figures 1.2 and 1.3. Once absorbed, Fe^{2+} is oxidized by hephastin located in the basolateral membrane. Ferric Fe is bound to the Fe binding protein transferrin for transport. Most absorbed Fe is brought to erythroid tissue in the bone marrow for synthesis of hemoglobin. Once transferrin reaches the desired tissue it is bound to

transferrin receptors (Tfr1 or Tfr2; Muckenthaler et al., 2008) and the transferrin and receptor complex are brought into the cell.

Once internalized, Fe is released from transferrin and apotransferrin, or transferrin that is not bound to Fe is returned to the blood stream. The Fe in the cytosol is brought to the mitochondria as Fe^{2+} to synthesize heme or Fe-S clusters in erythroid precursors (Dunn et al., 2006), or utilized in phagocytes for the production of lactoferrin, a protein similar to transferrin that is found in milk (Halliwell and Gutteridge, 1985). Iron not utilized immediately for heme production is bound to ferritin or hemosiderin for storage (Chua et al., 2007). These compounds are found mainly in lysosomes of the cells in the liver, spleen and bone marrow (Harrison and Arosio, 1996). Ferritin is the main storage form, and hemosiderin is a complex very similar to ferritin (NRC, 2005) although less soluble. Research indicates that ferritin in the lysosomes is processed through partial degradation of the protein component when Fe is in excess to produce hemosiderin (Harrison and Arosio, 1996). Ferritin concentrations in the serum were highly correlated with the total amount of stored Fe in humans (Walters et al., 1973). Miyata and Furugouri (1987) also examined the relationship between serum ferritin and Fe stored in dairy cows and found that serum ferritin was highly indicative of Fe nutritional status. This research group also determined that the suitability of this relationship for measuring Fe status depends on reproductive stage in dairy cows. Due to the inflammatory processes in the uterus and the mammary gland that follow parturition, uterine involution and tissue repair, serum ferritin is not an accurate measure of Fe status until 30 d after parturition (Furugouri et al., 1982).

Excretion and Toxicosis. The natural mechanisms to prevent Fe overload are through regulation of Fe absorption (Jacobs, 1980) as described above. Therefore, there is no mechanism

for Fe excretion to regulate Fe status and only a small amount of Fe is excreted in the feces and urine (Chua et al., 2007). The tissues most likely to be affected by excess Fe intake and absorption are the liver, heart and pancreatic beta cells. In dairy cattle, the signs of chronic Fe toxicosis include reduced feed intake, reduced growth, a decrease in the efficiency of feed conversion and diarrhea (NRC, 2005).

The body responds quickly to an increase in Fe intake by down-regulating Fe absorption mechanisms and increasing the amount of Fe stored in ferritin. In rats the synthesis of ferritin in the liver was maximal 6 h after injection of Fe (Drysdale and Munro, 1966).

Oxidative Stress

Excessive absorbed Fe can cause oxidative stress. During the processes of normal metabolism, reactive oxygen metabolites such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) are generated (Miller et al., 1993). These metabolites become a serious concern when they form radical oxygen species, such as the hydroxyl radical ($HO^\bullet$). Iron is a pro-oxidant, and free metal ions, especially the ferrous ion can create oxygen radicals like the hydroxyl radical from hydrogen peroxide and superoxide through the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^\bullet$; Sordillo and Aitkin, 2009). Under normal circumstances, proteins such as ferritin and transferrin sequester Fe in the body for storage. However, if the mechanisms controlling Fe absorption fail and excessive Fe is absorbed blood Fe concentration may exceed the capacity of Fe-binding proteins, leading to free Fe When the reactive oxygen metabolites are formed as a result of free Fe in the body, a consequence of Fe overload, oxygen radicals are produce. Unless oxygen radicals are controlled by antioxidant mechanisms apoptosis occurs (Halliwell, 1987).

This process is cumulative, because when cells are injured free Fe pools can be disturbed causing release of the free Fe into the cellular Fe pool, increasing the production of radical oxygen species (Halliwell, 1987). Halliwell and Gutteridge (1985) found that transferrin, lactoferrin and ferritin are not generally catalysts of oxygen radical formation; however, they suggested that in the situation of Fe overload, those proteins also might contribute to radical production.

The formation of hydroxyl radicals is a serious problem in the body. The oxygen radicals are destructive to lipids, proteins, and DNA (Miller et al., 1993). Radicals are species that contain one or more unpaired electrons (Halliwell, 1987). Because of this unique structure, these radicals remove electrons from other stable molecules, causing serious tissue damage. These damages induce complications in membrane permeability, the function of enzymes, synthesis of steroid hormones and problems in muscle tone (Miller et al., 1993). The amount of damage and the concentrations of Fe sufficient to cause peroxidative damage depend on the antioxidant status of the animal. Older animals are more susceptible than younger animals (Wu et al., 1990). Some signs of oxidative stress and cellular damage in dairy cows include reduced reproductive performance, which can be caused by a decrease in uterine contractibility, reduced immune function, mastitis, parturient paresis, increased incidence of retained placenta and udder edema (Miller et al., 1993).

Antioxidant status is an important component in the prevention of oxidative stress. Antioxidants prevent the formation of oxygen radicals and peroxides and prevent oxidative stress and cellular damage (Sordillo and Aitkin, 2009). Both oxygen radicals and peroxides are products of normal metabolism, and generally adequate antioxidants are to prevent damage. Vitamin E, or α-tocopherol, is an antioxidant that reduces radicals (Sordillo and Aitkin, 2009).

Superoxide dismutase is an enzyme that reduces the superoxide radical to hydrogen peroxide, which can then be oxidized by glutathione peroxidase to water and glutathione. However, in the presence of excess pro-oxidants, like free Fe, antioxidant systems can be overwhelmed and oxidative stress results. As the load of oxygen radicals increases, the antioxidants are depleted, leaving the animal in more danger. Calves fed excess Fe (750 mg Fe/kg of ferrous sulfate) had increased expression of superoxide dismutase mRNA in heart tissue compared to calves fed a control ration (65 mg Fe/kg), indicating that excess Fe caused oxidative stress (Hansen et al., 2010).

Excessive Fe in the diet caused damage to the intestinal epithelium, as evidenced by an increase in flux of labeled mannitol across the intestinal epithelium, and a decrease in epithelial resistance in the intestinal epithelium of Holstein bull calves fed excessive Fe (750 mg Fe/kg DM; Hansen et al., 2010). Although the cause of the damage was unclear in this experiment the authors hypothesized that results pointed toward oxidative stress through lipid peroxidation.

Fe, Cu and Zn Interactions

Iron interactions with other trace elements, specifically Cu and Zn were suspected for many years. Coup and Campbell (1964) initially noted that hypocupremia developed in cattle dosed daily with 30 mg Fe/kg body weight from ferric hydroxide ($Fe(OH)_3$) for 7 mo. Continued research showed that Fe supplementation decreased Cu storage in both cattle and sheep (Campbell et al., 1974). However, the majority of these studies supplemented Fe using ferrous

sulfate ($FeSO_4$), which has antagonistic effects on Cu as well, failing to distinguish the effect of Fe from the effect of S (Suttle, 1974, Prabowo et al. 1988).

Bremner et al. (1987) demonstrated that it does not take excessive concentrations of Fe in the diet to have a negative effect on Cu metabolism. They showed that concentrations of Fe commonly found in some forages are high enough to impact Cu metabolism. Dietary Fe concentrations of 250, 500, and 750 mg/kg decreased plasma and liver Cu concentrations without clinical signs in calves fed supplemental Fe as ferrous carbonate ($FeCO_3$). Researchers in Scotland demonstrated that 800 mg/kg DM of supplemental Fe, supplied as ferrous carbonate, was sufficient to cause severe Cu deficiency in calves after 16 wk (Humphries et al., 1983). The supplemented diets reduced hepatic Cu, and decreased plasma Cu, ceruloplasmin, and erythrocyte superoxide dismutase activity, a Cu and Zn-containing enzyme. Steers supplemented with 1,000 mg/kg DM of ferrous sulfate had decreased concentrations of Cu in liver and kidney tissue, and decreased apparent absorption of Cu compared with steers fed a control diet (Standish et al., 1971).

The most prevalent theory on the interaction between Cu and Fe absorption involves DMT-1 and competition for transport. Copper is absorbed via metal transporters Ctr1 and DMT-1, the main Fe transporter (Sharp, 2004). Additionally, ceruloplasmin, a Cu-containing protein is a ferroxidase in serum that is essential for changing valence of Fe that affects the ability of Fe to bind to specific proteins. In the case of Cu deficiency, ceruloplasmin is decreased, which can decrease Fe status, and result in anemia. Hephaestin also requires Cu, and it is required for the transport of Fe across intestinal epithelial cells (Sharp, 2004).

Steers supplemented with 1,000 mg/kg DM of ferrous sulfate had decreased concentrations of Zn in the liver compared with steers fed no supplemental Fe (100 mg/kg DM). (Standish et al., 1971), and as the concentration of ferrous sulfate added to the diet increased (0, 400, and 1,600 mg/kg DM) the concentration of Zn in the liver decreased (Standish et al., 1969). In contrast, in sheep fed 1,600 mg/kg DM of either ferrous sulfate or ferric citrate ($Fe_3(C_6H_5O_7)$), there was no change in liver Zn concentration (Standish and Ammerman, 1971). Supplementing cattle with 1000 mg Fe/kg DM decreased Cu status, but did not impact Zn status over time (Mullis et al., 2003). Non-heme-Fe inhibits the absorption of Zn when dosed together in humans, heme-Fe does not (Solomans and Jacob, 1981). Although both Fe^{2+} and Fe^{3+} inhibit Zn absorption, Fe^{2+} has a greater impact (Solomans et al. 1983).

Unlike the interaction of Fe and Cu, interactions of Fe and Zn are most likely not mediated through competition at DMT-1 because in a situation of Fe overload, DMT-1 expression and Fe uptake were reduced, but Zn absorption was not impacted in humans (Kordas and Stolzfus, 2004). Although the mechanism of interaction between these trace elements is unknown, there is evidence to suggest that the interaction between non-heme-Fe and Zn takes place in the small intestine (Solomans and Jacob, 1981).

Iron Chemistry Associated with Biology

Ferric iron is the common form in feedstuffs and has a low absorbability. The NRC (2001) estimated that the absorption coefficient of Fe from the diet is about 0.10 in dairy cattle, and this number decreases as the amount of Fe consumed increases. A small amount of Fe^{3+} is reduced to Fe^{2+} in the abomasum by hydrochloric acid. In fact, the reduction of Fe^{3+} to Fe^{2+} is

important in Fe absorption. Iron is bound to transferrin as Fe^{3+}, but transferred through the cell as Fe^{2+}, to be incorporated into heme or oxidized again to be stored, although the mechanisms for this are not known (Sharp and Srai, 2007). The ferrous (Fe^{2+}) form of Fe is highly available to the animal. One source estimated that Fe^{2+} from ferrous sulfate is 100% available to mammals (Hurrell, 1997). This form is soluble in water and colorless, and is oxidized to Fe^{3+} upon exposure to oxygen, creating ferric oxide (Fe_2O_3), or rust. Iron toxicity is especially a concern in areas where the Fe concentration of drinking water is high, because most of the Fe in water is presumably in the ferrous state (NRC, 2001).

The ratio of Fe^{2+} - to - Fe^{3+} in water depends on many factors including reduction-oxidation (redox) potential, pH and other elements and chemical compounds. The oxidation of Fe^{2+} to Fe^{3+} increases as both the pH and temperature of water increase (Roekens and Van Grieken, 1983). However, the rate of oxidation of Fe is only dependent on pH within the range of 4 to 8. At pH lower than 4 the rate of oxidation is very low, and above a pH of 8, the oxidation rate is so high that concentrations of Fe^{2+} are difficult to detect (Morgan and Lahav, 2007). However, the typical pH of natural groundwater normally falls within the range where the rate of Fe oxidation is dependent on pH.

Redox potential has a similar effect on the oxidation of Fe as pH. Redox potential is the tendency of a chemical species to lose or gain electrons. According to Hem and Cropper (1959) the redox potential of natural water falls between 0.3 to 0.5 volts, and as the redox potential increases, oxidation of Fe^{2+} also increases. However, in a more recent paper Nordstrom (2000) indicated that natural groundwater is not influenced by redox potential, only pH. In the case of natural water, redox potential has no practical meaning. In aqueous systems free electrons are

very rare, with extremely short half-lives, and are virtually nonexistent in natural groundwater (Nordstrom, 2000). When considering the redox potential it is important to consider the specific species, Fe^{2+} to Fe^{3+} conversion activity for example, and even this is affected by many factors, such as exposure to oxygen, pH of the solution and dissolved Fe concentration. The redox potential of Fe^{2+}/Fe^{3+} is difficult to measure, extremely variable (Pierre et al., 2002) and less influential in Fe oxidation than pH.

Overall, the most important factor in Fe^{3+} to Fe^{3+} oxidation is elemental oxygen (O_2) content of water, or exposure of the water to oxygen (e.g. in air). This is the reason that aeration is such a common treatment method for water with high Fe^{2+} content. As the water comes in contact with oxygen, Fe^{2+} is oxidized to Fe^{3+}, and complexes to form ferric oxide or ferric hydroxide via the Fenton reaction; this precipitates out of solution as a red solid (Stumm and Lee, 1961). This precipitate can then be filtered out of the water, decreasing the Fe content.

Fe Microbiology

Another problem caused by high Fe concentration in water is the presence of Fe-oxidizing bacteria. Iron bacteria are prokaryotes that harvest energy from the oxidation of Fe^{2+} to Fe^{3+}. Although the majority of Fe-oxidizing bacteria exist at very low pH, there are some bacteria that can oxidize Fe at a near neutral pH, enabling these bacteria thrive in groundwater. *Gallionella ferruginea* and *Sphaerotilus natans* are examples of these Fe-oxidizing bacteria and they are the species commonly found in water supply pipes (Madigan and Martinko, 2006). They can be identified by a large amount of Fe precipitate and a red biofilm present in pipes and water fixtures. Although there is no evidence to suggest that Fe-oxidizing bacteria pose any

threat to human or livestock health, they can cause a significant problem with the aesthetic appeal and palatability of drinking water for humans and reduce water flow and corrode plumbing equipment.

There is some evidence that excess Fe can contribute to bacterial infections in animals, as free Fe is required for bacterial growth (NRC, 2001). Baynes et al. (1986) found a correlation between elevated serum ferritin and acute pneumonia and sepsis. Free Fe also can have a negative impact on natural resistance to bacterial infection (Bullen et al., 2006).

Current Drinking Water and Nutritional Recommendations

Iron in Drinking Water for Dairy Cattle. Currently the quality recommendation for Fe content of drinking water for dairy cattle is 0.3 mg/L. This value comes from the EPA (2004) and is the same as the human limit to avoid health problems and a reduction in palatability. It is believed that dairy cattle can withstand higher concentrations without affecting water intake or health (NRC, 2005). However, there is no research to validate this supposition. There is some anecdotal information suggesting that dairy farmers who installed water treatment systems to decrease the Fe content of their drinking water observed improvements in cow health and milk production (Beede, Michigan State University, East Lansing, MI, personal communication). Some researchers believe that reduced milk yield and health problems from drinking water with high Fe content may result from reduced water intake (Beede, 2006; Socha et al., 2003).

Socha et al. (2003) estimated that a cow increases Fe intake by 83 mg/d by drinking water (27.7 gal/d) with an Fe concentration of 0.8 mg/L, which is the average concentration based on over 3,500 samples taken across the United States. However, this calculation does not

consider the potential impact of absorption, which can vary widely depending on age, total Fe consumption, and other factors. The current belief is that Fe in water may be much more available than Fe in feeds, due to the difference in valence (Fe^{2+} vs. Fe^{3+}). Beede (2009) estimated that a cow drinking 31 gal/d of water with an Fe concentration of only 0.3 mg/L (assuming 100% absorption) doubles her absorbed Fe intake when considered in addition to her Fe intake from an average TMR of 20.5 kg DM/d (absorbability of feed Fe assumed to be 0.1). Yet the research on Fe toxicity from the diet in lactating dairy cows is scarce (Coup and Campbell, 1964), and research about Fe toxicity from water in lactating dairy cows was not found in the scientific literature.

Research on Drinking Water with Iron

No research was found testing the effects of Fe in drinking water on normal mammals with adequate Fe status. However, researchers have investigated the possibility of using water as a vehicle to deliver Fe to humans and animals with an Fe deficiency. Researchers in Brazil tested the effects of delivering Fe in water to rats fed an Fe-free diet, and determined that the inclusion of ferric ammonium citrate, ferrous sulfate or ferrous gluconate increased hemoglobin concentrations in the treatment rats compared with control rats given deionized water (Ferreira et al., 1991). This initial evidence of the success of using water to deliver Fe to prevent anemia resulted in studies in humans in areas of the world that tend to have a problem with anemia. After the initial experiment with rats, the researchers tested water as a source of Fe in children with a high incidence of anemia in Brazil (Dutra de Oliveira et al., 1994). They treated the only water source of a daycare with children from age 2 to 6 yr with ferrous sulfate heptahydrate (20

mg/L Fe) for 8 mo. It was estimated that the children drank an average of 500 ml/d, for an estimated Fe intake of 10 mg/d from the water. Prior to treatment, 58% of the children had serum hemoglobin concentrations below 11 g/dL, the low end of the normal range. Following treatment, only 3% of the children had serum hemoglobin concentrations below 11 g/dL, with an average concentration of 13 g/dL, considered by the investigators to be an acceptable hemoglobin concentration. They also determined that the serum ferritin concentrations of the treated children increased from an average before treatment of 13.7 μg/dL to an average after 8 mo of treatment of 25.6 μg/dL. The same research group treated the drinking water of socioeconomically challenged families in Brazil to contain 10 mg Fe/L of ferrous sulfate and 100 mg/L ascorbic acid (Dutra de Oliveira et al., 1996). The ascorbic acid was added to decrease the color and turbidity of the treated water and to make it more palatable. They found that both hemoglobin and serum ferritin were increased after 4 mo, in both children and adults. When drinking fountains at a daycare in Brazil were supplemented with 10 mg/L Fe and 100 mg/L ascorbic acid, researchers found that children consuming the water had increased serum hemoglobin concentrations after 6 mo (Noguiera de Almeida et al., 2005). These children consumed about 500 ml/d of fortified water, for a total Fe consumption of 5 mg/d from the water.

No similar studies were found for cattle or other species of domestic livestock.

Iron Toxicity in Cattle

Dietary Fe toxicity in calves was documented by several different studies. In studies in which 130 d-old male Holstein calves were fed high concentrate diets supplemented with 1,000

mg/kg DM of ferrous carbonate, no differences were found between control and treated calves in the Fe concentration of the liver, kidney, pancreas, spleen, small intestine, muscle, ribs, or bile (Ho et al., 1984). Miller et al. (1991) reported that in young, pre-weaned heifers fed whole milk and a starter ration supplemented with 0, 500, 1,000, 2,000 or 4,000 mg/kg DM of ferrous carbonate (approximately 50% Fe) for 8 wk, weight gains and feed consumption were not reduced consistently. They found that the lowest average gain and intake were for calves that were fed ferrous carbonate at 2,000 mg/kg DM though the authors suggested that the difference in weight gain between 2,000 and 4,000 mg/kg DM did not have a significant meaning. They also determined that when no milk was fed during the last 2 wk of the trial, both sets of calves fed 2,000 and 4,000 mg/kg DM had reduced intake and weight gains when compared with the control calves (0 mg/kg DM). In contrast, Jenkins and Hidiroglou (1987) found no differences for calves fed milk replacer with supplemental ferrous sulfate heptahydrate at concentrations of 100, 500, 1,000 or 2,000 mg Fe/kg DM, but calves on the 5,000 mg Fe /kg DM treatment had lower DMI and average daily gain. Also, when compared with calves on the lowest dietary treatment, calves on the 5,000 mg Fe/kg DM treatment had increased non-heme Fe, ferritin and hemosiderin in the liver, kidney and spleen. Also, as milk replacer Fe concentration increased, there was a concurrent increase in Fe concentrations in blood plasma, bile, gall bladder, muscle, heart, spleen, liver and kidney.

Few studies have investigated the effects of high dietary Fe in adult ruminants. One study in Florida tested the effects of ferrous sulfate in beef steers (Standish et al., 1969). Researchers fed diets containing 0, 400 or 1,600 mg Fe/kg DM supplemental ferrous sulfate, and investigated the effects on average daily gain, feed-to-gain ratio, blood measurements and the

mineral content of various body tissues. They found that as the dietary Fe concentration increased, the amount of feed consumed by the steers decreased, as did the average daily gain. However, there was no difference between treatments in hemoglobin or hematocrit concentrations of the blood plasma. The Fe contents of the liver, spleen, kidney and heart were increased as dietary Fe increased. They also found an inverse relationship between the Fe content and the Cu and Zn content of the liver. As the dietary Fe concentration increased the Cu and Zn content of the liver decreased. When 1,600 mg Fe/kg DM of either ferrous sulfate or ferric citrate was fed to growing wethers, hematocrit decreased by the Fe treatments when compared with control, and hemoglobin was numerically lower (Standish and Ammerman, 1971). However, in agreement with Standish et al., (1969), the Fe concentrations of the liver and the spleen were greater in the wethers supplied excess dietary Fe.

Although most research on Fe toxicity of dairy cattle was completed in pre-ruminating dairy calves, one New Zealand study investigated the effects of pasture heavily irrigated with water with a high Fe content on non-lactating dairy cattle (Coup and Campbell, 1964). Iron content of the pasture available to the Fe treatment group increased through periodic irrigation, reaching concentrations ranging from 570 to 9,980 mg/kg (dry basis). Researchers were not positive whether the Fe was taken up by the grass, or was simply on the surface. This was achieved through irrigating the pasture with water with a high Fe concentration (17 mg/L) three times through the two-month research period for a total of 68 cm of water applied on the high Fe pasture. Pasture of the control group was not irrigated with the high Fe water. There was a loss of live weight in both groups, but animals in the control group, on pasture that never had Fe content above 420 mg/kg DM, lost less live body weight, and lost weight less rapidly than the cows on

high Fe pasture. They also found that the cows on high Fe pasture continuously had loose, abnormally colored manure (Coup and Campbell, 1964). In a separate experiment, the same researchers tested the effects of dosing a lactating dairy cow on pasture with 130 g of Fe^{3+} per day for 5 d in the form of ferric chloride. The dosed cow's (only one cow) milk yield decreased rapidly, as did her live body weight. Her feces also were dark in color, loose and malodorous (Coup and Campbell, 1964). Cattle dosed for 10 d twice daily with increasing amounts of ferric hydroxide, (0, 15, 30 and 60 g/d or approximately, 0, 7.5, 15 and 30 g Fe/d) had a dose-related decrease in milk and fat yield, body weight and reduced feed conversion efficiency (Coup and Campbell, 1964). No other research was found that tested the effects of high amounts of dietary Fe on lactating dairy cattle, nor the effects of high Fe in drinking water.

Therefore, because research on the effects of dietary Fe in lactating dairy cows is scant and with variable results and research on the effects of drinking water with high Fe concentrations is very limited, and non-existent in the case of lactating dairy cows, we have many questions. The only knowledge that we currently have about the effects of Fe^{2+} from drinking water on lactating dairy cows is through personal accounts (Beede, Michigan State University, East Lansing, MI, personal communication). The research that supports the current guidance for Fe content of drinking water for cattle is based on recommendations for humans, although it is suggested that cattle can tolerate higher concentrations (NRC, 2005). Overall, we hypothesize that indicators of Fe status will be impacted short-term when lactating dairy cows are dosed abomasally with Fe in a water solution. We also hypothesize that the preference of cows for drinking water will be affected by the concentration and valance of Fe in the water. Through two separate approaches we intend to characterize the lactating dairy cow's

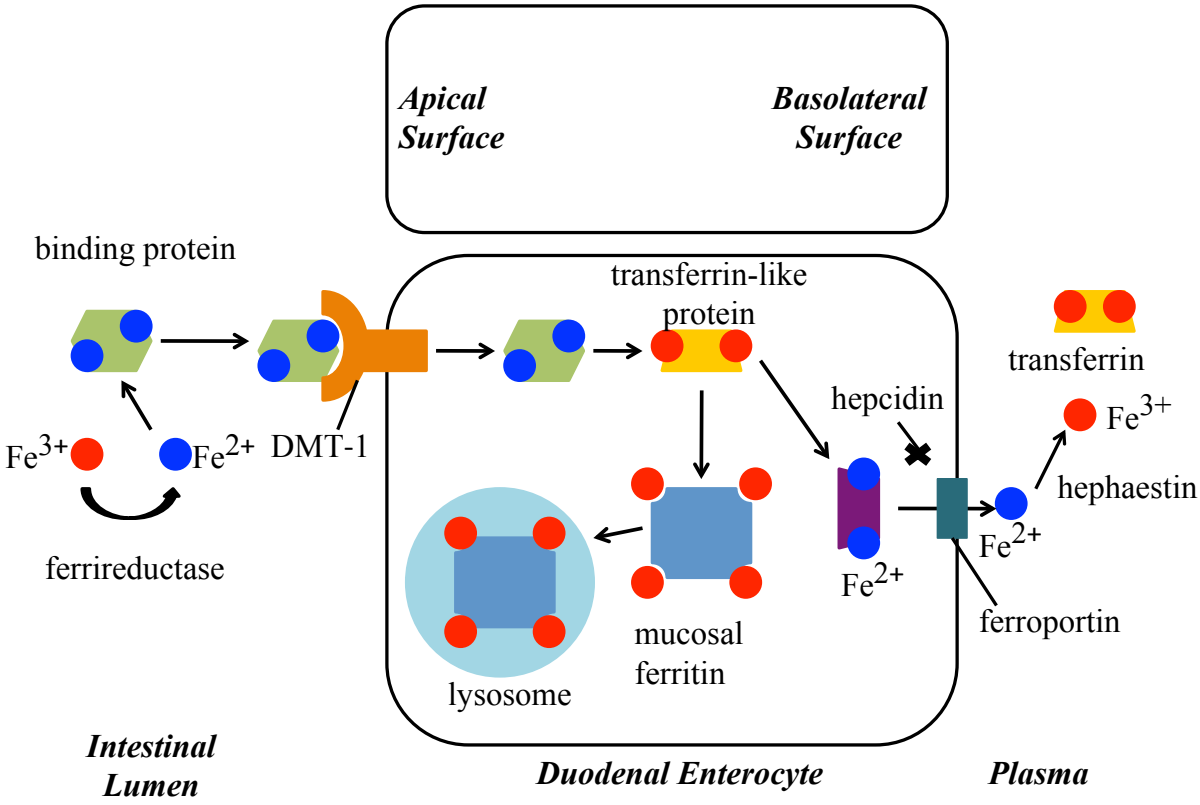
physiological response and preference for Fe at different concentrations, of different valences (Fe^{2+} vs. Fe^{3+}) and from different chemical salts delivered through drinking water.

APPENDIX A

FIGURES REFERENCED IN CHAPTER 1

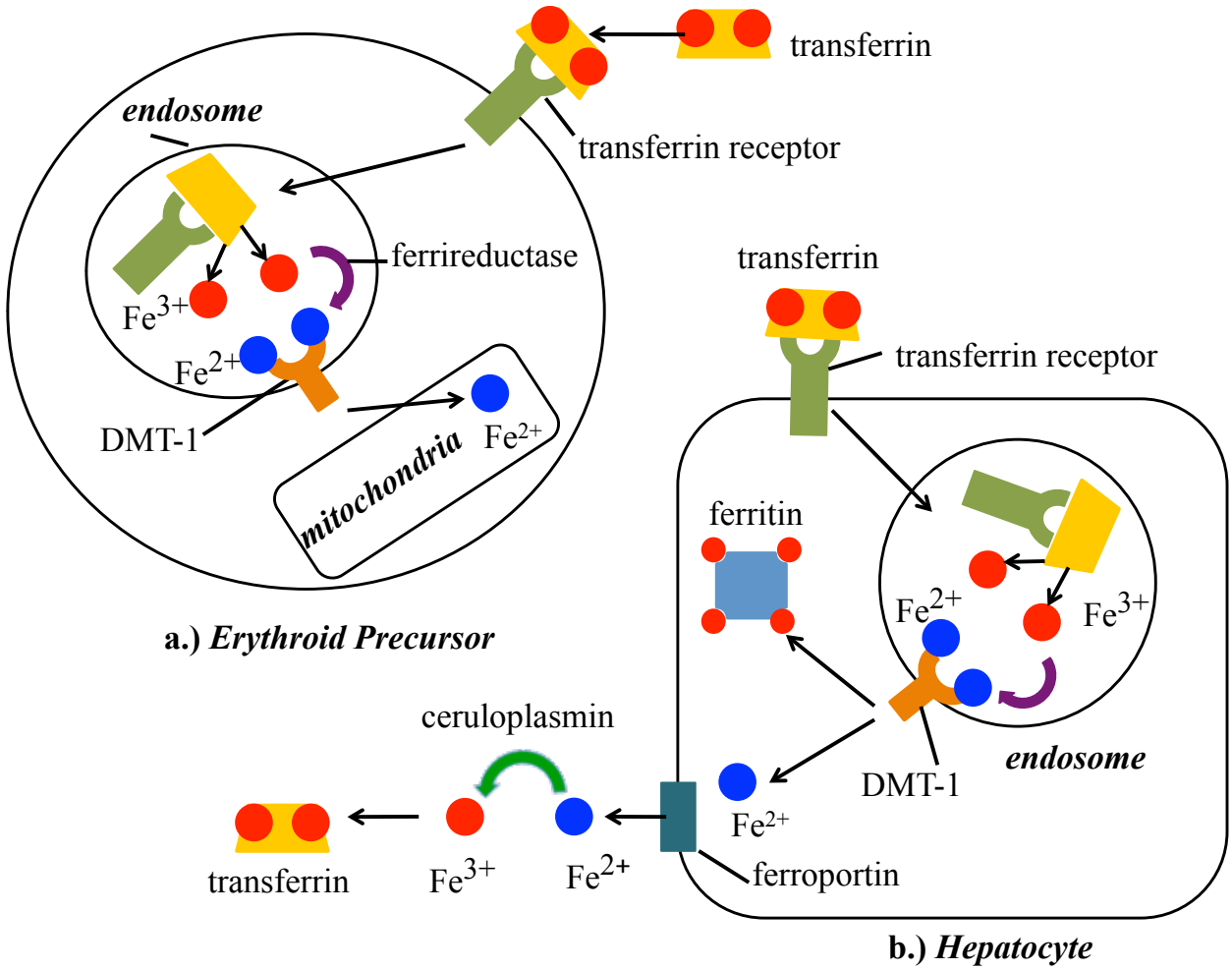
FIGURES

Figure 1.1. Mechanisms involved in Fe absorption



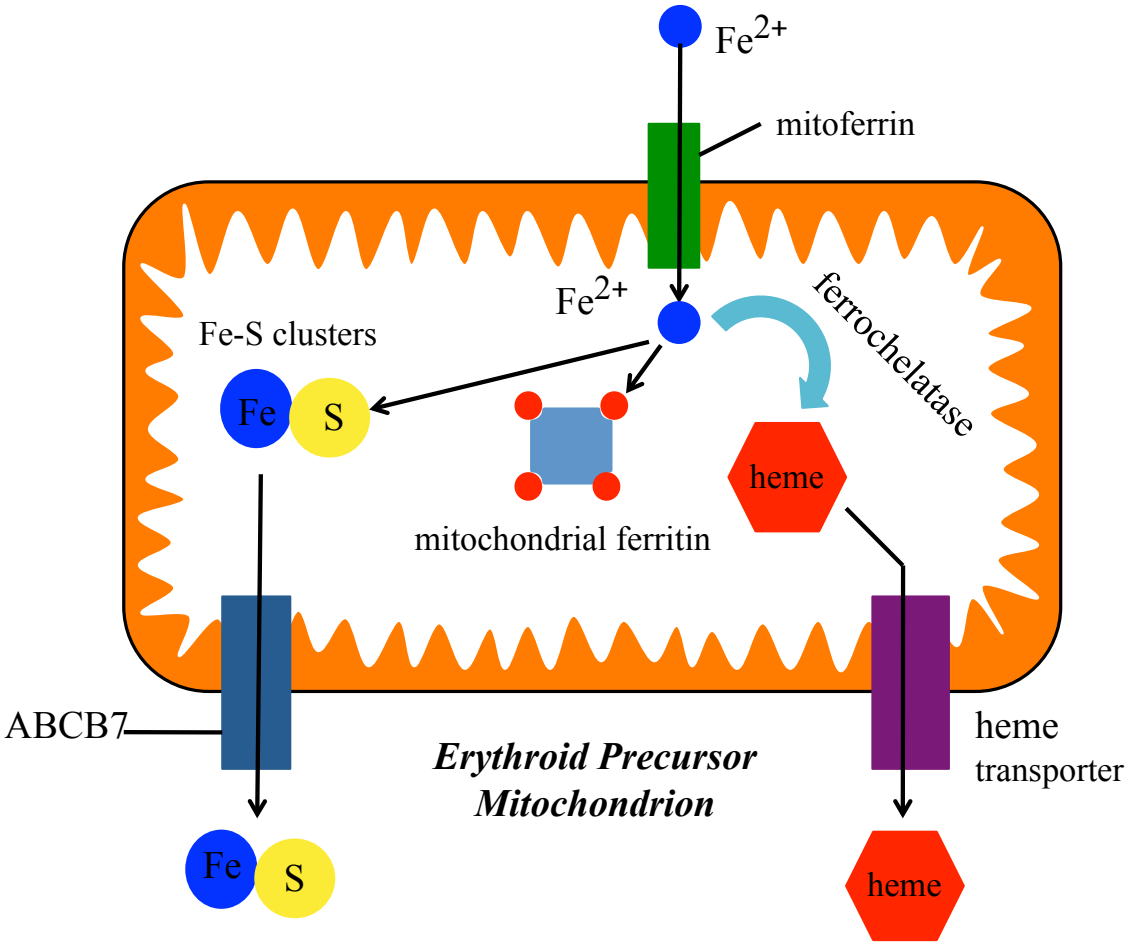
Dietary ferric iron (Fe^{3+}) is reduced by a ferrireductase to ferrous iron (Fe^{2+}) in the intestinal lumen. The Fe^{2+} binds to a binding protein and this complex binds to divalent metal transporter 1 (DMT-1) which is then internalized. If Fe status and stores are high, Fe is bound to mucosal ferritin, which is stored in the lysosome until the enterocyte is sloughed off and excreted in feces. If Fe status and body stores are inadequate, Fe travels through the enterocyte through unknown mechanisms, and exported from the enterocyte via the basolateral transporter ferroportin. Once in the bloodstream, Fe^{2+} is oxidized to Fe^{3+} by hephaestin and bound to transferrin for transport. In a situation of Fe overload hepcidin is released, which causes removal of ferroportin from the basolateral surface and degradation of ferroportin. [Adapted from Beard and Dawson (1997) and Sharp (2007)]. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.

Figure 1.2. Molecular mechanisms of Fe utilization in erythroid pre-cursers and hepatocytes



In both pre-erythrocytes and hepatocytes, transferrin is bound to a transferrin receptor (Trf1 or Trf2) on the cell plasma membrane and internalized through endocytosis into the endosome. The low pH in the endosome causes the release of Fe from transferrin. Ferrous iron (Fe^{3+}) is then reduced to ferrous iron (Fe^{2+}) by a ferrireductase, and exported from the endosome by DMT-1. **a.)** In erythroid precursors after release of Fe from the endosome, Fe is transported to the mitochondria for heme and Fe-S cluster synthesis. **b.)** In the hepatocyte Fe is bound to ferritin for storage, or can be exported as Fe^{2+} via ferroportin, oxidized to Fe^{3+} by ceruloplasmin and bound to transferrin again for transport. [Adapted from Chua et al. (2007) and Dunn et al. (2006)].

Figure 1.3. Formation of heme and Fe-S clusters in erythroid pre-curser mitochondrion



Ferrous iron (Fe^{2+}) is transported into the mitochondria via a transporter, possibly mitoferrin. The path of Fe inside the mitochondria is possibly determined by frataxin. The Fe can be used to synthesize heme by ferrochelatase, it can be sequestered in mitochondrial ferritin, or it can be used for Fe-S synthesis. Fe-S clusters are exported through the transporter ABCB7. Heme is transported from the mitochondria through a heme transporter (possibly ABCG2, FLVCR or ABC-me) [Adapted from Dunn et al. (2006)].

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CHAPTER 2

EFFECTS OF ABOMASALLY DOSED FERROUS (Fe^{2+}) OR FERRIC (Fe^{3+}) IRON ON SHORT-TERM IRON STATUS OF LACTATING DAIRY COWS

ABSTRACT

The majority of Fe naturally occurring in drinking water drawn from underground wells is in the more bioavailable ferrous (Fe^{2+}) valence. In contrast, the majority of Fe in feeds is of the ferric (Fe^{3+}) valence, and poorly absorbed by ruminants. Therefore, Fe from drinking water, though present in lower concentrations, could have a greater impact on Fe status, potential Fe toxicity and oxidative stress than feed Fe. Our objective was to evaluate the short-term effects of abomasally infused Fe^{2+} and the difference in short-term effects of Fe^{2+} and Fe^{3+} when administered at concentrations to simulate total daily Fe intake from high-Fe drinking water. In each experiment, 6 mid-lactation Holstein cows were assigned in a replicated 3 x 3 Latin Square balanced for treatment sequences. Treatments in Experiment 1 were 0, 0.75 and 1.5 mg of Fe from ferrous lactate [$Fe(C_3H_5O_3)_2$] per kg body weight (BW). Treatments were calculated to approximate 0, 4.5 and 9 mg Fe/L concentrations in drinking water, respectively, and were dosed directly into the absomasum in 1 L of deionized water. Experiment 2 differed only in the treatments administered: 0 mg Fe and 1.5 mg Fe/kg BW of either ferrous ($FeSO_4$) or ferric sulfate [$Fe_2(SO_4)_3$]. In each experiment blood samples were taken at 0 h and hourly for 12 h post-dosing, and liver biopsies were taken at 0, 18 and 36 h post-dosing. There were no effects of either concentration or valence of Fe dosed on serum Fe, unsaturated Fe binding capacity, percent Fe saturation, or Zn. In Experiment 1, cows infused with 0.75 mg/kg BW of ferrous lactate had increased serum total Fe binding capacity and hepatic Cu concentrations. Cows

infused with 1.5 mg Fe/kg BW had increased serum α-tocopherol concentrations. In Experiment 2, there was a tendency for cows administered ferric sulfate to have lower serum Fe than those infused with no Fe or ferrous sulfate. Cows infused with ferrous sulfate had lower serum Cu concentrations than those in other treatments. Treatments did not influence short-term Fe status, suggesting that it may take chronic exposure to Fe in drinking water, or greater Fe concentrations to impact Fe status and productivity of lactating cows.

INTRODUCTION

There is very little research describing non-dietary Fe toxicity in dairy cows. Lactating cows dosed intraruminally with 130 g Fe/d as ferric hydroxide [$Fe(OH)_3$] for 10 d presented clinical signs of extreme toxicity (Coup and Campbell, 1964). Excess dietary Fe can decrease weight gain and feed consumption in young ruminants and steers (Miller et al., 1991; Jenkins and Hidiroglou, 1987; Standish et al. 1969) apparently through Fe overload and oxidative stress. These same mechanisms may impact dairy cows consuming drinking water with high concentrations of Fe. Even though the concentration of Fe in most drinking water and amount consumed is much less than that from a typical dairy cow ration, Fe in water may be ferrous iron ($\mathbf{Fe^{2+}}$) (Colter and Mahler, 2006). Ferrous iron is more absorbable than Fe from the ration, which is typically in the ferric form ($\mathbf{Fe^{3+}}$) (NRC, 2001).

Iron metabolism is regulated through absorption, as very little Fe is excreted. Pathways of Fe absorption are down regulated in response to high Fe concentration from feed or water (Jacobs, 1980). As intake of Fe increases, Fe receptor divalent metal transporter 1 (**DMT-1**) on the apical surface of the duodenal enterocyte is internalized (Sharp, 2004). Additionally, hepcidin, a peptide hormone involved in Fe homeostasis, is released which inhibits the basolateral Fe transporter ferroportin and reduces Fe export from the enterocyte. Despite tight regulation, the mechanisms can be overwhelmed in cases of Fe overload.

Excess Fe also can lead to oxidative stress. Iron is a pro-oxidant (Sordillo and Aitken, 2009) and when Fe homeostasis is achieved, absorbed Fe is bound to Fe-binding proteins such as transferrin or sequestered in ferritin. However, when excess Fe is consumed and reaches the

lumen of the small intestine, high concentrations can damage the intestinal mucosa. Although most Fe is absorbed and incorporated into mucosal ferritin, and the rest excreted in feces, excess Fe can overwhelm absorptive mechanisms. Dairy calves exposed to high dietary Fe concentrations (750 mg/day of $FeSO_4$ for 56 d) had increased intestinal permeability (Hansen et al., 2010).

Multiple studies have used solutions of Fe in water to improve the Fe status of Fe-deficient rats and humans (Ferreira et al., 1991; Dutra de Oliveira et al., 1994). After determining that the inclusion of Fe^{2+} in drinking water increased hemoglobin concentration in rats (Ferreira et al., 1991), Fe^{2+} was supplied in drinking water to children with low Fe status, for an estimated total intake of 10 mg Fe/d. After 8 mo of supplementation, the percentage of children with deficient concentrations of hemoglobin decreased from 58 to 3% (Dutra de Oliveira et al., 1994). This evidence suggests that low concentrations of Fe^{2+}, delivered through water can impact measurements of Fe status.

Therefore, we hypothesize that the majority of Fe, naturally occurring in drinking water as Fe^{2+} could increase measurements of Fe status and potentially cause toxicity if present in great enough concentrations. Our objective was to evaluate the short-term effects of amount and valence of abomasally infused Fe on the Fe status of mid-lactation dairy cows. We expected that as the amount of Fe dosed increases, measurements of Fe status would increase, and Fe^{2+} would increase measurements of Fe status more than Fe^{3+}.

MATERIALS AND METHODS

The Institutional Animal Care and Use Committee at Michigan State University approved all experimental procedures (AUF # 03/10-024-00).

Animals, Experimental Design, and Treatments

Experiment 1. Six ruminally fistulated Holstein cows [219 ± 85 DIM (days in milk)] were assigned randomly in a replicated 3 x 3 Latin Square design involving 3 periods and 3 experimental treatments. There was a preliminary adjustment period of 2 wk prior to the first experimental period and 7 d between the experimental periods. Treatments were 0 (0Fe), 0.75 (0.75Fe) and 1.5 (1.5Fe) mg Fe^{2+}/kg BW from ferrous lactate [$Fe(C_3H_5O_3)_2$]. Treatments were made iso-lactate through lactic acid addition. Each treatment was administered as a single dose in 1 L of distilled, deionized water into the abomasum (Table 2.2). The 0.75Fe and 1.5Fe treatments were formulated to contain the total amount of Fe that a 600-kg lactating cow drinking 100 L of water/d would consume in a single day if drinking water with total Fe concentrations of 4.5 and 9 mg/L, from water according to total recoverable analysis (EPA method 200.7, 1994), or 1, and 2.25 mg/L from water according to direct Fe analysis (EPA method 200.7, 1994), respectively. One animal was removed from the experiment during period 2 due to illness, but recovered, and returned for period 3.

Experiment 2. Six ruminally fistulated Holstein cows (205 ± 22 DIM) were assigned randomly in a replicated 3 x 3 Latin Square design involving 3 experimental treatments and 3 periods. There was an adjustment period of 2 wk prior to the first experimental period and 7 d

between the experimental periods. Experimental treatments were 0 (Control) and 1.5 mg Fe/ kg BW from either ferrous (ferrous sulfate; $FeSO_4$) or ferric sulfate (ferric sulfate; $Fe_2(SO_4)_3$. Treatments were made iso-sulfate through sulfuric acid addition. Each treatment was administered as a single dose in 1.5 L of distilled, deionized water into the abomasum (Table 2.3). The ferrous sulfate and ferric sulfate treatments were formulated to contain approximately the total amount of Fe that a 600-kg lactating cow drinking 100 L of water/d would consume if drinking water with total Fe concentration of 9 mg/L, from water according to total recoverable Fe analysis (EPA method 200.7, 1994), or 2.25 mg/L from water according to direct Fe analysis (EPA method 200.7, 1994), respectively.

Preparation of Treatment Solutions.

Experiment 1. Cows were weighed weekly beginning 2 wk before period 1 to determine the amount of Fe each cow would receive, administered as a function of BW. Treatment solutions of 0, 0.75 or 1.5 mg Fe/kg BW from ferrous lactate were prepared by adding ferrous lactate to 125 mL of distilled, deionized water, heating and stirring well until the Fe compound dissolved. The amount of lactic acid required to make treatments iso-lactate was added at this time. This solution was diluted with 875 ml of distilled, deionized water to bring the total treatment solution to 1 L. The pH of the solution was measured and recorded (Table 2.2).

Experiment 2. Cows were weighed weekly beginning 2 wk before period 1 to determine the amount of Fe each cow would receive, administered as a function of BW. Treatment solutions of 0, 1.5 mg Fe/kg BW from ferrous or ferric sulfate were prepared by adding the

appropriate Fe compound to 200 mL of distilled deionized water, heating and stirring well until the Fe compound dissolved into solution. The amount of sulfuric acid required to make treatments iso-sulfate was added at this time. This solution was diluted with 1,350 mL of distilled, deionized water to bring the total solution volume to 1.5 L. The pH of the solution was measured and recorded (Table 2.3).

Common Treatment Administration and Sampling (Experiments 1 and 2).

Experimental periods were 36 h in length. Treatment solutions were administered as a single dose in approximately 1 min through a polyvinyl tube passed through the ruminal fistula and the reticulo-omasal orifice into the proximal abomasum. An insertion device, similar to that described by Gressley et al. (2006), was crafted from a 2.5 cm (inside diameter) polyvinyl chloride pipe (schedule 40 PVC), cut to approximately 23 cm in length and smoothed with sandpaper. One end was sanded into a smooth beveled edge to enable easy insertion into the reticulo-omasal orifice. Once the insertion device was placed into the abomasum, a dosing tube (1.58 cm outside diameter, 1.27 cm inside diameter) was inserted through the insertion device so that approximately 40 cm of the tube was located through the reticulo-omasal orifice beyond the rumen. Five hundred mL of water was initially poured via funnel through the dosing tube to confirm placement of the tube in the abomasum. After confirmation, 1 L treatment solution was poured into the abomasum. After all of the treatment solution was administered, 500 ml of additional water was administered through the tube into the abomasum to purge any remaining

treatment solution. In Experiment 2, 250 ml of water was used to confirm initial placement of the stomach tube in the abomasum, and the treatment solution was 1.5 L.

Jugular vein catheters were placed at least 24 h before the beginning of each experimental period in the contralateral vein from the previous period and removed at the end of each experimental period. Catheters were in place for approximately 75 h during each experimental period and all catheters were maintained twice daily by checking for patency and flushing with Na-heparin (100 IU/ml heparin in 0.9% sterile saline solution). Blood samples from indwelling jugular catheters were taken hourly -6, -5, -4, -3, -2, -1, 0 (just before treatment dosing), and hourly for the next 12 h. Pre-treatment blood sampling began at 1100 h, and continued until 1600 h. Cows were milked in the Dairy Teaching and Research Center (Michigan State University, East Lansing, MI) parlor at 1700 h, and treatment administration was completed at 1800 h. Orts were collected at 1000 h and feeding occurred at the same time (1115 h) each day in each period. Blood samples were allowed to clot and then spun in a centrifuge (1,000 *g*). Serum was harvested, transferred to microcentrifuge vials and stored at -10°C until analyses for Fe, Cu, Zn, α-tocopherol, and Fe-binding capacity. Whole blood samples were transferred directly to microcentrifuge tubes containing an anticoagulant and frozen at -80°C until analysis for glutathione peroxidase (**GSH-Px**) activity.

Liver samples were taken by biopsy 2 h before treatments were administered and then randomly among the 6 cows between 14 to 16 h after treatment administration and 36 h post-treatment administration. Liver biopsies were performed as outlined by Bradford and Allen (2005). At collection, cores were examined visually for contamination by adipocytes or excess

blood, and contaminated cores were discarded. Samples were stored in microcentrifuge vials at -10°C until analysis for Fe, Cu and Zn concentrations.

Common Feeding, Water and Milk Data Collection and Analysis.

A basal diet formulated to meet or exceed NRC (2001) recommendations for all nutrients (including Fe) was fed *ad libitum* in the initial adjustment phase beginning 2 wk before period 1 through the conclusion of period 3 (Table 2.4). Cows were fed once daily in their individual tie-stalls, and milked twice daily. Feed intakes, water intakes and milk yield and composition analysis are in Table 2.1.

Feed and water intakes (in-line water flow meters) were measured daily throughout the experiment (preliminary period through the end of period 3). Individual feed ingredients were sampled the week before each collection period began, and again between experimental periods. Each individual feed sample was weighed and dried in a forced-air oven at 55°C for 72 h until completely dry to determine DM percentage. Dried samples were ground through a 2 mm screen in a Wiley mill (Arthur H. Thomas, Philadelphia, PA). Similar feed ingredient samples were pooled by experiment and sent for analysis by Dairy One Laboratories (Ithaca, NY). Milk samples were taken weekly from one AM and PM milking and sent for composition analysis at Michigan DHIA Universal Laboratory Services (Lansing, MI). Drinking water was sampled daily from an in-line valve from the beginning of the preliminary period through the end of period 3. One sample each from the beginning and end of each experiment were analyzed by each of two separate methods for Fe. Raw water samples were sent to Midwest Laboratories

(Omaha, NE) for direct metals water analysis (Livestock water analysis, EPA 200.7 method (1994)). A second sample was acidified (pH < 2.0) with 1 mL nitric acid, and sent to the same laboratory for acid digestion and metals analyses (Total recoverable metals analysis using EPA 200.7 method (1994)). Water analyses are presented in Table 2.5. Internal water reference samples containing either 0, 1, 4 and 8 mg of Fe/L from ferrous lactate were placed as unknowns within the set of samples in triplicate for analysis. The intra-assay %CV was 4.0%.

Laboratory Analyses

Internal reference sample. Blood was taken from cows not in the experiments and serum was harvested using the same procedure as that from experiment cows. This pool of serum and whole blood was used as an internal reference sample to assess the variability within and among all assays. Internal reference samples were placed in assay sets every 10 samples to comprise approximately 10% of total samples analyzed. Once results were obtained for each analyte, the percent coefficient of variation (% CV) was calculated, and if the % CV was greater than 10, the samples, including additional internal reference samples, were re-analyzed.

Serum non-heme Fe and Fe binding capacity analyses. Analyses of serum Fe, unsaturated Fe binding capacity (**UIBC**), total Fe-binding capacity (**TIBC**) and percent Fe saturation were completed at the College of Veterinary Medicine Clinical Pathology Laboratory at Michigan State University. The analyses were completed on an Olympus AU640e chemistry immuno-analyzer (Olympus America Inc., Center Valley PA) utilizing Olympus reagents. The serum samples were incubated in an acidic medium and Fe dissociated into Fe^{3+} and apo-

transferrin molecules. Hydrochloric acid and sodium ascorbate were added to reduce Fe^{3+} to Fe^{2+}. Ferrous ions reacted with chromogen TPTZ ([2,4,6-Tri-(2-pyridyl)-5-triazine] to form a complex measured at a wavelength of 600/800 nm.

Serum UIBC was measured by adding a known amount of Fe^{3+} to a known sample amount to saturate available transferrin binding sites. The remaining unbound Fe reacts with nitroso-PSAP [2-nitroso-5-(N-propyl-N-sulfopropylamino)phenol] to form a complex measured colorimetrically. The TIBC is calculated as: Fe (μg/dL) + UIBC (μg/dL). Percent Fe saturation is calculated as: [Fe (μg/dl) / TIBC (μg/dL)] x 100. The intra- and inter-assay variation of the internal reference samples were: for Fe: 2.19% and 1.88%; and for UIBC: 6.11% and 5.5%, respectively.

Serum trace element analyses. Two hundred μL of each serum sample diluted with 5 mL of a solution containing 0.5% EDTA and Triton X-100, 1% ammonia hydroxide, 2% propanol and 20 ppb of scandium, rhodium, indium and bismuth as internal standards. An Agilent 7500ce (Agilent Technologies, Santa Clara, CA) Inductively Coupled Plasma-Mass Spectrometer (**ICP-MS**) was used for the analysis. Each element was calibrated using a 4-point linear curve of the analyte: internal standard response ratio. The intra- and inter-assay variations of the internal reference samples were: for Cu: 3.43% and 5.38% and for Zn: 3.94% and 5.45% respectively.

Serum α-tocopherol analysis. Fat-soluble vitamins were extracted from serum before being analyzed by liquid chromatography. Serum (0.5 mL) was diluted with deionized water containing 0.9% saline. Twenty μl of apocarotenal (as an internal standard), 1 ml of ethanol containing butylated hydroxytolulene and 1 ml of hexane were added to the diluted serum. Caps

were replaced and the samples were mixed with a vortex mixer for 10 min. Samples were then placed in a centrifuge and spun (1,000 *g*) for 10 min. After centrifugation, a positive displacement pipette was used to remove 0.5 ml of the top layer of hexane, which was then placed in a clean test tube and evaporated to dryness in a Rapidvap vacuum evaporation system (Labconco, Kansas City, MO), set at 95 rpm, and 35°C. Once dry, the residue was suspended with 0.5 ml of mobile phase, consisting of 70% acetyl nitrile, 10% methanol and 20% methylene chloride. Samples were transferred to vials for analysis. Samples were analyzed by high performance liquid chromatography (**HPLC**) at a wavelength of 292 nm. The sample (50 µL) was injected in to a column (4.6 x 77 mm, Waters Corporation, Milford, MA) at a flow rate of 1.2 mL/min into a photodiode array detector (Waters 996 Detector; Waters Corporation, Milford, MA). The absorbance was measured and area under the curve quantified. Quantification was by internal standard ratio and a multipoint calibration curve. The intra- and inter-assay variations of the internal reference samples were 6.21% and 6.77% respectively.

Whole blood GSH-Px activity analysis. Samples were thawed in a refrigerator for 70 min and analyzed in triplicate. Sixty-six µl of double Drabkins solution (400 mg potassium cyanide, 79.2 mg potassium ferricyanide, and 200 mL distilled, deionized water), 714 µL of distilled water, and 20 µL of sample were added to each culture tube in an ice bath, and mixed using a vortex mixer. First, 270 µl of reaction buffer was added to each well of a 96-well plate, consisting of 0.2M K_2PO_4 buffer, sodium azide solution, deionized water, 1.11 mg/dL NADPH, and glutathione reductase enzyme. Fifteen µL of 12 mg/mL reduced glutathione and 12 µL of diluted sample were added to each well. The well plate was incubated for 5 min at 25°C. After

incubation 3 µL of 12 mM H_2O_2 was added to each well, and the plate was read immediately using a Spectramax plate reader (Molecular Devices, Sunnyvale, CA) at 340 mn, every 9 s for 5 min. Hemoglobin was measured by adding 5 mL of cyanmethemoglobin to 20 µL of whole blood. The sample optical density was measured at 540 nm on a hemophotometer (Fisher Scientific Co, Pittsburgh, PA). Results were reported as g/100mL of blood. The units of GSH-Px activity were expressed as IU/g of hemoglobin. One IU is defined as the amount of enzyme that will cause the oxidation of 1.0 nmol of NADPH to $NADP^+$ per minute at 25°C. The inter-assay variation for the internal reference samples was 7.3%.

Liver tissue mineral element analysis. All liver tissue samples were prepared and analyzed at the same time. Samples were thawed and placed in a 24°C oven to dry overnight. Samples were removed and weighed. Three hundred µl of concentrated nitric acid were added to each sample and left overnight to digest in an oven at 32-37°C. After acid-digestion samples were diluted with deionized water to approximately 100 times their dry mass. After dilution, 200 µl of each sample were pipetted and diluted with 5 mL of a solution containing 0.5% EDTA and Triton X-100, 1% ammonia hydroxide, 2% propanol and 20 ppb of scandium, rhodium, indium and bismuth as internal standards. An Agilent 7500ce (Agilent Technologies, Santa Clara, CA) Inductively Coupled Plasma-Mass Spectrometer (ICP-MS) was used for the analysis.

Statistical Analyses

Data were analyzed by method of least-squares ANOVA using the Mixed Models procedure of SAS (Version 9.2, SAS Institute Inc.). Data of hourly measurements were analyzed

using the REPEATED statement, and the covariance structure that resulted in the lowest Bayesian information criterion (**BIC**) was used. Fixed effects in the model were treatment, hour and treatment by hour interaction. Random effects were cow and period. If the interaction was non-significant *(P* > 0.15), it was removed from the model. Additionally, if hour was non-significant *(P* > 0.10), it also was removed from the model. Mean of the pre-dosing samples from each cow was used as a covariate for each dependent variable. If the covariate term was not significant, it was removed. Residual distributions were tested for normality using the Shapiro-Wilk test, and outliers were identified using Cook's D statistic. Differences between treatments were identified using the PDIFF option with Tukey's adjustment. Least-squares means and SEM are presented. Significance for main effects was declared at $P < 0.05$, and a trend toward significant difference at $P < 0.10$. Significance for interaction effects was declared at $P < 0.10$, and a trend toward significance at $P < 0.15$. Pearson correlations were calculated for the relationships between independent response variables using the Correlation procedure.

RESULTS

Water Analyses

Average water sample Fe concentrations were 0.42 and 0.46 mg/L from direct Fe analysis, and 1.59 and 2.36 mg/L from total recoverable Fe analysis for Experiments 1 and 2, respectively. The Fe concentrations from the direct Fe analysis were approximately 25% of the total recoverable Fe concentrations. The direct Fe analysis is that used in the field to evaluate water quality in dairy farms (Beede, personal communication, Michigan State University, 2011).

Treatment by Hour Interactions

There were no treatment by hour interactions for any response variable. There was a tendency for a treatment by hour interaction in hepatic Cu concentration *(P* = 0.10) in Experiment 1. The 0.75Fe treatment tended to cause a greater decrease in hepatic Cu concentration than other treatments (Figure 2.1).

Experiment 1

Treatment effects. Our objective was to characterize the effects of varying amounts of ferrous lactate abomasally infused on Fe and antioxidant status. Lactating cows were administered 0, 0.75, or 1.5 mg of Fe from ferrous lactate per kg of BW, and Fe status responses were evaluated for 12 h. We found no effects of treatment on serum Fe, UIBC, Fe saturation, Zn or Cu, whole blood GSH-Px activity, or hepatic Fe and Zn concentrations (Table 2.6). Serum TIBC tended to be greater for cows on 0.75 Fe than cows on 0Fe ($P = 0.06$). Serum α-tocopherol

was greater in response to 1.5Fe than 0Fe ($P = 0.006$), and hepatic Cu concentrations were lower in cows administered 0.75Fe than cows administered 0Fe ($P = 0.02$).

Hourly effects. When data were pooled across treatments there were some effects of hour on response variables. Both serum Fe ($P = 0.04$; Figure 2.2) and Fe saturation ($P = 0.01$; Figure 2.3) decreased over time. Serum Zn concentrations decreased over the experimental period ($P < 0.0001$; Figure 2.4). Hepatic Cu concentrations decreased over time ($P = 0.02$; Figure 2.5), whereas hepatic Zn tended to decrease from 0 h to 36 h ($P = 0.09$; Figure 2.6). There were no hourly effects on serum TIBC, UIBC, α-tocopherol and Cu, whole blood GSH-Px activity, and hepatic Fe concentrations.

Correlations. The correlations between various independent serum and liver response variables were investigated (Tables 2.7, 2.8). There was a negative correlation between serum Fe and serum UIBC ($r = -0.46$; $P < 0.05$).

Experiment 2

Treatment effects. The objective was to evaluate the effects that Fe^{2+} or Fe^{3+} have on Fe and antioxidant status of lactating dairy cows. Iron was supplied as 0 mg of Fe or 1.5 mg of either ferrous or ferric sulfate per kg BW. Overall there were no effects of treatment on serum TIBC, UIBC, Fe saturation, α-tocopherol, Zn; whole blood GSH-Px; or hepatic Fe, Cu or Zn concentrations (Table 2.9). Serum Cu concentrations in response to ferrous sulfate were greater than serum Cu concentrations for cows on either Control or ferric sulfate treatments ($P =$

0.0002). Serum Fe concentrations tended to be lower for cows on the ferric sulfate treatment than Control cows ($P < 0.10$).

Hourly effects. Hourly effects pooled across treatment were found for serum Fe, UIBC, Fe saturation, α-tocopherol, Zn, and whole blood GSH-Px. Serum Fe decreased over the experimental period ($P = 0.01$; Figure 2.7) and UIBC ($P = 0.001$; Figure 2.8) and Fe saturation ($P < 0.0001$; Figure 2.9) also decreased. Serum Zn decreased slightly, but consistently throughout the experimental period ($P < 0.0001$; Figure 2.10). There was an hour effect on α-tocopherol ($P = 0.001$; Figure 2.11) though no consistent trend to increase or decrease was identified. Whole blood GSH-Px tended to decrease from 0 h to 6 h, but then increase slightly by h 12 ($P = 0.07$; Figure 2.12).

Correlations. The correlation between various independent serum and liver response variables was investigated (Tables 2.10, 2.11). There was a correlation between serum Fe and serum UIBC ($r = -0.54$; $P < 0.05$) and hepatic Cu and Zn ($r = 0.6535$; $P < 0.05$).

DISCUSSION

In our study, 0.75 mg Fe/kg BW and all 1.5 mg Fe/kg BW treatments were formulated to contain approximately the total amount of Fe that a 600-kg lactating cow drinking 100 L of water/d would consume if drinking water with total Fe concentrations of 4.5 and 9 mg/L, respectively. However, after receipt of water analyses we realized that these values are not necessarily accurate. The laboratory utilizes the EPA 200.7 method for direct metals for Livestock water analysis. However, there is an additional method that can quantify total recoverable Fe through the use of acid digestion. After receiving the two types of analyses, we noticed that the Fe values reported for the direct metals analysis were approximately 25% of the values for the total recoverable metals analysis. Based on this information, we suspect that the concentrations of Fe dosed may be more similar to the total Fe intake of an average cow drinking 100 L of water daily with Fe concentrations of 0, 1, or 2.25 mg/L respectively. This could be reason for anecdotal evidence that high concentrations of Fe in drinking water cause problems in cow health and productivity. Drinking water containing 2 mg Fe/L may actually contain 8 mg Fe/L.

Experiment 1

Serum Fe concentrations. The serum Fe concentrations of cows dosed with ascending amounts of ferrous lactate were not affected. This was unexpected as humans orally administered similar amounts of Fe^{2+} as ferrous sulfate (approximately 1.4 mg Fe/kg BW) in solution had increased serum Fe concentrations (approximately 100 µg/dL) within 2 h (Walker et al., 1989).

However, Ammerman et al. (1967) orally dosed sheep of adequate Fe status with 150 μC Fe^{59} from either ferrous sulfate or carbonate, providing 1.8 mg total Fe/kg BW, or ferric chloride or oxide, providing 0.8 mg total Fe/kg BW. They reported that the radio-labeled Fe could be detected in the serum and was maximal at 12 h post-dosing, independent of source, though no more than 0.25% of labeled Fe was detected in the serum from any source. It is likely that the systems involved in Fe regulation can easily handle small increases in Fe intake. Other research showed that serum Fe did not increase in response to a meal in humans (Sinnah et al., 1969).

Hansen et al. (2010) reported that although calves supplemented with 750 mg Fe/kg dietary DM of ferrous sulfate (approximately 22 mg Fe/kg BW) did not have increased concentrations of Fe in serum, Fe addition negatively affected ferroportin expression, and increased hepcidin expression in intestinal tissue. The results of our experiment suggest that the concentrations of Fe contained in poor quality water apparently are not great enough to overwhelm the regulation of Fe absorption and significantly affect short-term Fe status.

It is possible that pH of the treatment solutions had an effect on Fe absorption. The 1.5Fe treatment had a greater pH, as it did not contain supplemental acid (Table 2.2). Between the pH range of 4 to 8, as pH of an aqueous solution decreases, Fe^{2+} and the ratio of Fe^{2+}/Fe^{3+} in the solution increases (Morgan and Lahav, 2007). At a lower pH, the concentration of Fe^{2+} is greater, and Fe^{2+} is more available for absorption than Fe^{3+}. Solomans and Jacob (1981) administered 1 g ascorbic acid with ferric chloride to increase Fe absorption in humans. In rats administered cimetidine, a compound that inhibits gastric acid secretion, absorption of Fe was decreased (Kim et al., 1993) indicating that an acidic environment is essential for Fe absorption.

In our experiment the greater pH of the 1.5Fe treatment could explain the lack of difference between serum Fe concentrations of cows given 0.75Fe and 1.5Fe treatment even though 1.5Fe contained twice as much Fe.

Serum TIBC, UIBC and percent Fe saturation. Serum TIBC is an indirect measurement of total Fe-binding proteins present in the serum (mainly transferrin). Transferrin is the binding protein for Fe transport in serum. Serum UIBC is an indirect measurement of the Fe binding proteins present in the serum that are not bound to Fe. In general, there is an inverse relationship between serum Fe and serum UIBC (Furugouri, 1971). This was true in Experiment 1. There was a negative correlation between serum Fe and UIBC ($r = -0.46$; Table 2.7). Cows administered 0.75Fe tended to have greater serum TIBC than 0Fe-cows (Table 2.6). Serum Fe was numerically greater, and UIBC numerically lower for 0.75Fe cows, suggesting that this treatment had the greatest overall impact. This explains the treatment difference, as TIBC is reflective of both serum Fe and UIBC.

Serum and hepatic Cu concentrations. Copper is transported into the enterocyte through two main transporters, DMT-1 and copper transporter 1 (**Ctr1**) (Sharp, 2004). DMT-1 has a high affinity for Fe^{2+}, and when excess Fe is present it can prevent the binding of Cu to DMT-1, reducing Cu absorption (Sharp, 2004). Decreased absorption could cause depletion in liver Cu reserves, as Cu stores are mobilized to maintain Cu homeostasis. In Experiment 1 it was expected that 1.5Fe would decrease serum and liver Cu most, because it delivered the most Fe; however, this was not the case. There was a tendency for a treatment by hour interaction in hepatic Cu concentration, as 0.75Fe decreased hepatic Cu concentration over time to a greater

extent than other treatments (Figure 2.1), and cows administered 0.75Fe had lower liver Cu concentrations when data were pooled across hour (Table 2.6). According to Standish et al. (1969) when cattle were administered high concentrations of dietary Fe as ferrous sulfate, there was a decrease in hepatic Cu concentration. In Experiment 1, 0.75Fe caused a greater decrease in hepatic Cu concentration than other treatments (Table 2.6).

Serum and hepatic Zn concentrations. There was no effect of treatment on serum or liver Zn concentrations. There is some evidence for an interaction between Fe and Zn in ruminants; however this relationship is less well-formulated and consistent than the relationship between Fe and Cu (Campbell et al., 1974, Standish et al., 1969, Standish and Ammerman, 1971). It is likely that the interaction only manifests when animals have been exposed to high concentrations of Fe for a long period of time. Steers fed high dietary concentrations of ferrous sulfate (0, 400, 1,600 or 3,200 mg Fe/kg), had decreased liver Cu and Zn concentrations as dietary Fe concentration increased (Standish et al., 1969). Steers were exposed to high concentrations for 49 d before sampling. We suspect that over a longer period of time than Experiment 1, exposure to Fe could impact Zn status.

Antioxidant status and oxidative stress. In vitro, during Fe-induced oxidative stress, α-tocopherol concentration decreased rapidly (Yamamoto and Niki, 1988). Somewhat in contrast, in our study serum α-tocopherol concentrations of cows on 0Fe were lower than that of cows on 1.5Fe. Additionally we found no change in GSH-Px activity in response to treatment. In the body, GSH-Px and vitamin E work in concert to protect tissues from oxidative damage induced by Fe (Milchak and Bricker, 2002). Also, induced α-tocopherol deficiency decreased glutathione

(an antioxidant) and increased hydroperoxides in rats (Nickander et al., 1994). Both the lack of response of GSH-Px activity and increase in serum α-tocopherol in Experiment 1 suggest that supplemental Fe did not induce oxidative stress. Similarly, in a study examining the relationship of supplemental Fe and antioxidant status, 1,000 mg/kg of supplemental Fe from ferrous sulfate added to swine starter diets had no effect on either GSH-Px activity or serum α-tocopherol concentrations (Dove and Ewan, 1990). Hansen et al. (2010) reported no change in GSH-Px activity in response to high dietary Fe from ferrous sulfate (750 mg/kg) in liver or heart tissue. Our results suggest that the amounts of Fe administered in Experiment 1 were not enough to impact antioxidant status, or cause oxidative stress short-term.

Experiment 2

Serum Fe concentrations. We expected serum Fe concentrations of cows on both ferrous and ferric sulfate treatments to be greater than those on the Control treatment, and serum Fe concentrations of cows given ferrous sulfate to be greatest. In contrast, there was a tendency for serum Fe to be lower in cows infused with ferric sulfate compared with Control, and cows given ferrous sulfate were not different from cows given Control or ferric sulfate treatments (Table 2.9). As mentioned previously, Fe absorption is tightly regulated, and it is not known if Fe^{2+} or Fe^{3+} affect transporters involved in Fe metabolism differently. It is possible that Fe^{3+} has a similar effect on the presence of DMT-1 in the apical membrane as Fe^{2+}. However as Fe^{3+} must be reduced in the intestinal lumen before absorption, if there are fewer transporters available,

absorption could be decreased more than in response to Fe^{2+}. Decreased Fe absorption, and Fe export from the enterocyte, would result in lower serum Fe concentrations short-term.

Serum TIBC, UIBC and percent Fe saturation. There was no effect of treatment on concentrations of serum TIBC, UIBC or percent Fe saturation (Table 2.9). In contrast, calves fed 1,000 mg Fe/kg dietary DM supplemental ferrous carbonate had decreased serum UIBC and TIBC after 1 wk, but recovered to pre-feeding values after 2 wk (Ho et al., 1984). We expected ferrous sulfate to result in lower UIBC, and increase TIBC and Fe saturation percentage, but 1.5 mg Fe/kg BW from either Fe source did not impact measurements of Fe status in Experiment 2.

Serum and hepatic Cu concentrations. There was an effect of treatment on serum Cu concentrations. Both cows on the Control and ferric sulfate treatments had serum Cu concentrations that were greater than those on ferrous sulfate, and there were no differences in hepatic Cu concentrations (Table 2.9). Standish and Ammerman (1971) reported that wethers administered 1,600 mg Fe/kg dietary DM of ferrous sulfate had decreased serum concentrations of Cu, whereas the same dietary concentration of ferric citrate had no effect, similar to the results in our experiment. Campbell et al. (1974) found that cows intraruminally dosed weekly with 210 mg/kg BW of ferric hydroxide slurry had markedly decreased hepatic Cu stores, and serum Cu concentrations, but not liver Zn. The authors suggested that though Fe^{2+} is more bioavailable than ferric iron, Fe^{3+} is more effective at rapidly depleting hepatic Cu. This was not the case in our experiments as hepatic Cu concentrations of cows given ferric sulfate were not different from cows administered the Control treatment. It is likely that the difference between the current experiment and that of Campbell et al (1974) was due to different amounts of Fe administered.

Serum and hepatic Zn concentrations. Whereas there is some evidence for an interaction between Fe and Zn in absorption, the mechanisms are not known, and as mentioned previously, the interaction of Fe and Zn has not been demonstrated as consistently as that of Fe and Cu (Campbell et al., 1974, Standish et al., 1969, Standish and Ammerman, 1971). In Experiment 2 there was no treatment-related difference in either serum or hepatic Zn concentrations. Solomans and Jacob (1981) concluded that while both Fe^{2+} and Fe^{3+} (50 mg as ferrous sulfate or ferric chloride) administered to humans decreased absorption of a 25 mg dose of Zn, Fe^{2+} inhibited Zn uptake to a greater extent than Fe^{3+}. Our experimental results are not consistent with this observation, as we found no difference between Control, ferrous or ferric sulfate on Zn measurements. This is in agreement with Experiment 1, as amount of Fe^{2+} dosed also did not impact Zn status. Results from both Experiments 1 and 2 indicate Zn was not impacted by the amounts and valences of Fe administered in our studies.

In Experiment 2 we found a positive correlation between hepatic Cu and Zn concentrations (Table 2.11). This effect was likely heavily influenced by 2 individual cows that had markedly greater concentrations of Cu and Zn than other cows. A similar relationship was not found in Experiment 1.

Antioxidant status and oxidative stress. There was no effect of ferrous or ferric sulfate treatments on serum α-tocopherol concentrations or whole blood GSH-Px. Lack of treatment differences suggests that there may be no difference between Fe supplied through either feed or water short-term, on antioxidant status and oxidative stress. Braughler et al. (1986) found that Fe^{3+} alone did not induce lipid peroxidation of rat brain tissue *in vitro*. Although we expected

that ferrous sulfate would have a greater impact on oxidative stress than ferric sulfate, this was not the case. At the concentrations dosed, neither Fe^{2+} nor Fe^{3+} causes oxidative stress short-term.

We found a positive correlation between serum α-tocopherol concentration and whole blood GSH-Px (Table 2.10). This suggests a potential relationship between the concentration of α-tocopherol in the serum and GSH-Px; when α-tocopherol concentration was greater, enzyme activity also was greater. This pattern is contrary to the results reported by Chow et al. (1973) that rats fed deficient dietary concentrations of α-tocopherol had greater GSH-Px activity. However, this pattern was not found in Experiment 1, indicating that additional research should be completed in this area.

Hourly Effects Pooled Across Treatments for Experiments 1 and 2

In our experiments, because we sampled blood hourly for 12 h, we were able to characterize changes in response variables over time; by treatments and pooled across treatments. In both Experiments 1 and 2, there were no overall effects of hour pooled across treatments for serum α-tocopherol, whole blood GSH-Px, and or Fe concentrations. In experiment 1 we found no overall effect of hour pooled across treatments on serum TIBC, UIBC, α-tocopherol, and Cu concentrations, whole blood GSH-Px activity or hepatic Fe concentrations. In Experiment 2 there was no overall hourly effect on serum TIBC and Cu concentrations, and hepatic Fe, Cu and Zn concentrations.

Serum Fe and UIBC concentrations. There is very little research on diurnal variation in serum and plasma Fe concentrations in cattle. Mansion et al. (1981) evaluated the effect of time of day on variation in serum Fe in Friesian cows and heifers, and found that there was no significant pattern throughout the day. These researchers concluded that the main source of variation was the animal.

This is contrary to our findings in Experiments 1 and 2. In Experiment 1 serum Fe decreased from h 2 (2000 h) through the rest of the sampling period (0600 h), indicating that daily serum Fe was greatest before 2000 h (Figure 2.2). This was similar to results in Experiment 2 in which serum Fe concentrations decreased from h 4 (2200 h) through h 11 (0500 h) (Figure 2.7). Our findings are similar to those in humans completed by Wiltink et al. (1973). Those researchers evaluated changes in plasma Fe over a 24 h period and determined that concentrations were greatest from 1200 to 2000 h and lowest between 2400 and 0800 h. Diurnal variations in serum Fe concentrations in humans are not affected by food intake (Sinnah at al, 1969). Stengle and Schade (1957) found no significant variation in TIBC in humans, which is in agreement with both of our experiments. In Experiment 2, serum UIBC increased from hour 1 (1900 h) through hour 11 (0500 h) (Figure 2.8). This effect is likely related to the serum Fe decrease over time because those responses are correlated as shown in Experiments 1 and 2.

Serum percent Fe saturation. Serum percent Fe saturation is an indirect measurement of Fe saturation of transferrin and is calculated as percent saturation = [serum Fe/TIBC] x 100. It would be expected that as serum Fe concentrations changed, there would be a concurrent change in serum percent saturation. We found that serum Fe (Figures 2.2, 2.7) and percent Fe saturation

(Figures 2.3, 2.9) decreased over time in both experiments. Overall, our data suggest there may be a pattern in daily serum Fe concentration, serum UIBC, and percent saturation in lactating dairy cows.

Serum Cu concentrations. There was no hourly effect on serum Cu concentrations in either experiment. Mansion et al. (1981) found that serum Cu increased linearly from 0700 to 1600 h in dairy cows. Diurnal variation of serum Cu in cattle should be investigated further.

Serum Zn concentrations. Although no information exists about diurnal variation in cattle, studies completed in humans suggest that plasma Zn concentrations decrease from 0800 to 1900 h and increase overnight (McMillan and Rowe, 1982; Markowitz et al., 1985). Also daily variation may be linked to food intake patterns, but not Zn content of the diet (Hashim et al., 1996). This is different than our findings. There was a clear pattern in serum Zn concentrations in both experiments; serum concentrations decreased overnight (from 1900 to 0600 h) (Figures 2.4, 2.10). It is possible that serum concentrations peaked shortly after feeding (1100 h) and decreased slowly throughout the day. A thorough evaluation of diurnal variation in cattle is needed.

Serum α-tocopherol concentrations. There is limited research on the diurnal variation of fat-soluble vitamins, but Nierenberg and Stukel (1987) found that hourly variation of plasma tocopherols was minimal, and non-significant in humans. No previous research evaluating the variation of plasma or serum fat-soluble vitamin concentrations in cattle or other ruminants was found. There was no hourly effect on α-tocopherol in Experiment 1. There was an effect in

Experiment 2 (Figure 2.11), however the effect was influenced mainly through variability from hour to hour rather than being a consistent effect over time.

Whole blood GSH-Px activity. There was no effect of hour on whole blood GSH-Px activity in Experiment 1. In Experiment 2 there was a tendency for activity to decrease from h 0 (1800 h) to h 6 (0000 h) and then increase slightly at the end of the experimental period (Figure 2.12). In human subjects limited research suggests that glutathione concentrations are subject to diurnal variation; concentrations are maximal around 0230 h, and minimal around 1330 h. Information about daily variation in GSH-Px activity was not found. Plasma malonyldialdehyde (MDA) concentrations generally are found to be greatest from 1200 to 1600 h and lowest at 0400 h in healthy subjects (Akbulut et al., 2003), suggesting that lipid peroxidation follows a diurnal pattern. This is partial evidence for a pattern in GSH-Px activity, however verification of this potential pattern requires additional research.

Hepatic trace mineral concentrations. There is no information in the literature about variation in hepatic mineral concentrations during such a short time period, as no other researchers have taken liver tissue samples as frequently as in our study. In Experiment 1, there was a decrease in hepatic Cu (Figure 2.5) and a tendency for a decrease in hepatic Zn (Figure 2.6) from h 18 (1200 h) to h 36 (0600 h), whereas there was no change in hepatic Fe concentration. These patterns were not observed in Experiment 2. Additional work should be completed in this area in order to explore a potential pattern in variation.

CONCLUSIONS

In conclusion, there was little difference between 0, 0.75 and 1.5 mg Fe^{2+}/kg BW, and 1.5 mg Fe^{2+} or Fe^{3+}/kg BW of on measurements of Fe and antioxidant status, or oxidative stress. There was some impact on serum and hepatic Cu concentrations, though the effects were variable and warrant further investigation.

Overall, the amounts of Fe administered abomasally in these experiments apparently are not great enough to affect short-term Fe status. However, we suggest that chronic exposure to greater amounts of Fe in drinking water, or exposure to greater concentrations of Fe, could cause antioxidant depletion and oxidative stress indicative of situations described in the field. This is supported by the information discovered in our work that the actual total recoverable concentrations of Fe in drinking water are about 4 times greater than conventionally believed based on the routinely used Livestock water analysis. These much greater concentrations could lead to decreased water intake, feed intake and milk production. Alternatively, decreased drinking water palatability from Fe contamination, or a combination of both of these effects could cause a decrease in cow production and health.

APPENDIX B

FIGURES AND TABLES REFERENCED IN CHAPTER 2

TABLES

Table 2.1. Pre-experimental dry matter intake, milk yield, water intake and body weight of cows in Experiments 1 and 2

	Experiment			
	1		2	
	Mean	±SD[1]	Mean	±SD
DMI, kg	23	1.9	25	1.6
Water intake, L	79.6	30.2	102.5	7.6
Body weight, kg	768	82.5	658	51.2
Milk yield, kg	36.2	13.7	36.9	3.5
Milk composition, %				
Fat	3.10	0.56	3.68	0.48
True protein	2.90	0.11	2.99	0.12
Lactose	4.51	0.36	4.92	0.09
SNF[2]	5.37	0.42	5.85	0.10
SCC[3], 1000 ml	186	112.0	258	357.8
MUN[4], mg/dl	17.1	1.56	14.7	1.71

[1]SD = standard deviation.

[2]SNF = solids not fat.

[3]SCC = somatic cell count.

[4]MUN = milk urea nitrogen.

Table 2.2. Characteristics of treatment infusion solutions in Experiment 1

	Treatments[1]					
	0Fe		0.75Fe		1.5Fe	
Solution characteristics	Mean	±SD[2]	Mean	±SD	Mean	±SD
Total Volume, L	1.00	--	1.00	--	1.00	--
pH	2.56	0.09	3.73	0.06	5.31	0.03
Lactate, g	3.70	0.39	3.73	0.40	3.71	0.41
Fe, g	--	--	0.58	0.06	1.15	0.13

[1]Treatments: 0Fe = 0 mg Fe/kg BW; 0.75Fe = 0.75 mg Fe/kg BW from ferrous lactate [$Fe(C_3H_5O_3)_2$]; 1.5Fe = 1.5 mg Fe/kg BW from ferrous lactate.

[2]SD = standard deviation.

Table 2.3. Characteristics of treatment infusion solutions in Experiment 2

	Treatments[1]					
	Control		ferrous sulfate		ferric sulfate	
Solution characteristics	Mean	±SD[2]	Mean	±SD	Mean	±SD
Total volume, L	1.50	0.00	1.50	0.00	1.50	0.00
pH	1.63	0.06	2.12	0.08	2.48	0.11
Sulfate, g	2.54	0.19	2.54	0.20	2.54	0.19
Fe, g	0.00	0.00	0.99	0.08	0.98	0.08

[1]Treatments: Control = no Fe; ferrous sulfate = 1.5 mg Fe/kg BW from ferrous sulfate [$FeSO_4$]; ferric sulfate = 1.5 mg Fe/kg BW from ferric sulfate [$Fe_2(SO_4)_3$].

[2]SD = standard deviation.

Table 2.4. Ingredient and nutrient composition of diets fed in Experiments 1 and 2

	Experiment	
	1	2
Ingredient	----------Percent of dietary DM----------	
Chopped alfalfa hay	4.86	4.86
Corn silage	32.24	32.96
Whole cottonseed	5.95	5.86
Grass hay	2.08	2.11
Ground corn	14.42	14.29
Alfalfa haylage	9.14	8.68
High moisture corn	6.29	6.35
Soybean meal, 48% CP	14.04	13.74
Soyhulls	7.77	7.97
Mineral-vitamin mix[1]	3.22	3.18
Nutrient Composition		
DM %	52.9	53.80
	----------Percent of dietary DM----------	
Neutral detergent fiber	31.4	32.0
Acid detergent fiber	21.3	18.3
Crude protein	17.51	17.51
Ca	0.88	1.01
P	0.36	0.33
K	1.23	1.17
Na	0.30	0.31
S	0.22	0.22
Mg	0.22	0.24
	----------mg/kg----------	
Co	0.12	0.12
Cu	8.5	8.9
Fe	185	240
Mo	1.9	1.9
Mn	37	36
Zn	30	38

[1]Mineral-vitamin mix contained 47.5% limestone, 22.5% sodium bicarbonate, 10.1% urea (45%N), 8.2% magnesium sulfate, 7.5% sodium chloride, 1.6% biotin premix (1.4 g/kg), 1.3% trace mineral premix, 30 KIU/kg vitamin A, 8 KIU/kg vitamin D, 56 KIU/kg vitamin E and 0.3% selenium yeast, dry basis.

Table 2.5. Water quality constituent analyses of drinking water in Experiments 1 and 2

	Experiment		
	1	2	Caution Level[1]
Quality Constituent	----------mg/L----------		
TDS[2]	392	454	1,000
Ca	94.7	104	150
Cl	9	27.5	500
Cu	ND[3]	ND	0.3
Fe (direct)[4]	0.42	0.46	0.3
Fe (total recoverable)[5]	1.59	2.36	-
Mg	33	36	80
Mn[4]	0.05	0.15	-
NO_3-N	ND	ND	25
Na	5.2	14.8	150
SO_4	65	101	300
Zn[4]	1.33	1.02	-
Conductivity, mmhos/cm	0.603	0.698	1.5
pH	7.9	8.1	6.5-9

[1]Caution level from Midwest Laboratories (Omaha, NE).

[2]TDS = total dissolved solids.

[3]ND = not detected.

[4]Direct metals analysis of raw (without acid digestion) water by Livestock water analysis using EPA 200.7 method (1994) by Midwest Laboratories (Omaha, NE).

[5]Total recoverable metals analysis of acidified water after acid digestion using EPA 200.7 method (1994) by Midwest Laboratories (Omaha, NE).

Table 2.6. Effects of abomasal dosing of different amounts of Fe^{2+} from ferrous lactate on blood and liver measurements in Experiment 1

	Treatments[1]				
	0Fe	0.75Fe	1.5Fe	SEM[2]	*P*-value
Serum					
Fe, µg/dL	178	192	183	15.7	0.71
TIBC[3], µg/dL	469	485†	478	13.6	0.08
UIBC[4], µg/dL	301	293	298	19.2	0.35
Fe saturation[5], %	38	40	39	3.7	0.92
α-Tocopherol, µg/mL	5.694[b]	5.894[ab]	6.085[a]	0.2170	0.01
Zn, µg/mL	0.993	0.987	0.947	0.0385	0.66
Cu, µg/mL	0.991	1.003	0.979	0.0305	0.49
Whole blood					
GSH-Px[6], IU/g Hb [7]	2.46	2.54	2.49	0.051	0.47
Liver, µg/g dry weight					
Fe	287.9	281.0	273.7	33.88	0.69
Cu	703.3[a]	639.2[b]	663.0[ab]	19.63	0.03
Zn	134.3	133.9	148.2	14.01	0.64

[a,b]Means within a row with different superscripts differ *(P* < 0.05).

[1]Treatments: 0Fe = 0 mg Fe/kg BW; 0.75Fe = 0.75 mg Fe/kg BW from ferrous lactate [$Fe(C_3H_5O_3)_2$]; 1.5Fe = 1.5 mg Fe/kg BW from ferrous lactate.

[2]SEM = standard error of the mean.

[3]TIBC = total Fe binding capacity = Fe + UIBC.

[4]UIBC = unsaturated Fe binding capacity.

[5]Fe Saturation = (Fe/TIBC) x 100.

[6]GSH-Px = glutathione peroxidase activity.

[7]Hb = hemoglobin.

†Tended to be lower *(P* <0.10) than 0Fe.

Table 2.7. Pearson correlations coefficients of relationships between independent serum response variables pooled across treatment and hour for Experiment 1

	UIBC[1]	α-Tocopherol	Cu	Zn	GSH-Px[2]
Fe	-0.46214*	0.38375	0.21908	0.28852	-0.19542
UIBC	--	-0.37463	0.20208	0.04449	0.29176
α-Tocopherol	--	--	0.22133	-0.26678	0.01971
Cu	--	--	--	-0.08332	-0.01447
Zn	--	--	--	--	-0.1946

[1]UIBC = unsaturated Fe binding capacity.

[2]GSH-Px = glutathione peroxidase activity.

*$P < 0.05$

Table 2.8. Pearson correlations coefficients of relationships between independent liver response variables pooled across treatment and hour for Experiment 1

	Cu	Zn
Fe	0.55385	0.49768
Cu	--	0.53844
Zn	--	--

Table 2.9. Effects of abomasal dosing of ferrous sulfate or ferric sulfate on blood and liver measurements in Experiment 2

	Treatments[1]				
	Control	ferrous sulfate	ferric sulfate	SEM[2]	*P*-value
Serum					
Fe, µg/dL	169	161	149[†]	11.7	0.11
TIBC[3], µg/dL	425	420	426	4.2	0.36
UIBC[4], µg/dL	254	263	274	9.5	0.22
Fe saturation[5], %	40	38	35	2.6	0.14
α-Tocopherol, µg/mL	5.189	5.412	4.829	0.2258	0.13
Zn, µg/mL	0.852	0.807	0.772	0.0468	0.20
Cu, µg/mL	1.006[a]	0.950[b]	1.036[a]	0.0352	0.0002
Whole blood					
GSH-Px[6], IU/mg Hb[7]	2.24	2.19	2.24	0.065	0.46
Liver, µg/g dry weight					
Fe	348.6	338.6	380.1	27.75	0.21
Cu	647.5	629.3	661.1	25.54	0.31
Zn	152.3	154.1	164.1	7.818	0.28

[a,b]Means within a row with different superscripts differ *(P* < 0.05).

[1]Treatments: Control = no Fe; ferrous sulfate = 1.5 mg Fe/kg BW from ferrous sulfate [$FeSO_4$]; ferric sulfate = 1.5 mg Fe/kg BW from ferric sulfate [$Fe_2(SO_4)_3$].

[2]SEM = standard error of the mean.

[3]TIBC = total Fe binding capacity = Fe + UIBC.

[4]UIBC = unsaturated Fe binding capacity.

[5]Fe Saturation = (Fe/TIBC) x 100.

[6]GSH-Px = glutathione peroxidase activity.

[7]Hb = hemoglobin.

[†]Tended to be lower *(P* < 0.10) than ferrous sulfate.

Table 2.10. Pearson correlations coefficients of relationships between independent serum response variables pooled across treatment and hour in Experiment 2

	UIBC[1]	α-Tocopherol	Cu	Zn	GSH-Px[2]
Fe	-0.54061*	0.1578	0.02096	0.30743	-0.04863
UIBC	--	-0.38257	0.10746	-0.34677	0.01364
α-Tocopherol	--	--	-0.10519	-0.03308	0.49837*
Cu	--	--	--	-0.13694	-0.29371
Zn	--	--	--	--	-0.27009

[1]UIBC = unsaturated Fe binding capacity.

[2]GSH-Px = glutathione peroxidase activity.

* $P < 0.05$.

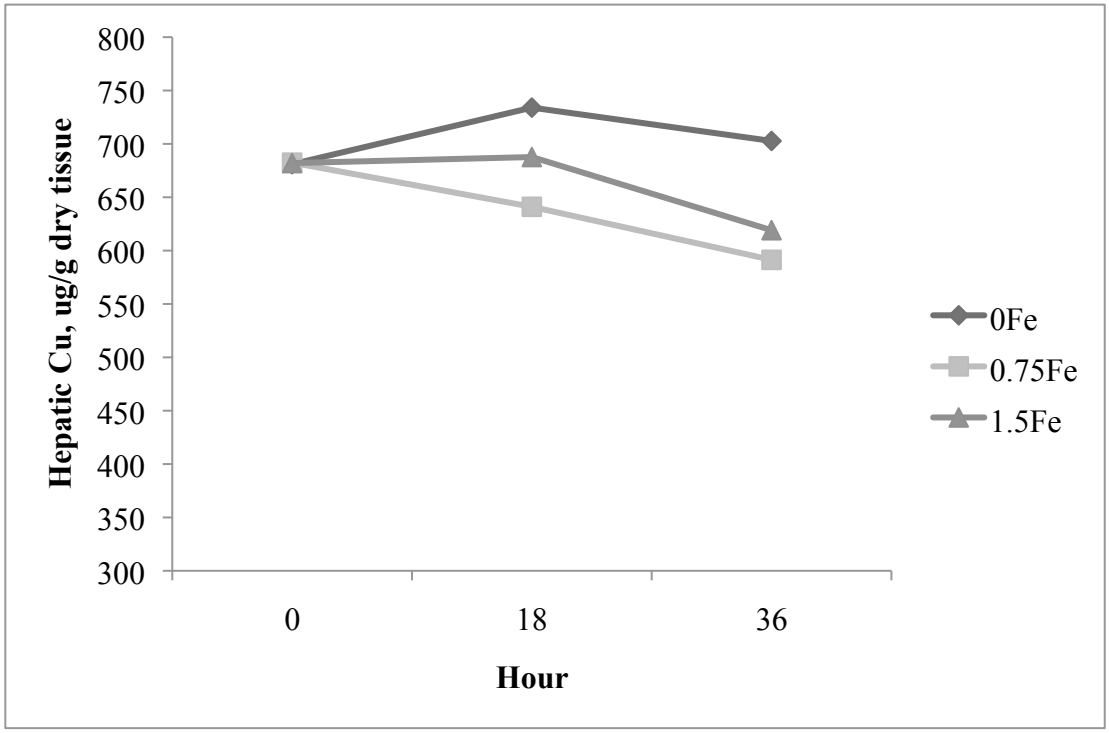
Table 2.11. Pearson correlations coefficients of relationships between independent liver response variables pooled across treatment and hour in Experiment 2

	Cu	Zn
Fe	0.04967	0.02190
Cu	--	0.80838*
Zn	--	--

* $P < 0.05$.

FIGURES

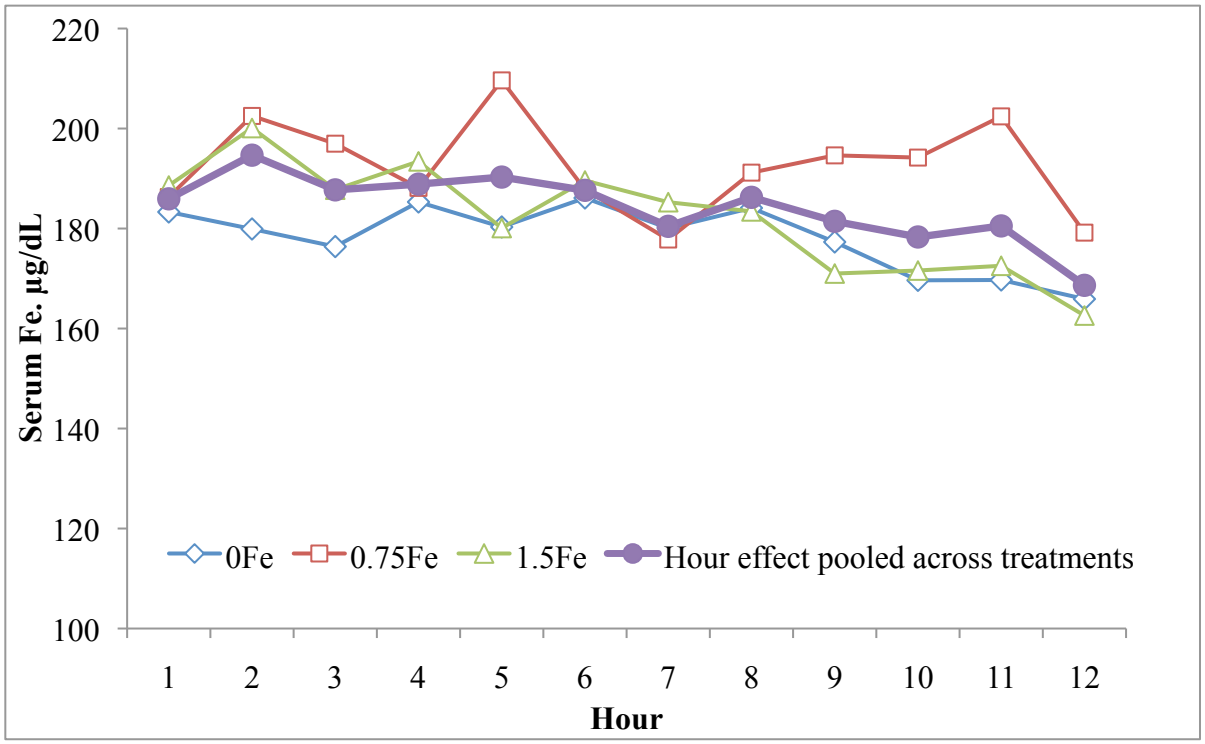
Figure 2.1. Treatment[1] by hour interaction response (SEM[2] = 26.55, $P = 0.10$) of hepatic Cu concentrations in Experiment 1



[1]Treatments: 0Fe = 0 mg Fe/kg BW; 0.75Fe = 0.75 mg Fe/kg BW from ferrous lactate [$Fe(C_3H_5O_3)_2$]; 1.5Fe = 1.5 mg Fe/kg BW from ferrous lactate.

[2]SEM = standard error of the mean.

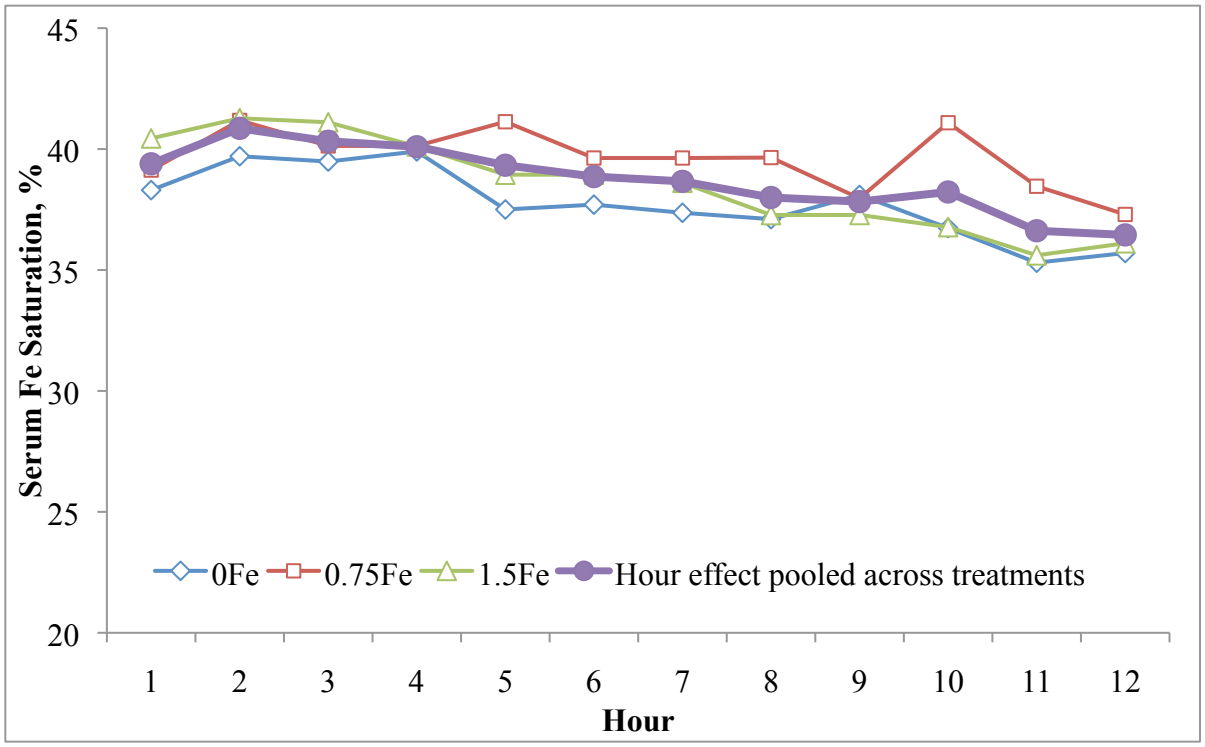
Figure 2.2. Treatment[1] by hour ($P = 0.63$) and pooled hour (SEM[2] = 17.55; $P = 0.04$) responses of serum Fe concentration in Experiment 1



[1]Treatments: 0Fe = 0 mg Fe/kg BW; 0.75Fe = 0.75 mg Fe/kg BW from ferrous lactate [$Fe(C_3H_5O_3)_2$]; 1.5Fe = 1.5 mg Fe/kg BW from ferrous lactate.

[2]SEM = standard error of the mean.

Figure 2.3. Treatment[1] by hour ($P = 0.82$) and pooled hour (SEM[2] = 3.94; $P = 0.008$) responses of serum Fe saturation[3] in Experiment 1

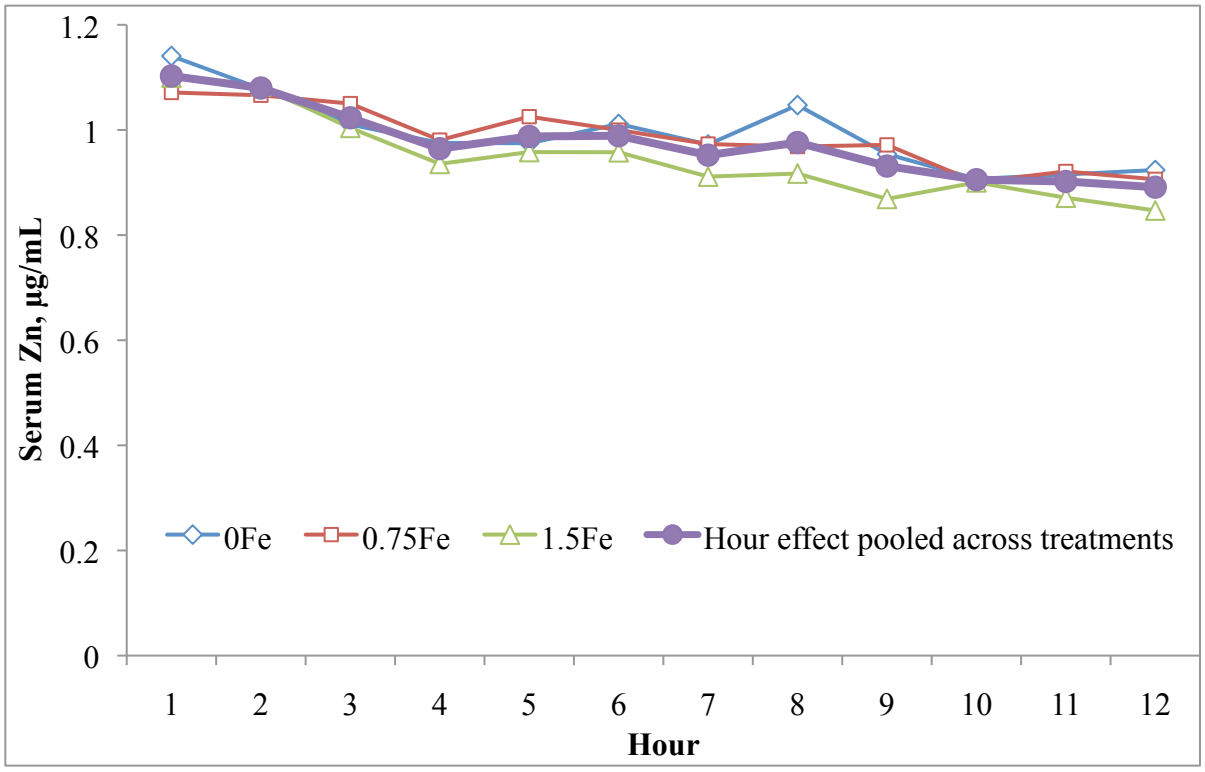


[1]Treatments: 0Fe = 0 mg Fe/kg BW; 0.75Fe = 0.75 mg Fe/kg BW from ferrous lactate [$Fe(C_3H_5O_3)_2$]; 1.5Fe = 1.5 mg Fe/kg BW from ferrous lactate.

[2]SEM = standard error of the mean.

[3]Fe Saturation = Fe/total Fe binding capacity.

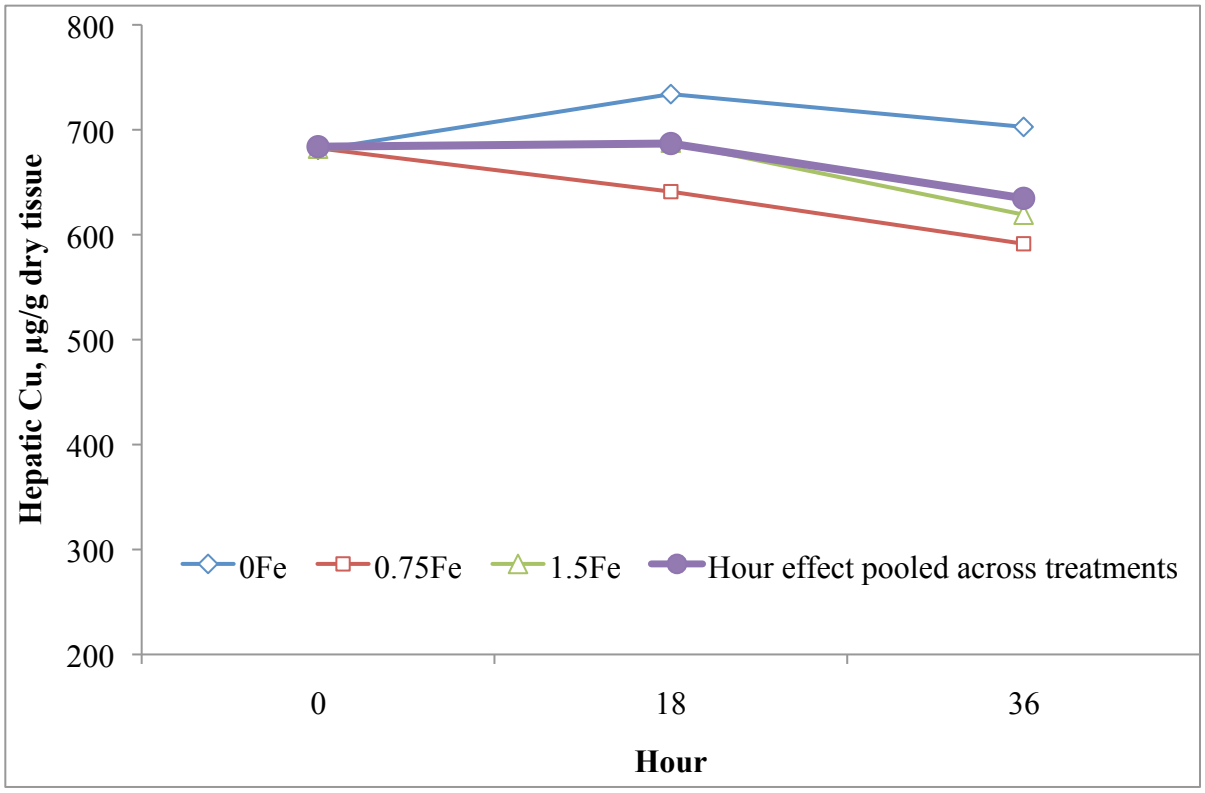
Figure 2.4. Treatment[1] by hour ($P = 0.87$) and pooled hour (SEM[2] = 0.06; $P < 0.0001$) responses of serum Zn concentration in Experiment 1



[1]Treatments: 0Fe = 0 mg Fe/kg BW; 0.75Fe = 0.75 mg Fe/kg BW from ferrous lactate [$Fe(C_3H_5O_3)_2$]; 1.5Fe = 1.5 mg Fe/kg BW from ferrous lactate.

[2]SEM = standard error of the mean.

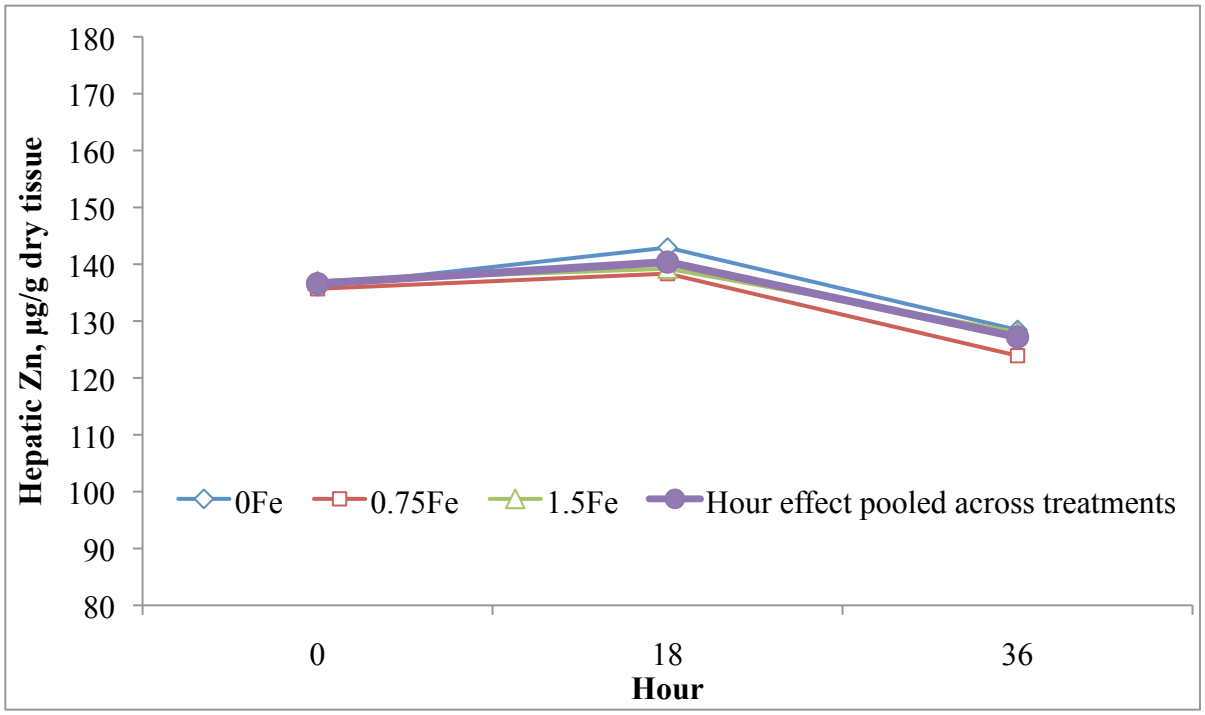
Figure 2.5. Treatment[1] by hour ($P = 0.62$) and pooled hour (SEM[2] = 26.55; $P = 0.02$) responses of hepatic Cu concentration in Experiment 1



[1]Treatments: 0Fe = 0 mg Fe/kg BW; 0.75Fe = 0.75 mg Fe/kg BW from ferrous lactate [$Fe(C_3H_5O_3)_2$]; 1.5Fe = 1.5 mg Fe/kg BW from ferrous lactate.

[2]SEM = standard error of the mean.

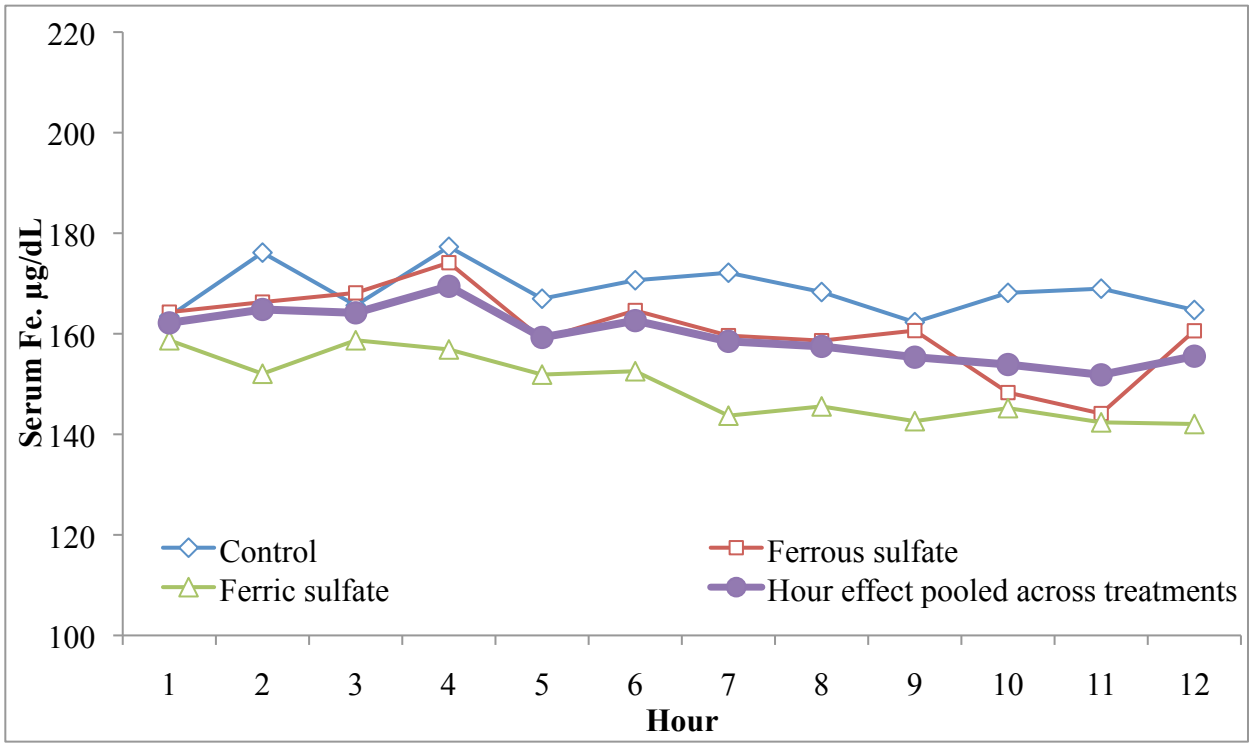
Figure 2.6. Treatment[1] by hour ($P = 0.99$) and pooled hour (SEM[2] = 7.14; $P = 0.08$) responses of hepatic Zn concentration in Experiment 1



[1]Treatments: 0Fe = 0 mg Fe/kg BW; 0.75Fe = 0.75 mg Fe/kg BW from ferrous lactate [$Fe(C_3H_5O_3)_2$]; 1.5Fe = 1.5 mg Fe/kg BW from ferrous lactate.

[2]SEM = standard error of the mean.

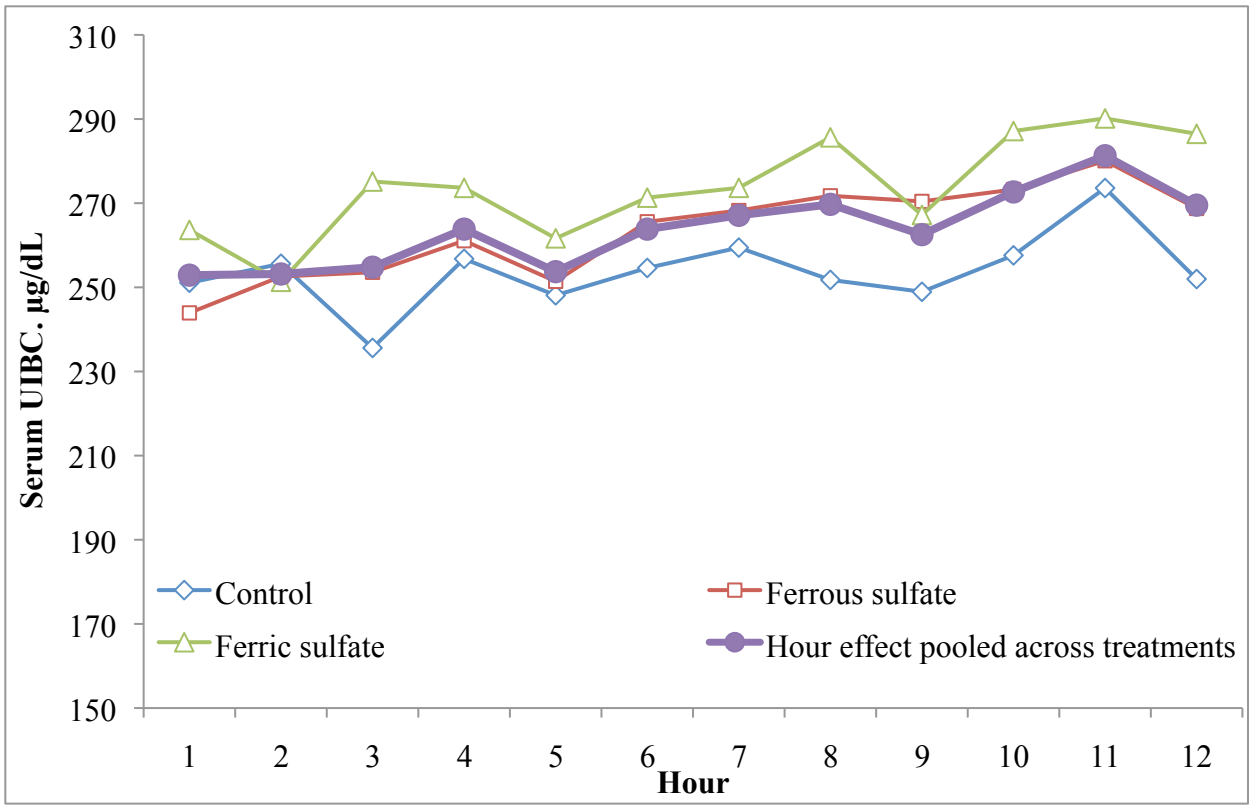
Figure 2.7. Treatment[1] by hour ($P = 0.85$) and pooled hour (SEM[2] = 13.03; $P = 0.01$) responses of serum Fe concentration in Experiment 2



[1]Treatments: Control = 0 mg Fe/kg BW; ferrous sulfate = 1.5 mg Fe/kg BW from ferrous sulfate [$FeSO_4$]; ferric sulfate = 1.5 mg Fe/kg BW from ferric sulfate [$Fe_2(SO_4)_3$].

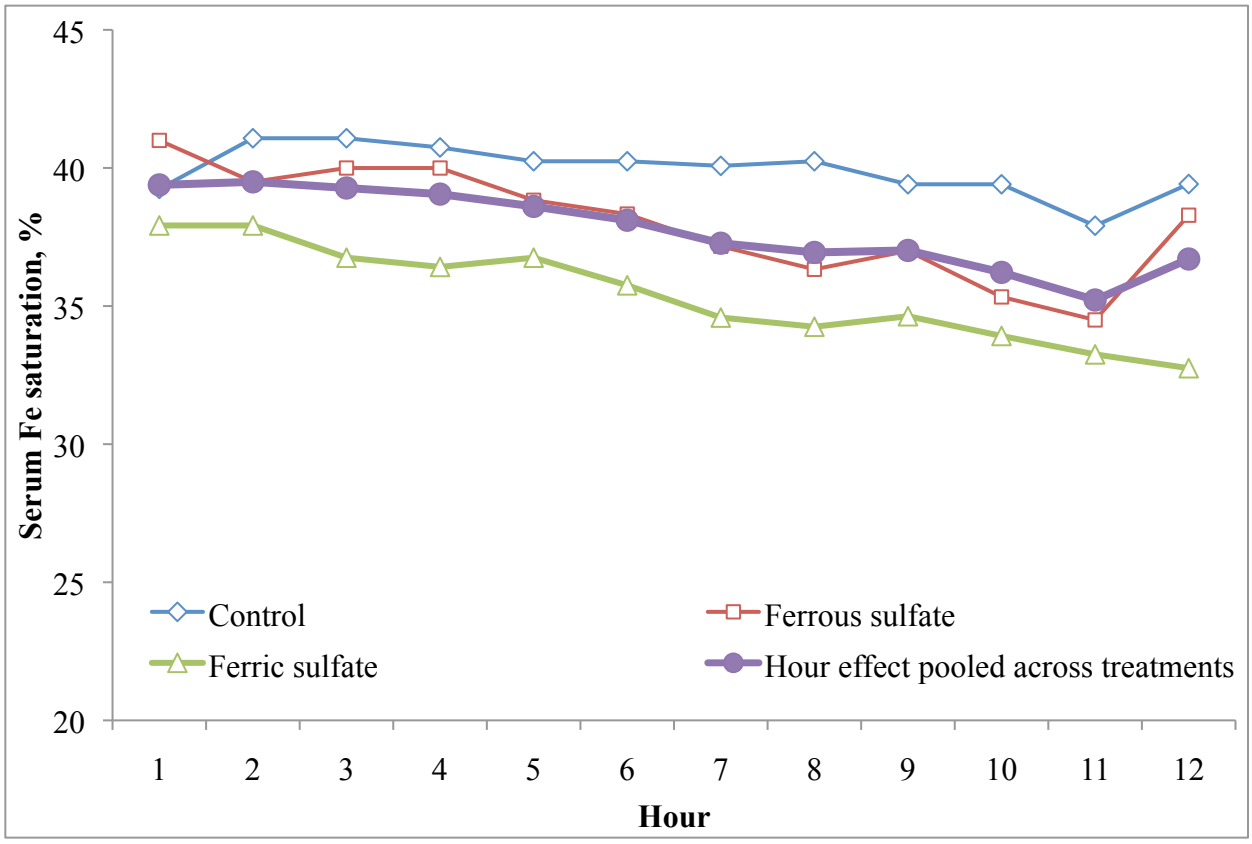
[2]SEM = standard error of the mean.

Figure 2.8. Treatment[1] by hour ($P = 0.93$) and pooled hour (SEM[2] = 12.94; $P = 0.001$) responses of serum unsaturated Fe-binding capacity in Experiment 2



[1]Treatments: Control = 0 mg Fe/kg BW; ferrous sulfate = 1.5 mg Fe/kg BW from ferrous sulfate [$FeSO_4$]; ferric sulfate = 1.5 mg Fe/kg BW from ferric sulfate [$Fe_2(SO_4)_3$].
[2]SEM = standard error of the mean.

Figure 2.9. Treatment[1] by hour ($P = 0.82$) and pooled hour (SEM[2] = 2.78; $P < 0.0001$) responses of serum Fe saturation[3] in Experiment 2

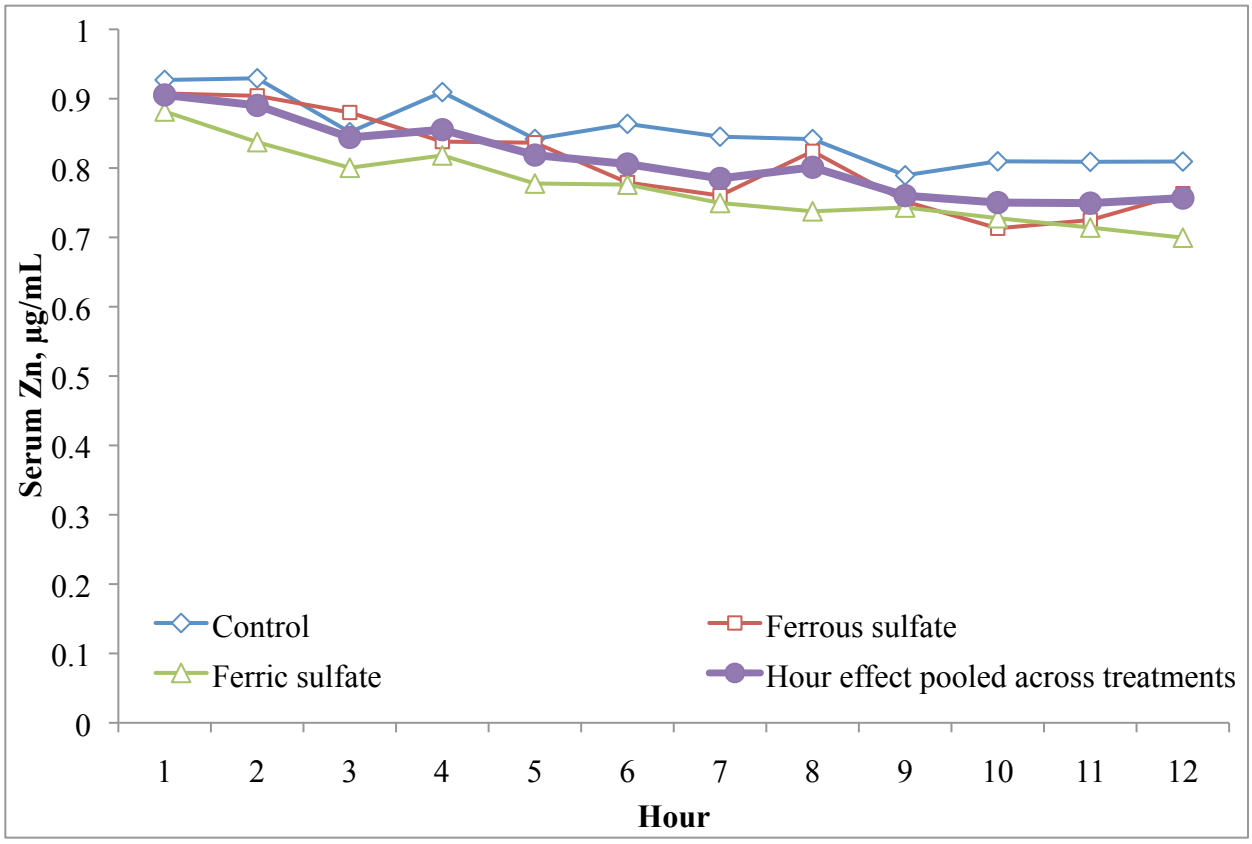


[1]Treatments: Control = 0 mg Fe/kg BW; ferrous sulfate = 1.5 mg Fe/kg BW from ferrous sulfate [$FeSO_4$]; ferric sulfate = 1.5 mg Fe/kg BW from ferric sulfate [$Fe_2(SO_4)_3$].

[2]SEM = standard error of the mean.

[3]Fe Saturation = Fe/total Fe binding capacity.

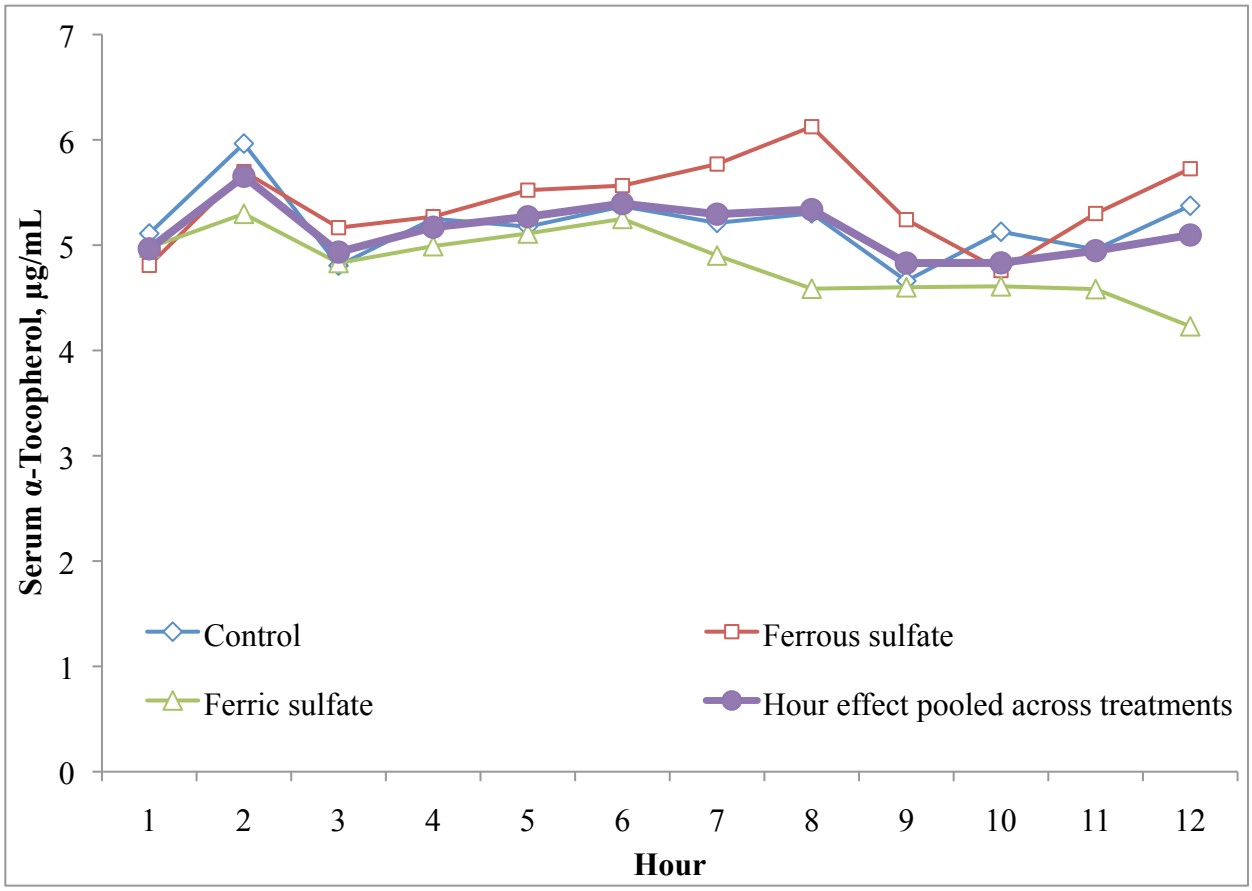
Figure 2.10. Treatment[1] by hour ($P = 0.87$) and pooled hour (SEM[2] = 0.05; $P < 0.0001$) responses of serum Zn concentration in Experiment 2



[1]Treatments: Control = 0 mg Fe/kg BW; ferrous sulfate = 1.5 mg Fe/kg BW from ferrous sulfate [$FeSO_4$]; ferric sulfate = 1.5 mg Fe/kg BW from ferric sulfate [$Fe_2(SO_4)_3$].

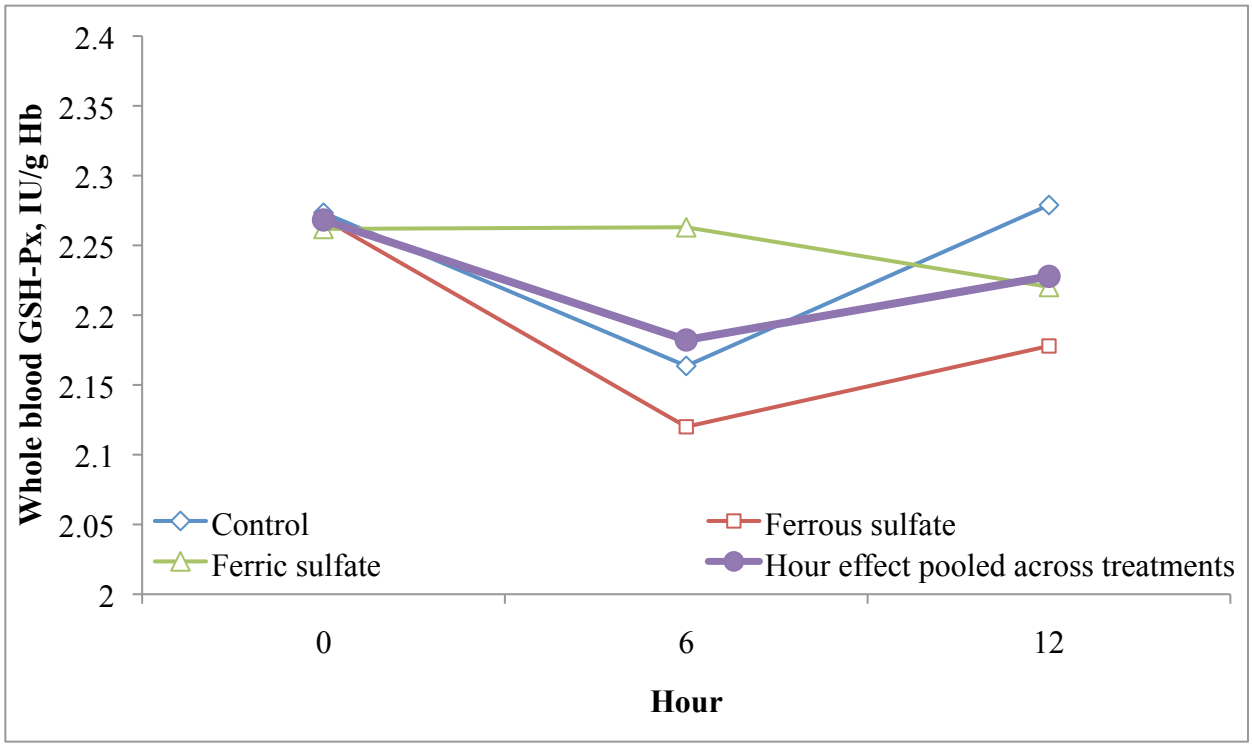
[2]SEM = standard error of the mean.

Figure 2.11. Treatment[1] by hour ($P = 0.19$) and pooled hour (SEM[2] = 0.33; $P = 0.001$) responses of serum α-Tocopherol concentration in Experiment 2



[1]Treatments: Control = 0 mg Fe/kg BW; ferrous sulfate = 1.5 mg Fe/kg BW from ferrous sulfate [$FeSO_4$]; ferric sulfate = 1.5 mg Fe/kg BW from ferric sulfate [$Fe_2(SO_4)_3$].
[2]SEM = standard error of the mean.

Figure 2.12. Treatment[1] by hour ($P = 0.33$) and pooled hour (SEM[2] = 0.07; $P = 0.08$) responses of whole blood glutathione peroxidase activity in Experiment 2



[1]Treatments: Control = 0 mg Fe/kg BW; ferrous sulfate = 1.5 mg Fe/kg BW from ferrous sulfate [$FeSO_4$]; ferric sulfate = 1.5 mg Fe/kg BW from ferric sulfate [$Fe_2(SO_4)_3$].
[2]SEM = standard error of the mean.

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CHAPTER 3

EFFECTS OF FERROUS (Fe^{2+}) OR FERRIC (Fe^{3+}) IRON CONTENT OF DRINKING WATER ON PREFERENCE AND BEHAVIOR OF LACTATING DAIRY COWS

ABSTRACT

Drinking water can contain high concentrations of Fe, mainly in the ferrous (Fe^{2+}) valence. Current recommended upper tolerable concentrations of Fe in drinking water for cattle (0.3 mg/L) come from human recommendations for palatability, but cattle may be able to tolerate higher concentrations. Our objective was to determine the effects that varying concentrations of ferrous (Fe^{2+}) or ferric (Fe^{3+}) iron, and Fe-salt source might have on lactating dairy cows' preference for drinking water offered as choices *ad libitum*. In four separate experiments, cows were offered pairs of water treatments for 22-h periods and water intake and drinking behavior were recorded. Experiment 1 treatments were: 0, 4, or 8 mg Fe/L from ferrous lactate [$Fe(C_3H_5O_3)_2$] . Cows did not show a preference between water with 0 or 4 mg Fe/L, and water intake was less with 8 compared with 0 or 4 mg Fe/L. Cows spent less time drinking water containing 8 mg Fe/L. Total time spent drinking was positively correlated with water intake when pooled across treatments. Experiment 2 treatments were: 0 or 8 mg Fe/L from either ferrous ($FeSO_4$) or ferric sulfate [$Fe_2(SO_4)_3$]. Water intake was not different among treatments. Experiment 3 treatments were: 0 or 8 mg Fe/L from either ferrous ($FeCl_2$) or ferric chloride ($FeCl_3$). Again, cows did not show any preference among the treatments. Experiment 4 treatments were: 0 or 8 mg Fe/L from ferrous lactate [$Fe(C_3H_5O_3)_2$] , sulfate ($FeSO_4$), or chloride ($FeCl_2$). Cows preferred water without added Fe, but did not exhibit any preference for

water containing any particular source of Fe with different anionic moieties. Cows spent less time drinking and drank less frequently when offered water containing 8 mg Fe/L from ferrous chloride compared with ferrous lactate or sulfate. Water intake was positively correlated with both drinking duration and frequency when pooled across treatments in Experiment 4. This research suggests that lactating dairy cows tolerate concentrations of Fe up to 4 mg/L without reducing water intake. Additionally, preference is not dependent on Fe valence or Fe source.

INTRODUCTION

Preference is the degree to which one alternative is preferred to another. A dairy cow's preference for drinking water is an indicator of palatability that can be measured by differences in water intake. Anecdotal information suggests that drinking water with Fe concentrations of 2 mg/L or greater may negatively impact milk production and cow health (Beede, Michigan State University, East Lansing, MI, personal communication). These might be effects mediated one of two ways: through changes in Fe and antioxidant statuses and oxidative stress, or through drinking water preference. In this research we focused on drinking water preference.

The upper tolerable limit for Fe concentration in water recommended for dairy cows [0.3 mg/L (direct water analysis)] is based on human palatability. Only one study investigated the effects of drinking water contaminated with Fe on ruminants. Horvath (1985) offered sheep simulated acid mine water and measured water intake. Acid mine drainage is water contaminated with byproducts of mining activity that often has a high Fe concentration (approximately 25-500 mg/L). Sheep were offered the same treatments 2 d in a row for 15 min twice daily. Treatments concentrations of 75 mg Fe/L from ferric sulfate, or 145 mg Fe from ferric chloride had no impact on water intake. However, it was not clear if the animals were offered water for the other 23.5 h of the day. Therefore water consumption may not have been reflective of preference in an average setting. Additionally, Fe concentrations of treatments ranged from 75 to 145 mg Fe/L, which are much greater than would be found typically in drinking water for dairy cows.

Palatability, as reflected by reduced DMI and subsequently ADG from supplemental Fe in rations was characterized in calves and sheep. Calves fed rations supplemented with 750 mg Fe/kg DM from ferrous sulfate ($FeSO_4$) had reduced DMI and ADG (Hansen et al., 2010). Dietary Fe concentrations of 1,600 mg Fe/kg from ferrous sulfate or ferric citrate ($FeC_6H_5O_7$)

decreased DMI of sheep when compared with non-supplemented diets, although ferrous sulfate had a greater negative impact on DMI than ferric citrate (Standish and Ammerman, 1971). However, dairy cow intake of pasture irrigated with water containing a high concentration of ferric hydroxide [$Fe(OH)_3$] (17 mg Fe/L) was not different than that of cows on non-irrigated pasture (Coup and Campbell, 1964).

The World Health Organization states that Fe concentrations greater than 0.3 mg/L will affect taste preference in humans (WHO, 2006), but concentrations of 1 to 3 mg Fe/L from anaerobic well water can be acceptable (WHO, 2003). Anaerobic water will contain mostly ferrous iron (**Fe^{2+}**) (Colter and Mahler, 2006), and this suggests that Fe^{2+} may be more palatable than ferric iron (**Fe^{3+}**).

The objective of our research was to determine if water intake responses of lactating dairy cows differ when offered water with ascending concentrations of Fe^{2+}, different Fe valences (Fe^{2+} or Fe^{3+}) and from different Fe sources (salts). We hypothesize that as the concentration of Fe^{2+} in water increases, water intake will decrease; lactating cows will prefer to drink water with Fe^{2+} when presented with both high Fe^{2+} and high Fe^{3+} drinking water, but will prefer water with no supplemental Fe (low basal Fe concentration in control water) to any other source.

MATERIALS AND METHODS

The Institutional Animal Care and Use Committee at Michigan State University approved all experimental procedures (AUF # 03/10-024-00).

Animals, Experimental Design, and Treatments

Experiment 1. Six mid-lactation Holstein cows (181 ± 44 DIM) situated in tie-stalls were assigned randomly in a replicated 3 x 3 Latin Square involving 3 periods, 3 treatments, in 3 possible treatment pair combinations. Experimental treatments were: 0 (0Fe), 4 (4Fe), or 8mg Fe/L (8Fe) from ferrous lactate [$Fe(C_3H_5O_3)_2$] added to on-site tap water (Table 3.2). Treatments were formulated to contain 0, 4 or 8 mg Fe/L of total recoverable Fe (prepared by acid digestion) or 0, 1 and 2 mg/L of Fe by direct metals analysis (raw water), both analyzed by EPA method 200.7 (1994). Treatments were offered as pairs. Each cow was offered one of these three possible pairs (0Fe and 4Fe; 0Fe and 8Fe; 4Fe and 8Fe) in each of three experimental periods; pairs were balanced for left-right effect.

Experiment 2. Six mid-lactation Holstein cows (187 ± 44 DIM) situated in tie-stalls were assigned randomly in a replicated 3 x 3 Latin Square design involving 3 experimental periods, 3 experimental treatments, in 3 possible treatment pair combinations. Experimental treatments were: 0 (Control), and 8 mg Fe/L from either ferrous (ferrous sulfate; $FeSO_4$) or ferric sulfate (ferric sulfate; [$Fe_2(SO_4)_3$]) added to on-site tap water (Table 3.3). Treatments were formulated to contain 0 and 8 mg Fe/L of total recoverable Fe (prepared by acid digestion) or 0 and 2 mg/L of Fe by direct metals analysis (raw water), both analyzed by EPA method 200.7 (1994). Treatments were offered as pairs. Each cow was offered one of these three possible pairs (control

and ferrous sulfate; control and ferric sulfate; ferrous sulfate and ferric sulfate) in each of three experimental periods; pairs were balanced for left-right effect

Experiment 3. Six mid-lactation Holstein cows (180 ± 16 DIM) situated in tie-stalls were assigned randomly in a replicated 3 x 3 Latin Square design involving 3 experimental periods, 3 experimental treatments, in 3 possible treatment pair combinations. Experimental treatments were: 0 (Control), and 8 mg Fe/L from either ferrous (ferrous chloride; $FeCl_2$) or ferric chloride (ferric chloride; $FeCl_3$) added to on-site tap water (Table 3.4). Treatments were formulated to contain 0 and 8 mg Fe/L of total recoverable Fe (prepared by acid digestion) or 0 and 2 mg/L of Fe by direct metals analysis (raw water), both analyzed by EPA method 200.7 (1994). However, a mistake was made in treatment formulation, and the ferrous chloride treatment actually contained approximately 12.5 mg/L (25% more Fe). Treatments were offered as pairs. Each cow was offered one of these three possible pairs (control and ferrous chloride; control and ferric chloride; ferrous chloride and ferric chloride) in each of three experimental periods; pairs were balanced for left-right effect.

Experiment 4. Twelve mid-lactation Holstein cows (135 ± 18 DIM) situated in tie-stalls were assigned randomly in a 6 x 6 Latin Square involving 6 experimental periods, 4 experimental treatments, in 6 possible treatment pair combinations. Experimental treatments were: 0 (control), and 8 mg Fe/L from either ferrous lactate (ferrous lactate), sulfate (ferrous sulfate) or chloride (ferrous chloride) added to on-site tap water (Table 3.5). Treatments were formulated to contain 0 and 8 mg Fe/L of total recoverable Fe (prepared by acid digestion) or 0 and 2 mg/L of Fe by direct metals analysis (raw water), both analyzed by EPA method 200.7 (1994). However, a mistake was made in treatment formulation, and the ferrous chloride treatment actually contained approximately 12.5 mg/L (25% more Fe). Each cow was offered

one of these six possible pairs (control and ferrous lactate; control and ferrous sulfate; control and ferrous chloride; ferrous lactate and ferrous sulfate; ferrous lactate and ferrous chloride; ferrous sulfate and ferrous chloride) in each of six experimental periods; pairs were balanced for left-right effect.

Water Intake Measurement and Behavior Assessment

The day that treatment water intake measurement began, water containers were removed approximately 2 h before the experimental period commenced. Containers were scrubbed with detergent, rinsed, and filled with treatments according to treatment assignments. Water containers were then placed on the left and right edges of the bunks. Cows were removed from the tie-stall and taken to the milking parlor approximately 1 h after water containers were removed. Therefore, cows were without drinking water for 2 h before water treatments were offered. During their absence, the remaining 50% of the ration, withheld during morning feeding, was added to the remaining feed in the bunks. Cows returned to their tie-stalls simultaneously, immediately after milking, to individual access to feed and two water containers. At this time (1600 h) the period of water intake measurement and manual recording of cow drinking behavior began.

Every 2 h containers were removed from all feed bunks at the same time, and behavior recording was paused. Each container was skimmed with a pool skimmer to remove as much feed as possible that was dropped into the containers by cows during the experimental period. Containers were weighed and the weight was recorded. If the weight was less than approximately 45 kg, the container was refilled in increments of 5 or 10 L, until the weight reached greater than that number. Containers were then replaced in each feed bunk at the same

time and the behavior recording recommenced. Behavior recording ended 12 h after the experimental period began. Containers were weighed, refilled and replaced in the bunks.

Initially, some cows pushed containers from the bunk in order to access feed. If containers were pushed more than 20 cm from the bunk, they were pushed back into the bunk within cow's reach. If the cow routinely pushed the containers away, the containers were banded to the bunks with rubber straps. Straps did not interfere with drinking. If the containers contained less than approximately 10 L of water before the scheduled refilling time, containers were removed from all bunks, and the containers were refilled so that containers none were ever empty and cows were always able to drink from either container.

Containers were left in the bunks for cows to access for 22 h. They were checked at 0900 h, weighed and refilled. Containers were removed and weighed at the end of the experimental period (1400 h).

Behavior Assessment. Drinking behaviors were recorded for the initial 12 h of the experimental period and categorized for analyses. Categories were lapping, drinking, and 'other'. Drinking was defined as a cow fully or partially submerging her muzzle in water to consume water. Lapping was defined as a cow consuming water without submerging her muzzle. The 'other' category included all behaviors associated with containers when cows were not actively consuming water. Non-consumption behavior interaction examples included splashing, sniffing and pushing containers away. Frequency of interaction with water containers also was evaluated. Behavior measurements were recorded to the nearest second. The beginning of one behavior signified the end of the previous behavior, so only beginning time of behavior was recorded. If a behavior was associated with a water container, the designated treatment identifier was recorded.

Other common behaviors recorded included eating, ruminating, urinating, defecating, standing up, lying down, resting, and standing.

Preparation of Treatments

Treatments were prepared in concentrated form (100-times final treatment concentration) in 1 L polypropylene bottles so 1 L of concentrated solution was diluted to 100 L of final treatment solution. During preparation of final water treatments, concentrated solutions were diluted with on-site tap water (water composition can be found in Table 3.7) until the proper concentration, and a volume (50 L) was reached. Additional treatment information is presented in Tables 3.2, through and 3.5.

Water containers

Water was offered in two, 75 L capacity white Rubbermaid Brute™ (Rubbermaid Commercial Products, Winchester, VA) storage tote containers (W: 38.4 cm, x H: 44.1 cm x L: 70.8 cm) each placed in the left or right side of individual cow's feed bunks with feed situated between the containers. There was a vacant tie-stall between cows to avoid cow social interactions that potentially might affect water intake.

Training

Cows were blocked from access to water in the milking parlor lane, so the only source of water was the source provided in the tie-stall throughout the pre-experimental and experimental periods. Nine d prior to the beginning of each experiment, cows were offered water from the on-site tap water source in the experimental water containers for 48 h to ensure that all cows became accustomed to the containers. Containers were removed from the bunks, weighed and refilled

every 6 h during training. Every 24 h containers were removed, emptied, scrubbed with detergent, rinsed well and refilled. There were no issues with cows refusing to drink from containers. Following the initial training period cows were placed in tie-stalls with individual automatic water cups, as in normal settings, and water intake was measured with in-line water flow meters for 5 d to establish baseline water intake. Forty-eight h immediately prior to actual data collection cows were moved to stalls without automatic water cups, and water was accessible only in the experimental water containers to re-accustom the cows to the containers and establish baseline intake from water containers. All cows were exposed to water containers for the same length of time during pre-experimental acclimation periods and always located with an unoccupied stall on each side.

Common Feeding, Water, and Milk Data Collection and Analyses

A basal diet formulated to meet or exceed NRC (2001) recommendations for all nutrients (including Fe) was fed *ad libitum* throughout each experiment (Table 3.6). Cows were fed once daily in their individual tie-stalls, and milked twice daily. Milk yield and water and feed intake were recorded daily for the entire experimental period, including adjustment periods. Pre-experimental feed intake, water intake, and milk yield and composition are presented in Table 3.1 for characterization of experimental animals.

Feed intakes were measured daily throughout the experiment. Individual feed ingredients were sampled the week before each collection period began, and again between experimental periods. Each individual feed sample was weighed and dried in a forced-air oven at 55°C for 72 h until completely dry to determine DM percentage. Dried samples were ground through a 2 mm screen in a Wiley mill (Arthur H. Thomas, Philadelphia, PA). Similar feed

ingredient samples were pooled by experiment and sent for analysis by Dairy One Laboratories (Ithaca, NY). Milk samples were taken once per experiment from one AM and PM milking each and sent for composition analysis at Michigan DHIA Universal Laboratory Services (Lansing, MI). Water was sampled once per period from each treatment. Similar samples were pooled by experiment and one sample per treatment in each experiment was analyzed by each of two separate methods for Fe. Raw water samples were sent to Midwest Laboratories (Omaha, NE) for direct metals water analysis (Livestock water analysis, EPA 200.7 method (1994)). A second sample was acidified (pH < 2.0) with 1 mL nitric acid, and sent to the same laboratory for acid digestion and metals analyses (total recoverable metals analysis using EPA 200.7 method (1994)). Water analyses are presented in Tables 3.2 through 3.5. Internal water reference samples containing either 0, 1, 4 and 8 mg of Fe/L from ferrous lactate were placed as unknowns within the set of samples in triplicate for analysis. The intra-assay %CV was 4.0%.

Statistical Analyses

Data were analyzed by method of least-squares ANOVA using the Mixed Models procedure of SAS (Version 9.2, SAS Institute Inc.). Data were analyzed as a Latin Square design with a split plot in which treatments were sub-plots within cow, period, and treatment pair whole plots. Differences in cumulative and non-cumulative (2 h time periods) water intakes were compared between treatments. Data of hourly measurements were analyzed using the REPEATED statement, and the covariance structure that resulted in the lowest Bayesian information criterion (BIC) was used. Fixed effects in the model included treatment, hour, and treatment by hour interaction. Random effects included cow and period. If the interaction term was non-significant ($P > 0.15$), intake was analyzed separately by hour. Residual distributions

were tested for normality using the Shapiro-Wilk test, and outliers were identified using Cook's D statistic. During evaluation of the full model, including interaction terms, intake data were transformed by taking the square root to accommodate the assumptions of normality. During analyses of individual hour water intake responses and total behavior responses, if required, data also were transformed to either square root or natural log if required to accommodate the assumptions of normality. Results were back transformed for presentation. Differences between treatments were identified using the PDIFF option with Tukey's adjustment. Least-squares means are presented. Significance for main effects was declared at $P < 0.05$, and a trend toward significance at $P < 0.10$. Significance for interaction effects was declared at $P < 0.10$, and a trend toward significance at $P < 0.15$. Pearson correlation coefficients were calculated for the relationships between water intake and behavior responses using the Correlation procedure.

RESULTS AND DISCUSSION

Treatment Effects on Water Intake

Experiment 1. Our objective was to examine the effects of drinking water Fe concentration on preference. Cows were offered pairs of treatments as 0, 4 or 8 mg Fe/L supplied as ferrous lactate in drinking water. We were concerned initially that cows would be induced to drink water containing additional Fe because it was novel to them, however, this was not the case. There was no interaction in either cumulative ($P = 0.96$) or non-cumulative water intake ($P = 0.98$) over time. There was an overall effect of treatment ($P < 0.05$) at the end of the total experimental period (22 h) (Table 3.8). At the end of the experimental period (22 h) intake of 4Fe tended to be greater than intake of 8Fe ($P = 0.07$), but not different from 0Fe. Horvath (1985) found that sheep offered 145 mg Fe/L from ferric sulfate $Fe_2(SO_4)_3$ had reduced water intakes, but when supplemental Fe concentration was reduced to 75 mg Fe/L from the same source, intake was not different from Control. We expected that 0Fe would be more acceptable to cattle than 4Fe water, but 4 mg Fe/L did not impact drinking water preference. There was no difference in total intakes based on which pair of treatments was offered. These results suggest that the 0.3 mg Fe/L tolerable concentration for lactating dairy cows may be too severe. However, further evaluation is warranted to evaluate the potential contribution of valence and source of Fe in drinking water.

Experiments 2 and 3. In Experiment 2, our objective was to determine the effect of Fe^{2+} or Fe^{3+} on drinking water preference. Cows were offered pairs of water treatments with 0 or 8 mg Fe/L as either ferrous or ferric sulfate. There was no interaction between treatment and time for cumulative ($P = 0.97$) or non-cumulative ($P = 0.69$) water intake over the 22 h period. Overall, there was no difference between total intakes for any treatments offered ($P = 0.28$;

Table 3.9). However, the total intake of cows on the Control treatment was at least 10 L greater than other treatments. Throughout the 22 h period water intakes for Control and ferric sulfate were numerically similar, until the final measurement. There was no interaction between treatment and hour. There also was no difference in total water intake due to pair of treatments offered.

In Experiment 3, we evaluated the difference between the effects of Fe^{2+} or Fe^{3+} on drinking water preference using a different Fe source. Cows were offered pairs of water treatments as 0 or 8 mg Fe/L as either ferrous or ferric chloride. However, the ferrous chloride treatment actually contained 12.5 mg Fe/L. There was no effect of treatment on total water intake (Table 3.10). No interaction occurred between treatment and hour for cumulative ($P = 1.0$) or non-cumulative ($P = 0.54$) water intakes. There was no impact of pair of treatments offered on water intake. Although not statistically different, water intake for ferrous chloride was numerically lower than the other treatments throughout the experimental period. These results are similar to results found in Experiment 2. In both experiments intakes of waters containing Fe^{2+} were numerically lower than other treatments offered. We suspect that the absence of significant differences was due to insufficient statistical power (approximately 49% and 40% for Experiments 2 and 3 respectively), as the variation among cows was greater than expected, and earlier published data were not available in the literature with variance estimates.

During both Experiments 2 and 3, it was noted that there was a change in the appearance of Fe^{2+} (ferrous sulfate and ferrous chloride) and Fe^{3+} (ferric sulfate and ferric chloride) treatments. Throughout the experiments, Fe^{2+} treatment colors increased from clear to reddish-brown due to exposure to air, presumably oxidation. The treatment waters remained cloudy throughout the study with suspended matter, presumably containing Fe. The Fe^{3+} treatments also

increased in color, however, the precipitate formed by Fe appeared to sink to the bottom more instead of staying suspended as occurred with the Fe^{2+} treatments. This left red sediment at the bottom of the water containers, but the upper portion of water still relatively clear. This leads us to suspect that cows had numerically greater intakes for Fe^{3+} than Fe^{2+} because Fe was more evenly distributed in the Fe^{2+} treatments. We suggest that the presumably more even distribution of Fe^{2+} had a negative effect on drinking water palatability.

Experiment 4. Iron has a strong tendency to form organic and inorganic salt complexes (ferrous lactate, ferrous sulfate, ferric oxide) with other elements (Hem and Cropper, 1959). It is not known if Fe salt complexes have an effect on palatability of drinking water. In Experiment 4, our objective was to determine if cow preference for drinking water was dependent on Fe source supplied. Cows were offered pairs of water treatments containing 0 or 8 mg Fe/L as ferrous lactate, ferrous sulfate, or ferrous chloride. However, the ferrous chloride treatment actually contained 12.5 mg Fe/L. There was no interaction between treatment and time for both cumulative ($P = 0.20$) and non-cumulative ($P = 0.58$) water intakes. There was an overall effect of treatments on total water intake ($P < 0.0001$; Table 3.11). Cows preferred the control water source to every supplemental Fe water treatment. There was no difference in intake by cows offered different sources of Fe ($P > 0.15$). This result is contrary to results of Horvath (1985) in which wethers offered 145 mg Fe/L of Fe^{3+} from either ferric sulfate or ferric chloride preferred water containing ferric chloride. Digesti and Weeth (1976) also found that water palatability, measured as total water intake, was reduced more by sodium sulfate than an equal anionic concentration of sodium chloride (concentrations ranged from 275 to 4,400 mg/L). In our study, the concentrations of lactate, sulfate and chloride were relatively lower, and likely did not have nearly as much impact on palatability as the concentrations of Fe. These results also suggest that

other contaminants (the anion of the salt) in water do not decrease the palatability of water with high Fe concentration.

Water Analyses

Water samples from treatments were collected throughout the experiments. They were prepared for analysis of Fe by each of two different methods: 1) the direct metals analysis method of raw water (without acidification and acid digestion) known as the Livestock water analysis; and, 2) the total recoverable metals analysis by acidification of the water and acid digestion before analysis. Midwest Laboratories (Omaha, NE) analyzed both sample preparations for Fe by using EPA 200.7 method (1994). The concentrations determined by the direct analysis method did not compare to the concentrations of solutions formulated and prepared for the experiments (Tables 3.2 through 3.5, 3.7). In general, the concentration values by direct metals analysis of Fe were 7 - 25% of the concentrations by the total recoverable metals analysis. The direct Fe analysis is that used in the field to evaluate water quality in dairy farms (Beede, Michigan State University, East Lansing, MI, personal communication). In our experiments cows drank less water with Fe concentrations of 8 mg Fe^{2+}/L as determined by the total recoverable metals analysis. Therefore, these results indicate that in the field, 2 mg of Fe/L may be closer to 8 mg Fe/L and perhaps could negatively impact water intake. In short, the amounts of Fe that we are offering to cows through high-Fe drinking water could be near 4-fold of what we had believed previously if one is using the Fe concentration values from the direct metals analysis (Livestock water analysis).

Treatment Effects on Drinking Behavior

Drinking behavior was measured for the first 12 h of the experimental periods in each experiment. Our objective was to determine if Fe concentration, valance and source impacted drinking water behavior durations, total time spent interacting with each treatment, and frequency of interactions with each treatment. The main behaviors assessed were drinking, lapping and non-consumption interactions.

Experiment 1. We hypothesized that as concentrations of Fe increased, total duration and frequency of drinking and lapping would decrease, and time spent interacting with the container, but not consuming water would increase. Cows spent more time drinking 0Fe and 4Fe than the 8Fe treatment ($P = 0.02$; Table 3.12). Water intake also was greater for both 0Fe and 4Fe water treatments than 8Fe. There were no other treatment effects on duration of other behaviors. There was no difference in the frequency of occurrence of any of the drinking behaviors. Total time spent at water containers and visit frequencies were not different due to treatment.

Experiment 2. Coinciding with our hypothesis related to water intake, we suspected that cows would spend more time drinking from the Control water than supplemental Fe treatments, and more time drinking water of the ferrous sulfate treatment than the ferric sulfate treatment. We also hypothesized that time spent interacting with the containers, not consuming water, would be greatest for ferric sulfate. There was no treatment effect on duration or frequency of drinking behaviors (Table 3.13). There also was no effect of treatment on total time spent and frequency of visits to water containers.

Experiment 3. Similar to our hypothesis in Experiment 2, we expected that drinking duration and frequency would be greater for ferrous chloride than ferric chloride, and greatest for the Control treatment water. We also expected that cows would spend more time interacting

with, but not consuming ferric chloride water. There was no treatment effect on duration or frequency of drinking behaviors (Table 3.14). There also was no effect of treatment on total time spent at each water container or frequency of visits.

Experiment 4. We expected that cows would spend more time drinking and drink more often from Control water, and there would be no differences in behaviors among Fe sources. There was no effect of treatment on frequency or duration of lapping or non-consumption behaviors (Table 3.15). There was an effect of treatment on drinking duration ($P = 0.005$) and frequency ($P = 0.03$). Cows spent more time drinking from the Control, ferrous lactate and ferrous sulfate treatments than the ferrous chloride treatment ($P = 0.02$), and tended to spend more total time drinking from the ferrous lactate treatment than the ferrous chloride treatment ($P = 0.07$). However, this effect may be due to the additional iron contained in the ferrous chloride treatment. Overall, cows drank more frequently from the Control treatment than supplemental Fe treatments. Treatment did not impact the total time spent at each container, nor the number of visits per container during the 12 h observation period.

Correlations between Drinking Behavior and Water Intake

The relationships between various drinking behavior durations and frequencies were investigated for individual treatments and pooled across treatments for each experiment. Correlations were completed for 12 h total behavior durations and corresponding 12 h total intake.

Experiment 1. Relationships and correlation coefficients between drinking behaviors and water intake were visually compared, and were consistent across treatments (Table 3.16). Overall, there was no correlation between lapping duration and total water intake. However,

when correlations were investigated by treatment, water intake of Control treatment tended to be negatively correlated with lapping duration (r = -0.53; $P < 0.10$), whereas lapping duration was positively correlated with water intake of ferrous sulfate (r = 0.65; $P < 0.05$) and not related for ferric sulfate (r = 0.28; $P > 0.15$). Overall, drinking duration (r = 0.62; $P < 0.05$) and drinking frequency (r = 0.56; $P < 0.05$) were positively correlated with total 12-h water intake. No correlations were observed for lapping or non-consumption behavior frequencies or durations.

Experiment 2. The correlation coefficients and relationships between drinking behaviors and water intake were consistent among treatments, after visual evaluation (Table 3.17). When behaviors and intakes were pooled across treatments, both drinking duration (r = 0.70; $P <0.05$) and drinking frequency (r = 0.52; $P < 0.05$) were positively correlated with water intake. There were no other relationships between drinking behaviors and water intake.

Experiment 3. There were no differences in drinking behavior durations or frequencies. However, an interesting trend was identified between treatments. Most of the correlations between behavior and water intake were negative for the ferrous chloride treatment (Table 3.18). This was in contrast to Control and ferric chloride treatments, in which all relationships between behavior and water intake were positive for these treatments. The differences could be due to the excess Fe or the valence of the ferrous chloride treatment. Overall, there were no correlations between water intake and behavior when responses were pooled across treatments.

Experiment 4. Correlation coefficients and relationships between individual treatment water intakes and drinking behaviors were visually examined, and were consistent across treatments (Table 3.19). Overall there were no relationships found. Various drinking behaviors were not correlated with treatment water intake.

Overall, Fe concentration and source had the most impact on drinking duration. Research completed by Dado and Allen (1994) indicates that lactating cows spend approximately 18.5 min/d drinking and drink 4.3 L/min. In our experiments cows spent approximately 8.8 min/12 h drinking (17.6 min/24 h) and drank 8.3 L/min. However, our estimate does not include time spent lapping. The differences between our research and Dado and Allen (1994) can be attributed to differences in cow water intake (110 L/d and 77.6 L/d), milk production (45 kg/d and 33 kg/d), and the season that the experiments took place (August and January), respectively. In all studies across all experiments the maximum difference in total time spent drinking was only approximately 200 s (difference between drinking duration of Control and ferrous chloride treatments in Experiment 4). However, this could translate to a decrease of 29.3 L/d. This decrease is illustrated in the positive correlation between time spent drinking and total water intake among all experiments.

CONCLUSIONS

Our results indicate that the upper tolerable concentration of Fe in drinking water for cattle should be re-evaluated. Total recoverable Fe concentration of 4 mg/L did not affect negatively the drinking water preference of lactating dairy cows when compared with water containing no supplemental Fe. Water intake was less with total recoverable Fe of 8 mg Fe^{2+}/L. However, as we have shown, the total recoverable concentrations of Fe in drinking water are about 4 times greater than conventionally believed based on the typical Livestock water analysis generally used in the field. This implies that drinking water with 2 mg Fe/L on farm, analyzed by a standard direct analysis method, could have the same negative effects as total recoverable Fe concentration of 8 mg/L. Additionally, cows did not exhibit a preference between Fe^{2+} and Fe^{3+} in water, and did not have a preference for Fe source comparing ferrous lactate, sulfate and chloride. Drinking duration was negatively impacted by greater Fe concentration and was dependent on source. This research suggests that potential effects of high Fe in drinking water on production and health could be due to reduced water intake due to reduction in palatability of drinking water.

APPENDIX C

FIGURES AND TABLES REFERENCED IN CHAPTER 3

TABLES

Table 3.1. Pre-experiment dry matter intake, milk yield and composition, and water intake of cows in Experiment 1 through 4

	Experiment			
	1-3[1]	±SD[2]	4	±SD
DMI, kg DM	23	3.2	23	3.5
Water intake, L	109.6	16.40	108.3	18.75
Milk yield, kg	47.0	5.81	45.3	7.03
Milk Composition, %				
Fat	3.02	0.13	3.26	0.71
True protein	2.74	0.15	2.81	0.46
Lactose	4.64	0.08	4.57	0.73
SNF[3]	5.53	0.09	5.46	0.87
SCC[4], 1000 ml	150	187.5	20	34.52
MUN[5], mg/dl	20.2	2.77	25.2	4.37

[1]Milk composition results were pooled for Experiments 1, 2, and 3.

[2]SD = standard deviation.

[3]SNF = solids not fat.

[4]SCC = somatic cell count.

[5]MUN = milk urea nitrogen.

Table 3.2. Characteristics of water treatments in Experiment 1

	Treatments[1]		
	0Fe	4Fe	8Fe
Concentrations of constituents (mg/L)			
Fe, formulated	0.0	4.0	8.0
Fe, direct analysis[2]	0.01	0.83	2.04
Fe, total recoverable[3]	0.40	4.04	8.47
Lactate, formulated	0.0	13.6	27.23

[1]Treaments: 0Fe = 0 mg Fe/L; 4Fe = 4 mg Fe/L from ferrous lactate [$Fe(C_3H_5O_3)_2$]; 8Fe = 8 mg Fe/L from ferrous lactate [$Fe(C_3H_5O_3)_2$].

[2]Direct metals analysis of raw (without acid digestion) water by Livestock water analysis using EPA 200.7 method (1994) by Midwest Laboratories (Omaha, NE).

[3]Total recoverable metals analysis of acidified water after acid digestion using EPA 200.7 method (1994) by Midwest Laboratories (Omaha, NE).

Table 3.3. Characteristics of water treatments in Experiment 2

	Treatments[1]		
	Control	Ferrous sulfate	Ferric sulfate
Concentrations of constituents (mg/L)			
Fe, formulated	0.0	8.0	8.0
Fe, direct analysis[2]	0.26	1.25	0.7
Fe, total recoverable[3]	1.56	8.73	7.75
Sulfate, formulated	0.0	13.77	20.65

[1]Treatments: Control = 0 mg Fe/L; ferrous sulfate = 8 mg Fe/L from $FeSO_4$; ferric sulfate = 8 mg Fe/L from $Fe_2(SO_4)_3$.

[2]Direct metals analysis of raw (without acid digestion) water by Livestock water analysis using EPA 200.7 method (1994) by Midwest Laboratories (Omaha, NE).

[3]Total recoverable metals analysis of acidified water after acid digestion using EPA 200.7 method (1994) by Midwest Laboratories (Omaha, NE).

Table 3.4. Characteristics of water treatments in Experiment 3

	Treatments[1]		
	Control	Ferrous chloride	Ferric chloride
Concentrations of constituents (mg/L)			
Fe, formulated	0.0	12.5	8.0
Fe, direct analysis[2]	0.06	2.04	0.3
Fe, total recoverable[3]	1.19	12.1	7.02
Chloride, formulated	0.0	18.79	32.12

[1]Treatments: Control = 0 mg Fe/L; ferrous chloride = 8 mg Fe/L from $FeCl_2$; ferric chloride = 8 mg Fe/L from $FeCl_3$.

[2]Direct metals analysis of raw (without acid digestion) water by Livestock water analysis using EPA 200.7 method (1994) by Midwest Laboratories (Omaha, NE).

[3]Total recoverable metals analysis of acidified water after acid digestion using EPA 200.7 method (1994) by Midwest Laboratories (Omaha, NE).

Table 3.5. Characteristics of water treatments in Experiment 4

	Treatments[1]			
	Control	Ferrous lactate	Ferrous sulfate	Ferrous chloride
Concentrations of constituents (mg/L)				
Fe, formulated	0.0	8.0	8.0	12.5
Fe, direct analysis[2]	0.04	1.6	1.88	1.82
Fe, total recoverable[3]	0.31	7.58	7.27	11.2
Lactate, formulated	0.0	27.22	0.0	0.0
Sulfate, formulated	0.0	0.0	13.77	0.0
Chloride, formulated	0.0	0.0	0.0	18.14

[1]Treatments: Control = 0 mg Fe/L; ferrous lactate = 8 mg Fe^{2+}/L from $Fe(C_3H_5O_3)_2$; ferrous sulfate = 8 mg Fe^{2+}/L from $FeSO_4$; ferrous chloride = 8 mg Fe^{2+}/L from $FeCl_2$.

[2]Direct metals analysis of raw (without acid digestion) water by Livestock water analysis using EPA 200.7 method (1994) by Midwest Laboratories (Omaha, NE).

[3]Total recoverable metals analysis of acidified water after acid digestion using EPA 200.7 method (1994) by Midwest Laboratories (Omaha, NE).

Table 3.6. Ingredient and nutrient composition of diets fed in Experiments 1 through 4

	Experiment	
	1-3[1]	4
Ingredient	----------Percent of dietary DM----------	
Chopped alfalfa hay	3.74	3.93
Corn silage	35.63	33.61
Whole cottonseed	5.70	6.20
Energy booster	0.40	0.42
Grass hay	4.83	5.17
Ground corn	9.52	10.09
Alfalfa haylage	9.86	8.89
High moisture corn	6.98	7.41
Soybean meal, 48% CP	14.64	15.19
Soyhulls	5.25	5.55
Mineral-vitamin mix[2]	3.27	3.45
Nutrient Composition		
DM, %	49.40	46.60
	----------Percent of dietary DM----------	
NDF	32.90	34.50
ADF	22.20	22.60
CP	17.84	17.95
Ca	1.11	1.06
P	0.35	0.37
K	1.26	1.34
Na	0.31	0.35
S	0.22	0.37
Mg	0.24	0.25
	----------mg/kg----------	
Co	0.11	0.12
Cu	10.1	10.9
Fe	257	179
Mo	1.8	2.2

Table 3.6. (cont'd)

	Experiment	
	1-3[1]	4
Nutrient Composition	----------mg/kg----------	
Mn	42	46
Zn	41	42

[1]Feed ingredients were pooled for experiments 1-3 for the values reported in this table.

[2]Mineral-vitamin mix contained 47.5% limestone, 22.5% sodium bicarbonate, 10.1% urea (45%N), 8.2% magnesium sulfate, 7.5% sodium chloride, 1.6% biotin premix (1.4 g/kg), 1.3% trace mineral premix, 30 KIU/kg vitamin A, 8 KIU/kg vitamin D, 56 KIU/kg vitamin E and 0.3% selenium yeast.

Table 3.7. Water quality constituent analysis of Control drinking water in Experiments 1 though 4

	Experiment				
	1	2	3	4	Caution level[1]
Quality Constituent					
TDS[2]	377	462	411	387	1,000
Ca	86	114	99	92	150
Cl	21	15	25	16	500
Cu	ND[3]	ND	ND	ND	0.3
Fe (direct Fe analysis)[4]	0.07	0.26	0.06	0.04	0.3
Fe (total recoverable)[5]	0.4	1.56	1.19	0.31	-
Mg	30	38	31	31	80
Mn	0.06	0.75	0.06	0.03	-
NO_3-N	ND	ND	ND	ND	25
Na	9	7	11	7	150
SO_4	53	122	59	61	300
Zn	0.17	0.14	0.06	0.14	-
Conductivity, mmhos/cm	0.58	0.71	0.63	.59	1.5
pH	7.8	7.7	7.7	7.6	6.5-9.0

[1]Caution levels from Midwest Laboratories (Omaha, NE)

[2]TDS = total dissolved solids

[3]ND = not detected

[4]Direct metals analysis of raw (without acid digestion) water by Livestock water analysis using EPA 200.7 method (1994) by Midwest Laboratories (Omaha, NE).

[5]Total recoverable metals analysis of acidified water after acid digestion using EPA 200.7 method (1994) by Midwest Laboratories (Omaha, NE).

Table 3.8. Cumulative water intake (L) by hour of cows in Experiment 1

	Treatments[1]			
	0Fe	4Fe	8Fe	SEM[2]
Hour				
2	10.9	10.5	8.3	1.61
6	24.5	21.8	17.9	2.31
12	35.7	35.6	26.9	3.22
17	47.3[a]	45.5[ab]	33.7[b]	4.33
22	60.4[ab]	61.9[a]	46.1[b]	4.80

[a-b]Means within a row with different superscripts tended to differ ($P < 0.10$).

[1]Treaments: 0Fe = 0mg Fe; 4Fe= 4 mg Fe/L from ferrous lactate [$Fe(C_3H_5O_3)_2$]; 8Fe = 8 mg Fe/L from $Fe(C_3H_5O_3)_2$.

[2]SEM = standard error of the mean.

Table 3.9. Cumulative water intake (L) by hour of cows in Experiment 2

	Treatments[1]			
	Control	Ferrous sulfate	Ferric sulfate	SEM[2]
Hour				
2	10.5	11.2	13.2	2.15
6	23.9	19.2	26.1	3.89
12	38.3	29.2	36.3	6.00
17	47.1	34.0	41.6	6.91
22	68.1	50.6	56.1	7.78

[1]Treatments: Control = 0 mg Fe/L; ferrous sulfate = 8 mg Fe/L from $FeSO_4$; ferric sulfate = 8 mg Fe/L from $Fe_2(SO_4)_3$.

[2]SEM = standard error of the mean.

Table 3.10. Cumulative water intake (L) by hour of cows in Experiment 3

	Treatments[1]			
	Control	Ferrous chloride	Ferric chloride	SEM[2]
Hour				
2	10.8	7.8	13.6	2.03
6	24.8	16.2	26.9	4.15
12	36.8	28.5	38.0	5.80
17	43.7	34.2	42.5	6.43
22	63.8	47.4	67.3	9.35

[1]Treatments: Control = 0 mg Fe/L; ferrous chloride = 8 mg Fe/L from $FeCl_2$; ferric chloride = 8 mg Fe/L from $FeCl_3$.

[2]SEM = standard error of the mean.

Table 3.11. Cumulative water intake (L) by hour of cows in Experiment 4

	Treatments[1]				
	Control	Ferrous lactate	Ferrous sulfate	Ferrous chloride	SEM[2]
Hour					
2[3]	11.6[a]	7.3[ab]	7.3[ab]	6.8[b]	1.36
6	32.3[a]	22.5[b]	19.4[b]	17.3[b]	2.69
12	49.8[a]	34.5[b]	31.3[b]	27.3[b]	3.66
17	55.1[a]	39.7[b]	35.5[b]	32.7[b]	4.16
22	71.9[a]	53.3[b]	45.8[b]	43.0[b]	5.11

[a-b]Means within a row with different superscripts differ ($P < 0.05$).

[1]Treatments: Control = 0 mg Fe/L; ferrous lactate= 8 mg Fe^{2+}/L from $Fe(C_3H_5O_3)_2$; ferrous sulfate = 8 mg Fe^{2+}/L from $FeSO_4$; ferrous chloride = 8 mg Fe^{2+}/L from $FeCl_2$.

[2]SEM = standard error of the mean.

[3]Hour 2 data were square root transformed for analysis, and LS means and SEM were back-transformed for presentation in this table.

Table 3.12. Cumulative drinking behavior durations and frequencies of cows in Experiment 1

	Treatments[1]				
	0Fe	4Fe	8Fe	SEM[2]	*P*-value
Behavior Duration (s)					
Drinking	210[a]	214[a]	124[b]	28.7	0.01
Lapping	196	226	234	84.8	0.65
Other[3, 4]	30	28	39	18.8	0.76
Behavior Frequency					
Drinking	14.3	13.8	11.7	1.49	0.17
Lapping	14.7	15.6	16.3	2.63	0.57
Other[3]	8.8	8.1	8.5	.96	0.56

[a-b]Means within a row with different superscripts differ ($P < 0.05$).

[1]Treaments: 0Fe = 0 mg Fe; 4Fe= 4 mg Fe/L from ferrous lactate [$Fe(C_3H_5O_3)_2$]; 8Fe = 8 mg Fe/L from $Fe(C_3H_5O_3)_2$.

[2]SEM = standard error of the mean.

[3]Other = interacting with water container but not consuming water.

[4]Data were square root transformed for analysis and LS means and SEM were back-transformed for presentation in this table.

Table 3.13. Cumulative drinking behavior durations and frequencies of cows in Experiment 2

	Treatments[1]				
	Control	Ferrous sulfate	Ferric sulfate	SEM[2]	*P*-value
Behavior Duration (s)					
Drinking[3]	182	119	156	50.5	0.558
Lapping	210	187	182	71.7	0.82
Other[4, 5]	14	15	15	8.0	0.99
Behavior Frequency					
Drinking	15.4	13.1	14.6	2.17	0.58
Lapping	13.8	14.2	14.1	2.41	0.95
Other[4, 5]	7.8	7.9	7.6	0.68	0.91

[1]Treatments: Control = 0 mg Fe/L; ferrous sulfate = 8 mg Fe/L from $FeSO_4$; ferric sulfate = 8 mg Fe/L from $Fe_2(SO_4)_3$.

[2]SEM = standard error of the mean.

[3]Data were natural log transformed for analyses, and means back-transformed for presentation in this table.

[4]Other = interacting with water container but not consuming water.

[5]Data were square root transformed for analysis and LS means and SEM were back-transformed for presentation in this table.

Table 3.14. Cumulative drinking behavior durations and frequencies of cows in Experiment 3

	Treatments[1]				
	Control	Ferrous chloride	Ferric chloride	SEM[2]	*P*-value
Behavior Duration (s)					
Drinking[3]	221	96	199	69.6	0.22
Lapping[3]	131	127	194	66.5	0.67
Other[3, 4]	46	19[†]	56	30.9	0.09
Behavior Frequency					
Drinking	23.5	19.9	31.3	5.81	0.21
Lapping	22.6	22.8	27.6	3.61	0.40
Other[4]	9.0	9.1	13.7	2.8	0.16

[1]Treatments: Control = 0 mg Fe/L; Ferrous chloride = 8 mg Fe/L from $FeCl_2$; ferric Chloride = 8 mg Fe/L from $FeCl_3$.

[2]SEM = standard error of the mean.

[3]Data were natural log transformed for analyses, and means back-transformed for presentation in this table.

[4]Other = interacting with water container but not consuming water.

[†]Tended to be lower than ferric chloride ($P < 0.09$).

Table 3.15. Cumulative drinking behavior durations and frequencies of cows in Experiment 4

	Treatments[1]					
	Control	Ferrous lactate	Ferrous sulfate	Ferrous chloride	SEM[2]	*P*-value
Behavior Duration (s)						
Drinking[3]	316[a]	222[ab]	200[ab]	106[b†]	49.5	.004
Lapping[4]	213	205	291	257	67.1	.37
Other[3, 5]	143	123.6	113	124	35.1	0.93
Behavior Frequency						
Drinking	11.2[a]	8.7[ab]	8.6[ab]	6.7[b]	2.15	0.03
Lapping[4]	8.4	8.8	9.8	9.7	2.11	.80
Other[4, 5]	4.1	3.9	3.9	3.9	.91	.99

[a-b]Means within a row with different superscripts differ ($P < 0.05$).

[1]Treatments: Control = 0 mg Fe/L; ferrous lactate= 8 mg Fe^{2+}/L from $Fe(C_3H_5O_3)_2$; ferrous sulfate = 8 mg Fe^{2+}/L from $FeSO_4$; ferrous chloride = 8 mg Fe^{2+}/L from $FeCl_2$.

[2]SEM = standard error of the mean.

[3]Data were natural log transformed for analyses, and means back-transformed for presentation in this table.

[4]Data were square root transformed for analysis and LS means and SEM were back-transformed for presentation in this table.

[5]Other = interacting with water container but not consuming water.

[†]Tended to differ ($P < 0.10$) from ferrous lactate.

Table 3.16. Pearson correlation coefficients for the relationships between drinking behavior and 12 h total water intake in Experiment 1

Behavior * Water Intake	Experiment			
	0Fe	4Fe	8Fe	Pooled across treatments
Drinking				
Duration	0.65371*	0.51433†	0.47432	0.62254*
Frequency	0.74011*	0.34511	0.31158	0.55553*
Lapping				
Duration	-0.53377†	0.64785*	0.27885	0.08379
Frequency	-0.01356	0.56509†	0.26319	0.22740
Other				
Duration	0.31802	-0.02181	-0.11572	0.06227
Frequency	0.49544	0.00658	-0.18433	0.14325

†$P < 0.10$.

*$P < 0.05$.

Table 3.17. Pearson correlation coefficients for the relationships between drinking behavior and 12 h total water intake in Experiment 2

Behavior * Water Intake	Experiment			
	Control	Ferrous sulfate	Ferric sulfate	Pooled across treatments
Drinking				
Duration	0.68347*	0.56967†	0.84163*	0.70464*
Frequency	0.41922	0.66291*	0.58211*	0.52143*
Lapping				
Duration	0.43651	0.25246	0.31928	0.31817
Frequency	0.62491*	0.08924	0.35114	0.28454
Other				
Duration	-0.08649	-0.35700	-0.10997	-0.15631
Frequency	-0.11826	-0.11070	0.13946	-0.06315

†$P < 0.10$.

*$P < 0.05$.

Table 3.18. Pearson correlation coefficients for the relationships between drinking behavior and 12 h total water intake in Experiment 3

Behavior * Water Intake	Experiment			
	Control	Ferrous chloride	Ferric chloride	Pooled across treatments
Drinking				
Duration	0.49667	0.38998	0.52901†	0.42773
Frequency	0.48297	-0.07224	0.54007†	0.41004
Lapping				
Duration	0.29902	-0.21694	0.61865*	0.35768
Frequency	0.43217	-0.46821	0.62511*	0.33905
Other				
Duration	0.76286*	-0.36625	0.58239†	0.38706
Frequency	0.53779†	-0.40534	0.56597†	0.34541

†$P < 0.10$.

*$P < 0.05$.

Table 3.19. Pearson correlation coefficients for the relationships between drinking behavior and 12 h total water intake in Experiment 4

Behavior * Water Intake	Treatment				
	Control	Ferrous lactate	Ferrous sulfate	Ferrous chloride	Pooled across treatments
Drinking					
Duration	0.45451*	0.32436†	0.42433*	0.42816*	0.42142
Frequency	0.39526*	0.48484*	0.43335*	0.32048†	0.41149
Lapping					
Duration	0.10954	0.05615	0.22615	0.38021*	0.11925
Frequency	-0.03270	0.12386	0.08579	0.30659†	0.06866
Other					
Duration	0.27511	0.26315	0.28772†	0.25306	0.28659
Frequency	0.22752	0.03329	0.09249	0.39405*	0.18444

†$P < 0.10$.

*$P < 0.05$.

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CHAPTER 4

CONCLUSIONS AND IMPLICATIONS

The majority of Fe naturally occurring in drinking water drawn from underground wells is in the more bioavailable ferrous (Fe^{2+}) valence. Consumption of water with high Fe concentrations could negatively impact cow health and productivity, as anecdotal information suggests. We hypothesized that drinking water with greater than normal Fe concentrations could affect cows in two ways; through changes in Fe status, trace mineral interactions, and increases in oxidative stress; and/or, through decreased water intake due to cow preference. Our objectives were to test each of these possibilities through a series of experiments.

In chapter 2, we presented results from two different experiments designed to investigate the short-term hourly effects (0 – 12 h) of dosing in the abomasum increasing Fe concentrations of each valence on short-term Fe status, antioxidant status, and measurements of oxidative stress. Overall, administration to lactating dairy cows with 0, 4, or 8 mg Fe/kg BW from ferrous lactate; or, 0 or 8 mg Fe/kg BW from either ferrous or ferric sulfate had minimal impacts on measurements of Fe status. In the first experiment, TIBC tended to be greater for cows given 0.75 mg Fe/kg BW, and in the second experiment, 1.5 mg Fe/kg BW from ferric sulfate tended to reduce serum Fe concentrations. No other treatment effects on any other measurements of Fe status were detected.

Hepatic Cu concentrations declined in response to 0.75 mg Fe/kg BW administered as ferrous lactate. Additionally there was a decrease in serum Cu concentrations in response to 1.5 mg Fe/kg BW from ferrous sulfate. These results suggest that the amount of Fe consumed by cows from drinking water under farm conditions could potentially cause an interaction with Cu metabolism. Campbell et al. (1974) reported that intraruminally-dosing dairy cows with high

concentrations (210 mg Fe/kg BW) of Fe leads to Cu deficiency. We suggest that Cu deficiency may still develop in dairy cows consuming drinking water contaminated by Fe over a longer period of time.

Overall results of the first two experiments suggest that at the amounts and concentrations tested, Fe contained in water does not impact short-term Fe status. However, we suggest that after prolonged consumption, high Fe water may negatively impact cow health similar to high dietary Fe. We suspect that continual exposure of dairy cows to Fe from water could decrease DMI, and increase oxidative stress and intestinal permeability, similar to the effects that high dietary Fe^{2+} in calves (Hansen, et al., 2010). Prolonged exposure also could lead to Cu deficiency. Further research should investigate the effects of chronic Fe consumption from drinking water on Fe metabolism, and cow health and productivity long-term.

In Chapter 3, we presented results from four drinking water preference experiments evaluating the effects of Fe concentration, valence and source on water intake and drinking behavior of lactating dairy cows. When cows were offered 0, 4, or 8 mg Fe/L from ferrous lactate, there was no difference in water intake among cows offered 0 and 4 mg/L, but water intake of cows offered the 8 mg Fe/L treatment was lower than either 0 or 4 mg/L. Intake of water containing 8 mg Fe^{2+}/L was least presumably due to reduced palatability.

In two separate experiments, 8 mg Fe/L from either a Fe^{2+} or Fe^{3+} source did not affect cow preference for drinking water as measured by water intake. However, we wonder if the lack of differences may have been due to insufficient statistical power due to larger than anticipated variance, as there were numerical decreases in both experiments in water intake of cows offered the Fe^{2+} versus the Control and Fe^{3+} treatments. During the experimental period, observers noticed that a red precipitate formed in the containers with water of both Fe^{2+} and Fe^{3+}

treatments. However, in the Fe^{2+} treatments, the precipitant remained suspended in the water, whereas the precipitant with Fe^{3+} treatments sank to the bottom of the containers leaving the upper portion of water relatively clear. We believe that the numerical differences in water intake were due to the presumably more even distribution of Fe in the treatment water with Fe^{2+} compared with Fe^{3+}. Overall, we believe that there is not much difference in cow preference between Fe^{2+} and Fe^{3+} of equal concentrations in drinking water.

We suggested that 8 mg Fe^{2+}/L in drinking water has a negative effect on water intake based on results from the initial experiment described in Chapter 3. This idea is supported by results from the final experiment described in Chapter 3. The objective was to determine if Fe source affected water intake. Twelve cows were offered pairs of treatments: 0, or 8 mg Fe/L as ferrous lactate, ferrous sulfate or ferrous chloride. Eight mg Fe/L reduced water intake from all three sources, but there were no differences among waters offered with different Fe sources. This indicates that 8 mg Fe^{2+}/L has a negative impact on water intake. The long-term impact of water with high Fe concentrations on cow health and productivity may be due to a decrease in palatability and preference. Nonetheless, the sources of Fe (or Fe salt compound) tested did not affect preference for water with high Fe concentrations.

The currently recommended upper tolerable concentration for Fe in drinking water for cattle is 0.3 mg/L. However, the NRC (2005) suggests that cattle may be able to tolerate higher concentrations. In our research, Fe concentration of 4 mg/L did not affect total water intake or drinking behavior of lactating dairy cows. These results suggest that the current upper tolerable concentration for cattle may be lower than practically needed, in agreement with the suggestion of NRC (2005).

It is important to note that the water treatments offered in our preference experiments containing 0, 4, and 8 mg Fe^{2+}/L (total recoverable Fe in which acid digestion of samples proceeds detection), corresponded to Fe concentrations of approximately 0, 1, and 2 mg Fe/L according to the direct metal analysis (Livestock water analysis) of Midwest Laboratories, (Omaha, NE). We found that the amount of Fe in raw water (prepared for analysis without acidification and acid digestion) was approximately 7 to 25% the concentration of total recoverable Fe. Both sample types were analyzed by the EPA 200.7 detection method (1994) after preparation. Therefore, in our research we did not test conditions similar to the field circumstances in which Fe concentrations in water were in the 4 to 10 mg/L range by the Livestock water analysis method (direct metal analysis), which may in fact be translate to total recoverable Fe concentrations as great as 16 to 40 mg/L.

Overall, our research suggests that the current caution level (0.3 mg/L) for Fe in drinking water for dairy cows may be lower than practically needed, and that cattle may be able to tolerate greater concentrations. In our research 4 mg Fe/L (total recoverable Fe) or 1 mg Fe/L (direct water analysis) from Fe^{2+} did not impact dairy cow preference for drinking water. Further research needs to be completed to determine if prolonged exposure and consumption of water with 4 mg total recoverable Fe/L or greater has negative impacts on cow health.

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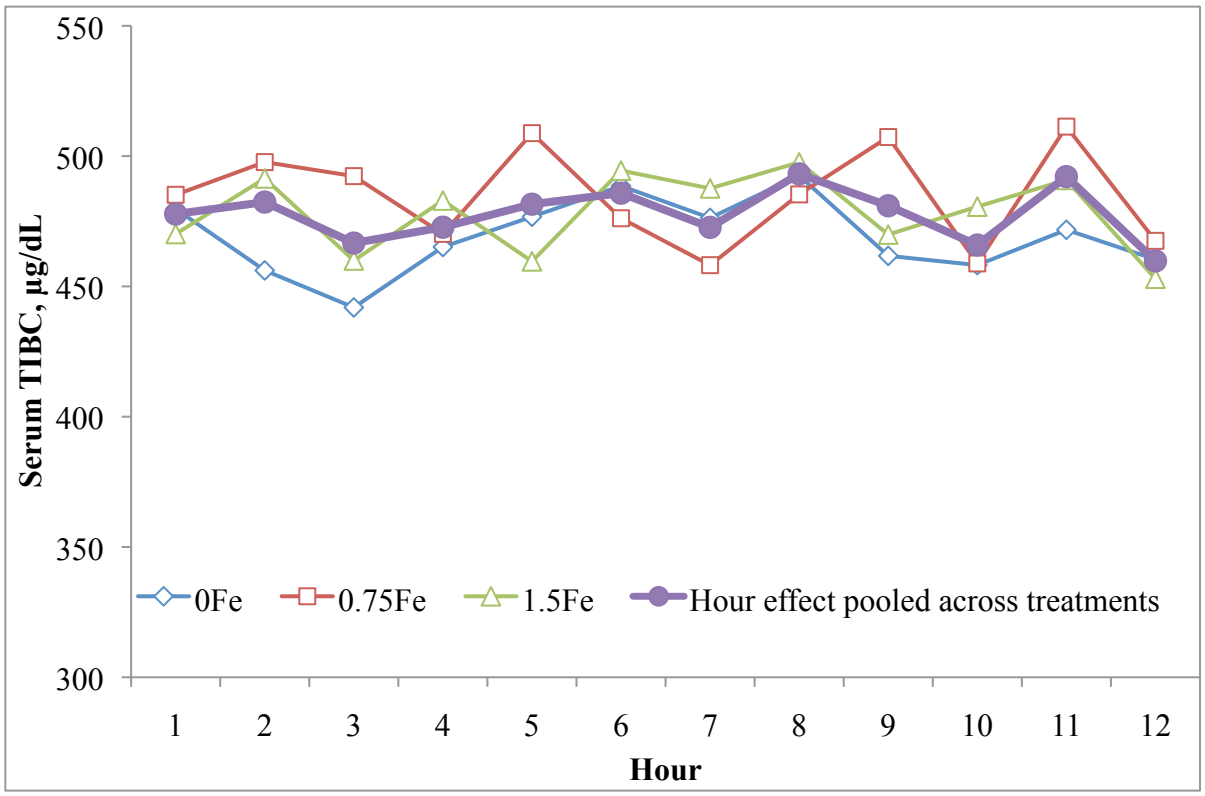
APPENDIX D

ADDITIONAL FIGURES FROM CHAPTER 2

FIGURES

Figure 4.1 Treatment[1] by hour (P = 0.34) and pooled hour (SEM[2] = 19.61; *P* = 0.17) responses of serum total Fe binding capacity[3] in Experiment 1

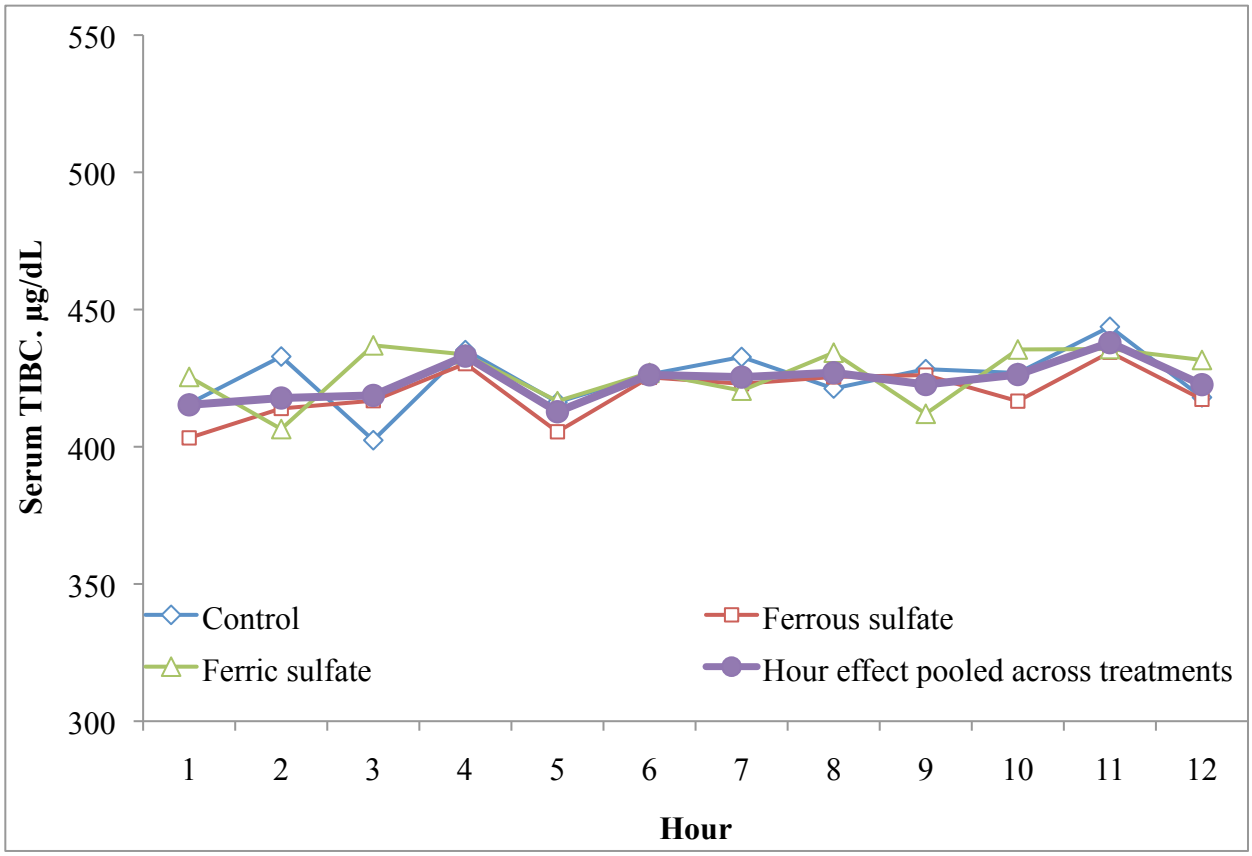


[1]Treatments: 0Fe = 0 mg Fe; 0.75Fe = 0.75 mg Fe/kg BW from ferrous lactate [$Fe(C_3H_5O_3)_2$]; 1.5Fe = 1.5 mg Fe/kg BW from ferrous lactate.

[2]SEM = standard error of the mean.

[3]Total Fe binding capacity = Fe + unsaturated Fe binding capacity.

Figure 4.2. Treatment[1] by hour ($P = 0.88$) and pooled hour (SEM[2] = 11.43; $P = 0.21$) responses of serum total Fe binding capacity[3] in Experiment 2

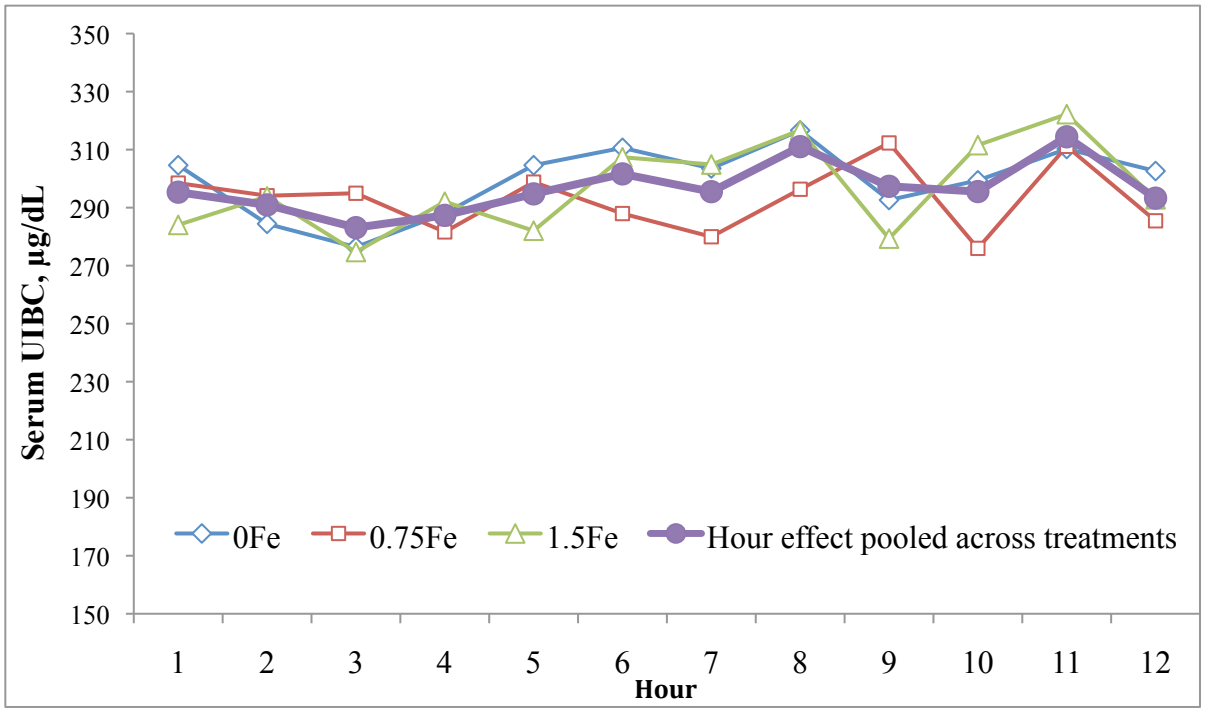


[1]Treatments: Control = no Fe; Ferrous sulfate = 1.5 mg Fe/kg BW from ferrous sulfate [$FeSO_4$]; Ferric sulfate = 1.5 mg Fe/kg BW from ferric sulfate [$Fe_2(SO_4)_3$].

[2]SEM = standard error of the mean.

[3]Total Fe binding capacity = Fe + unsaturated Fe binding capacity.

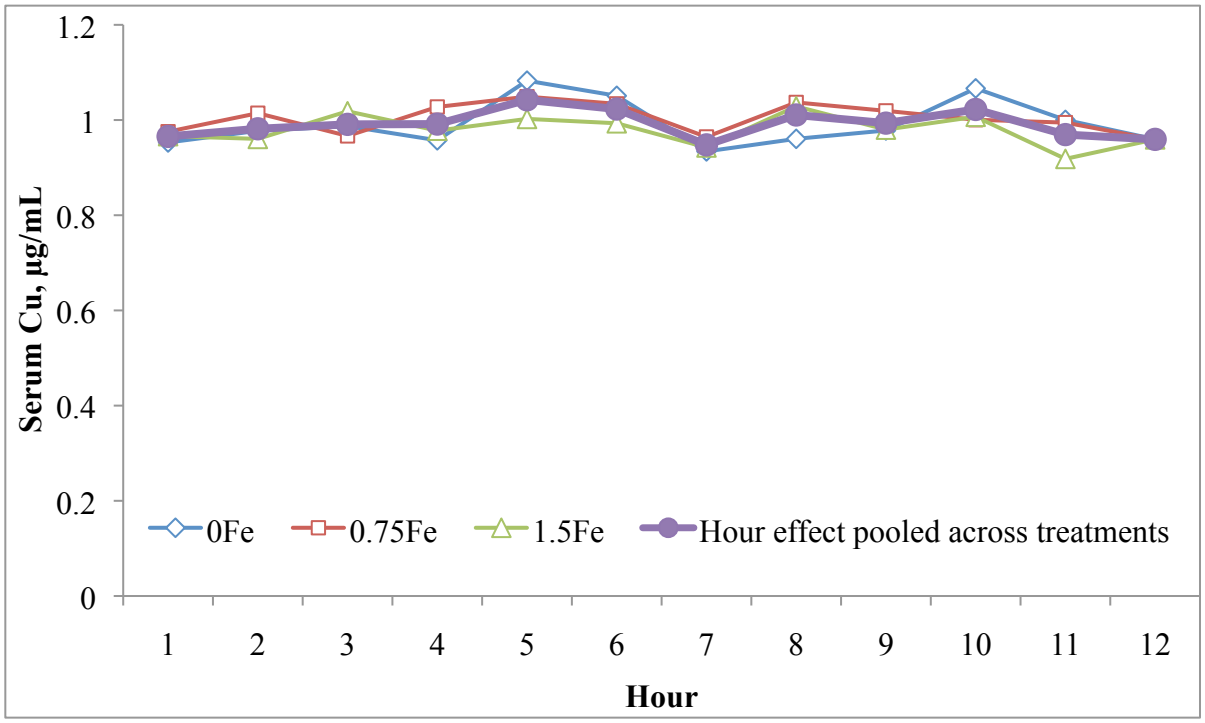
Figure 4.3. Treatment[1] by hour ($P = 0.63$) and pooled hour (SEM[2] = 22.37, $P = 0.14$) responses of serum unsaturated Fe binding capacity in Experiment 1



[1]Treatments: 0Fe = 0 mg Fe; 0.75Fe = 0.75 mg Fe/kg BW from ferrous lactate [$Fe(C_3H_5O_3)_2$]; 1.5Fe = 1.5 mg Fe/kg BW from ferrous lactate.

[2]SEM = standard error of the mean.

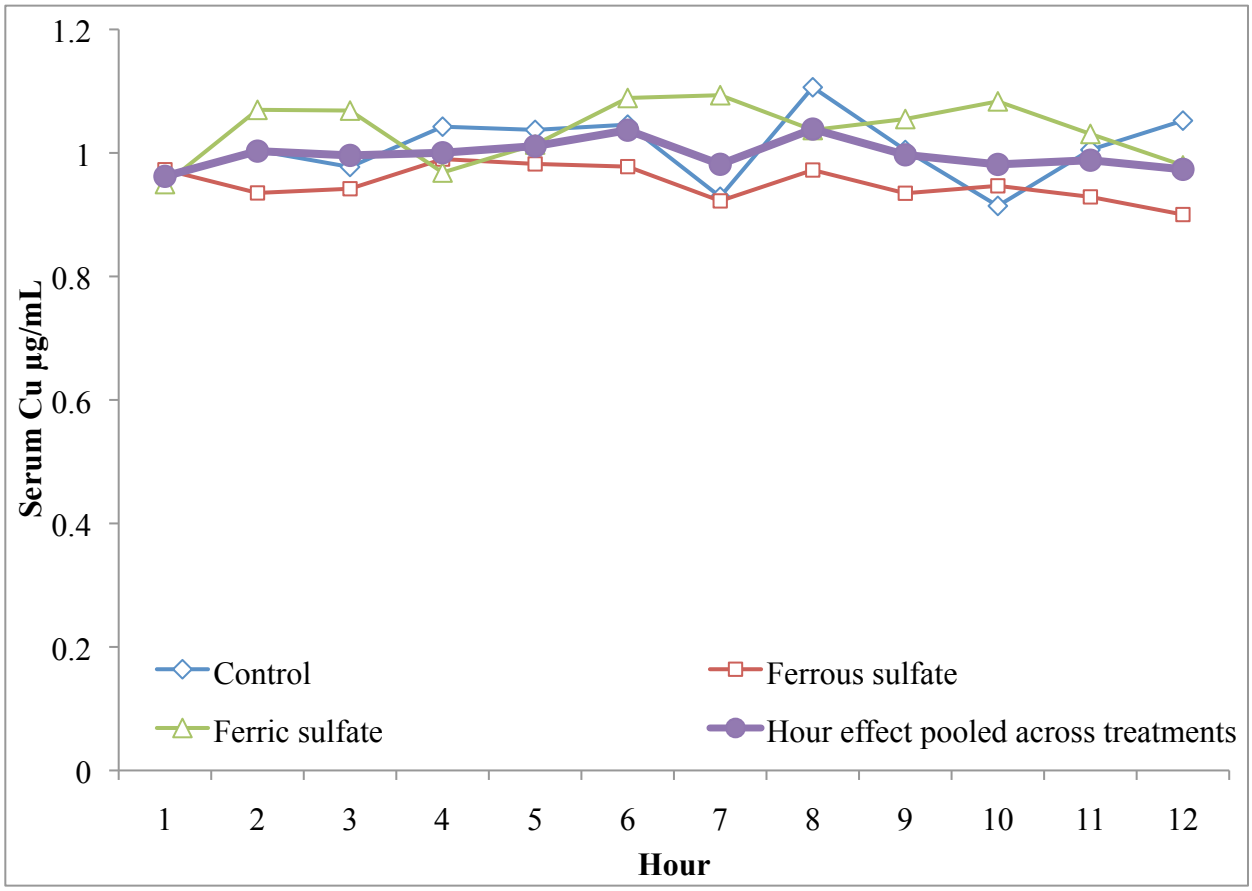
Figure 4.4. Treatment[1] by hour ($P = 0.99$) and pooled hour (SEM[2] = 0.06; $P = 0.37$) responses of serum Cu concentration in Experiment 1



[1]Treatments: 0Fe = 0 mg Fe; 0.75Fe = 0.75 mg Fe/kg BW from ferrous lactate [$Fe(C_3H_5O_3)_2$]; 1.5Fe = 1.5 mg Fe/kg BW from ferrous lactate.

[2]SEM = standard error of the mean.

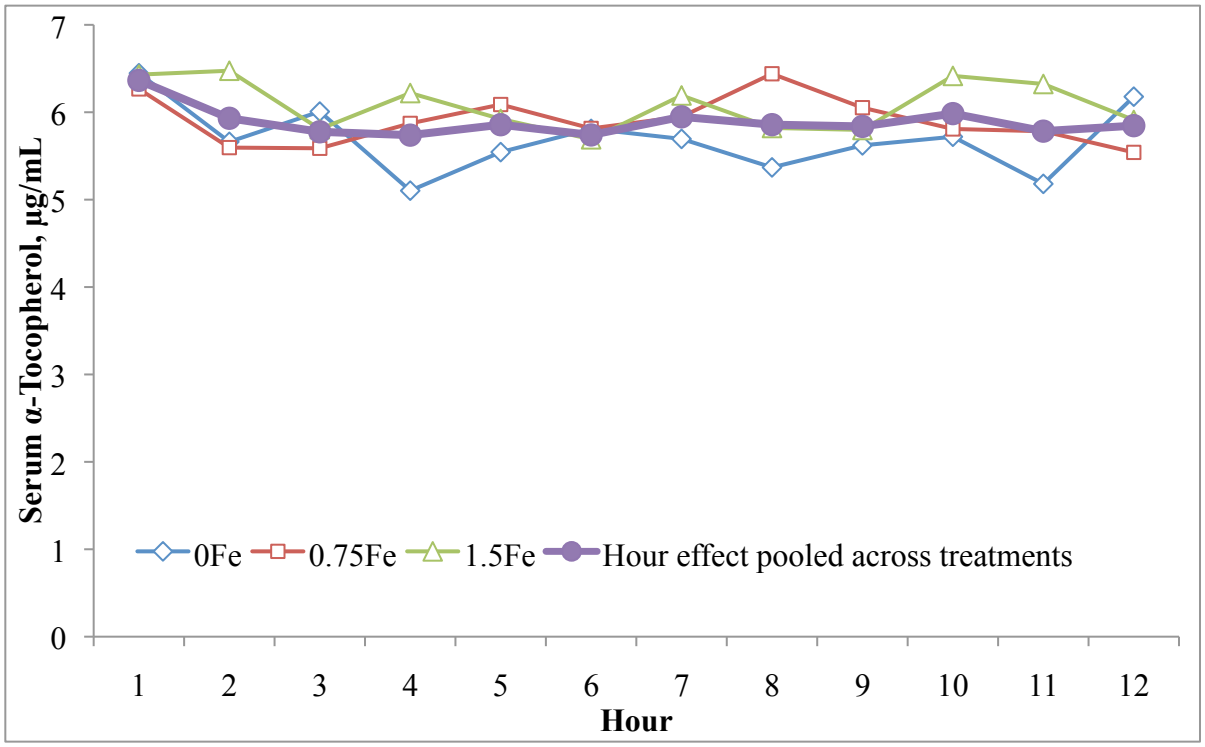
Figure 4.5. Treatment[1] by hour ($P = 0.61$) and pooled hour (SEM[2] = 0.06; $P = 0.82$) responses of serum Cu concentration in Experiment 2



[1]Treatments: Control = no Fe; Ferrous sulfate = 1.5 mg Fe/kg BW from ferrous sulfate [$FeSO_4$]; Ferric sulfate = 1.5 mg Fe/kg BW from ferric sulfate [$Fe_2(SO_4)_3$].

[2]SEM = standard error of the mean.

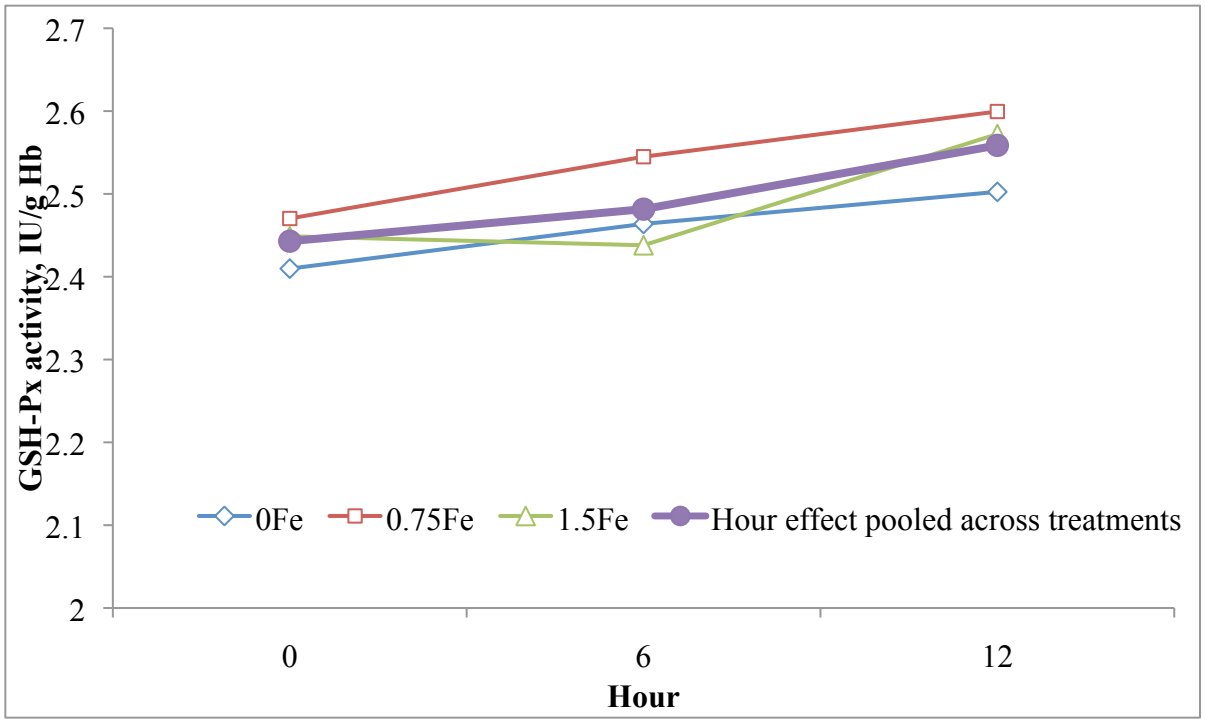
Figure 4.6. Treatment[1] by hour ($P = 0.21$) and pooled hour (SEM[2] = 0.34; $P = 0.36$) responses of serum α-Tocopherol concentration in Experiment 1



[1]Treatments: 0Fe = 0 mg Fe; 0.75Fe = 0.75 mg Fe/kg BW from ferrous lactate [$Fe(C_3H_5O_3)_2$]; 1.5Fe = 1.5 mg Fe/kg BW from ferrous lactate.

[2]SEM = standard error of the mean.

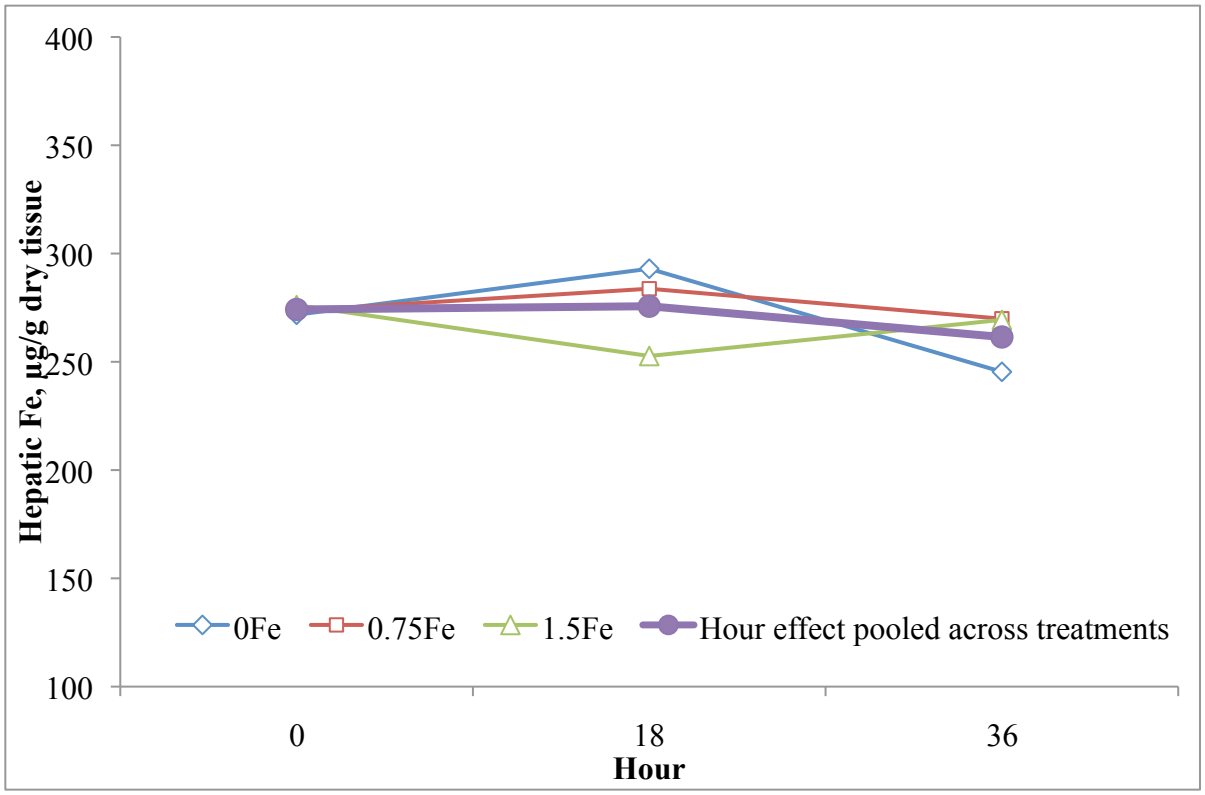
Figure 4.7. Treatment[1] by hour (P = 0.97) and pooled hour (SEM[2] = 0.09; *P* = 0.21) responses of whole blood glutathione peroxidase activity in Experiment 1



[1]Treatments: 0Fe = 0 mg Fe; 0.75Fe = 0.75 mg Fe/kg BW from ferrous lactate [$Fe(C_3H_5O_3)_2$]; 1.5Fe = 1.5 mg Fe/kg BW from ferrous lactate.

[2]SEM = standard error of the mean.

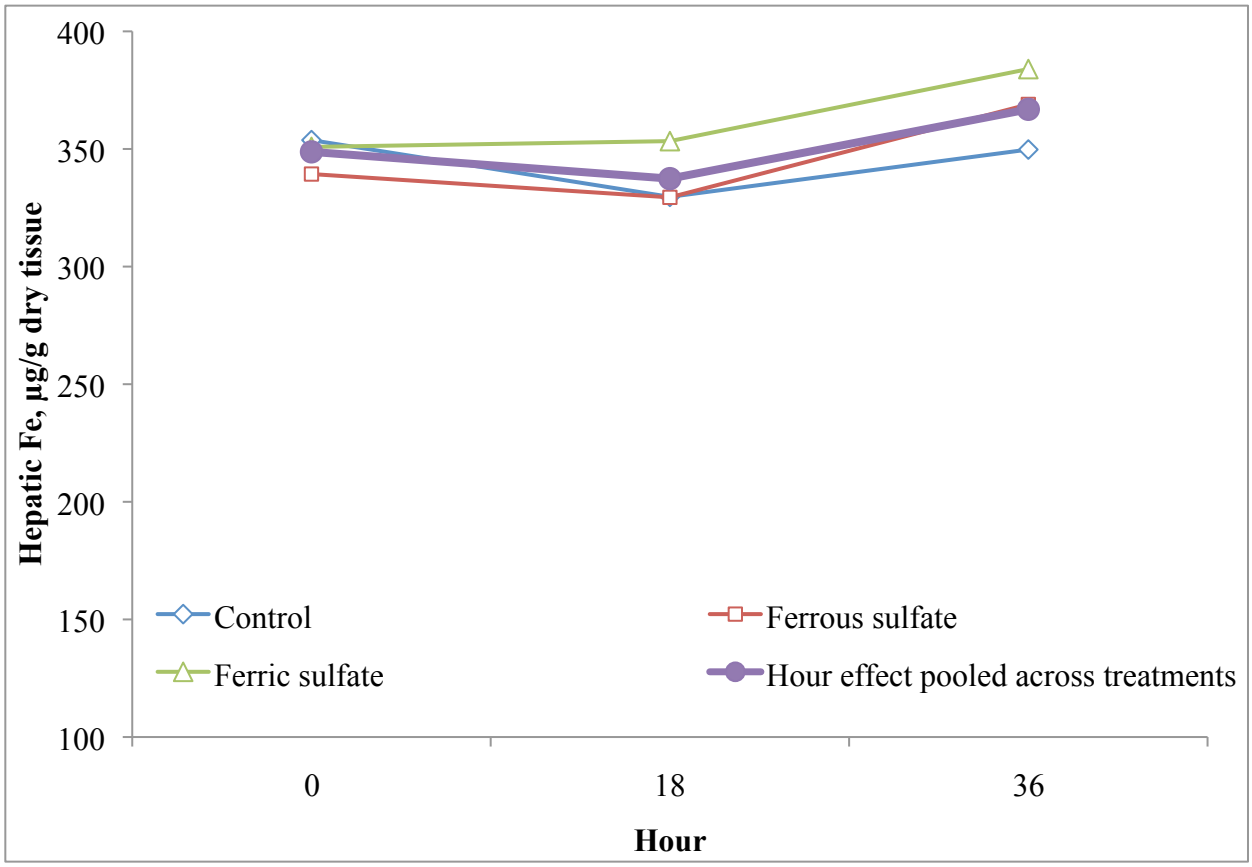
Figure 4.8. Treatment[1] by hour ($P = 0.69$) and pooled hour (SEM[2] = 21.69; $P = 0.45$) responses of hepatic Fe concentration in Experiment 1



[1]Treatments: 0Fe = 0 mg Fe; 0.75Fe = 0.75 mg Fe/kg BW from ferrous lactate [$Fe(C_3H_5O_3)_2$]; 1.5Fe = 1.5 mg Fe/kg BW from ferrous lactate.

[2]SEM = standard error of the mean.

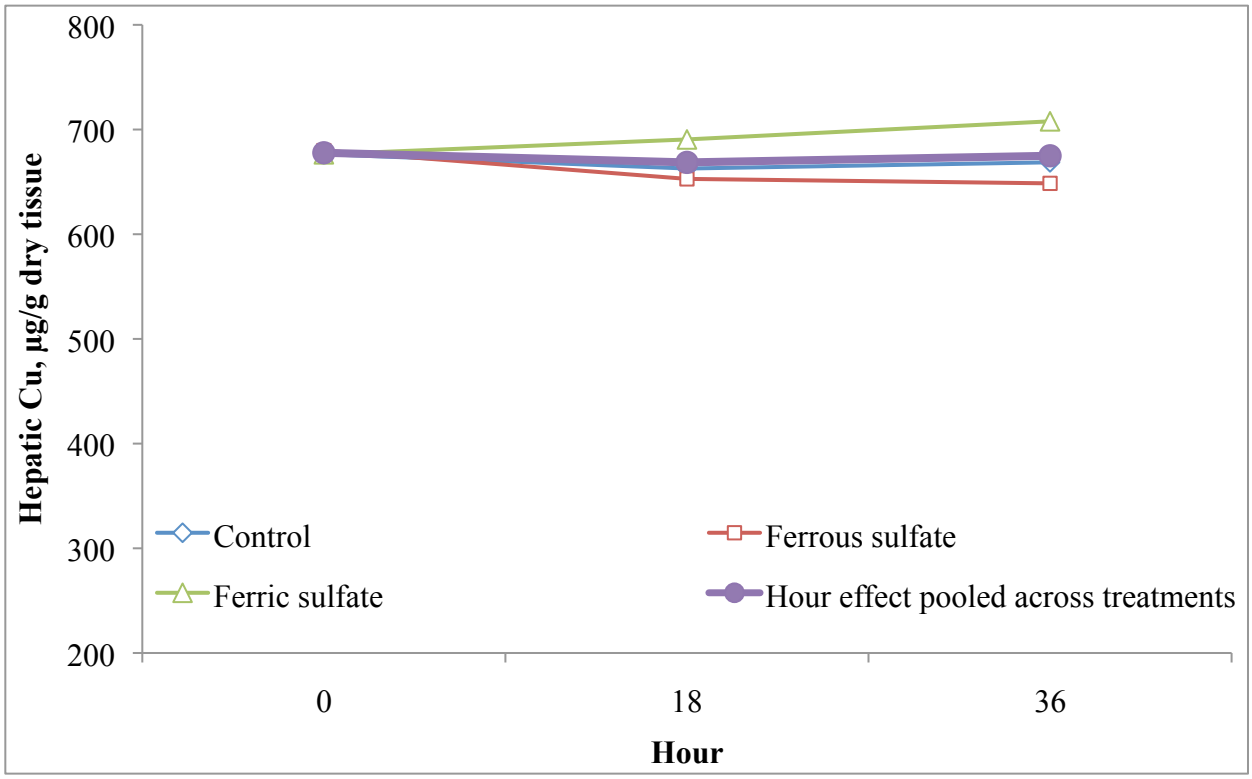
Figure 4.9. Treatment[1] by hour (P = 0.69) and pooled hour 2 (SEM[2] = 30.04; *P* = 0.57) responses of hepatic Fe concentration in Experiment



[1]Treatments: Control = no Fe; Ferrous sulfate = 1.5 mg Fe/kg BW from ferrous sulfate [$FeSO_4$]; Ferric sulfate = 1.5 mg Fe/kg BW from ferric sulfate [$Fe_2(SO_4)_3$].

[2]SEM = standard error of the mean.

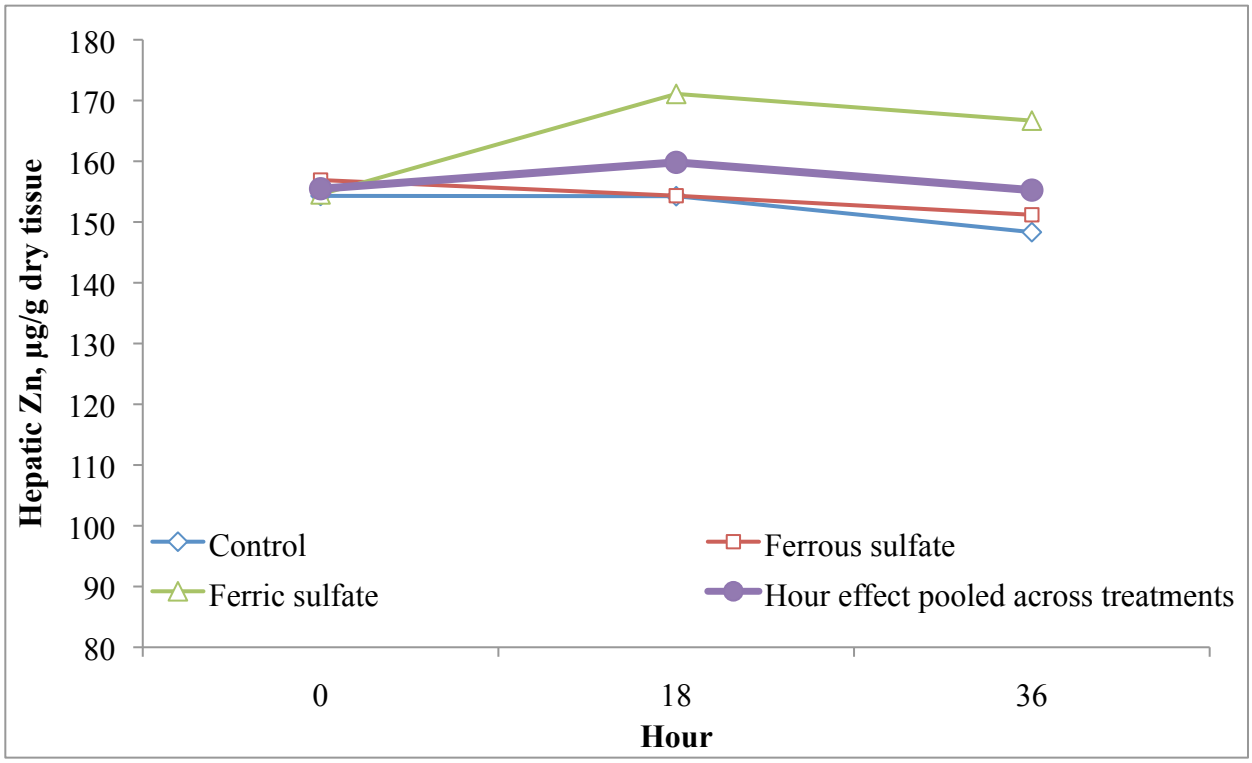
Figure 4.10. Treatment[1] by hour ($P = 0.57$) and pooled hour (SEM[2] = 25.54; $P = 0.86$) responses of hepatic Cu concentration in Experiment 2



[1]Treatments: Control = no Fe; Ferrous sulfate = 1.5 mg Fe/kg BW from ferrous sulfate [$FeSO_4$]; Ferric sulfate = 1.5 mg Fe/kg BW from ferric sulfate [$Fe_2(SO_4)_3$].

[2]SEM = standard error of the mean.

Figure 4.11. Treatment[1] by hour ($P = 0.57$) and pooled hour (SEM[2] = 9.50; $P = 0.38$) responses of hepatic Zn concentration in Experiment 2



[1]Treatments: Control = no Fe; Ferrous sulfate = 1.5 mg Fe/kg BW from ferrous sulfate [$FeSO_4$]; Ferric sulfate = 1.5 mg Fe/kg BW from ferric sulfate [$Fe_2(SO_4)_3$].

[2]SEM = standard error of the mean.

APPENDIX E

DEVELOPMENT OF STUDY PROTOCOL TO MEASURE DRINKING WATER PREFERENCE AMONG LACTATING DAIRY COWS

DEVELOPMENT OF STUDY PROTOCOL TO MEASURE DRINKING WATER PREFERENCE AMONG LACTATING DAIRY COWS

ABSTRACT

This work was undertaken to develop methods for subsequent drinking water preference experiments to evaluate the preferences of dairy cows for various drinking water treatments with different concentrations and sources of Fe. The objective was to develop a procedure and temporal sequence as a study protocol to measure water intake and animal behavior among lactating dairy cows given two treatment choices simultaneously. This experiment was used to determine the optimal time to withhold drinking water before offering different water treatments to determine preference, and the length of time to measure water intake and animal behavior when treatments are offered. In protocol development drinking water with a high concentration of NaCl (2%), generally disliked by cows, and Control water from the on-site tap source were employed to create definitive preference choice. Cows were withheld from water access for 2 h during afternoon milking and treatment preparation. They returned from the parlor to both water treatments, and the experimental period began. Both treatments were offered to each cow each period, for three periods. Periods were designed originally to last for 12 h but were extended to 22 h. Water intakes were measured every 2 h for 12 h and then at 17 and 22 h, and drinking behavior was recorded for the initial 12 h of each period. Cows initially preferred NaCl water to tap water, but approximately 8 h into the first period, the control water was preferred, and continued to be preferred through the remainder of the experiment. Twenty-two hours was adequate for cows to establish preference for a water treatment. Cows had adequate thirst drive to establish preference through water intake, and cows tested both treatments. A method was developed to observe and manually record cow drinking behavior that allowed us to successfully

identify differences in behavior as affected by treatment. Cows spent more time drinking from Control water than 2% NaCl, and more time lapping from NaCl than Control.

INTRODUCTION

Researchers have used two-choice preference testing in experiments with cattle and other species (Goatcher and Church, 1970a, b; Coppock et al., 1974). In general, two-choice preference testing is used to determine differences in palatability of feed ingredients and supplements. However, Goatcher and Church (1970a, b) completed a series of studies to determine the taste responses of ruminants to various stimuli using water as a carrier. They evaluated flavors including sucrose, NaCl, and acids. These experiments determined the upper and lower discrimination thresholds, and preference and rejection thresholds for different tastes using weaned Holstein heifer calves and other ruminants. The researchers were not concerned about the intake response of animals to individual flavors in water, but to the flavors themselves. They also offered tastes that are not usually found in water.

Additionally, Goatcher and Church (1970b) utilized weaned heifer calves in their experiments that require much less water as a function of bodyweight on a daily basis compared to high-producing lactating dairy cows. Although the researchers were successful in evaluating taste responses in their experiments, we wanted to tailor our experiments more directly to our objectives and experimental animal. In order to properly test our hypotheses in future experiments, we wanted to ensure that the length of experimental periods and water withholding period would allow us to successfully determine differences in total water intake between treatments for lactating dairy cows.

Therefore, the aim of this work was to determine the optimal experimental period length and water withholding period, and to develop a protocol for measuring drinking behavior and evaluate cow preference for different water treatments.

MATERIALS AND METHODS

The Institutional Animal Care and Use Committee at Michigan State University approved all experimental procedures (AUF # 03/10-024-00).

Animals, Experimental Design, and Treatments.

Four mid-lactation Holstein cows (134 ± 22 DIM) were assigned in a completely randomized design. Experimental treatments were: drinking water from the on-site tap source (Control; Table 5.3), or water from the same on-site source containing 2% NaCl (NaCl). Treatments were offered as a pair. Each cow was offered both treatments in each of three experimental periods, and pairs were balanced for left-right effect.

Water Intake Measurement and Behavior Assessment

The day that treatment water intake measurement began, water containers were removed approximately 2 h before the experimental period commenced. Containers were scrubbed with detergent, rinsed, and filled with treatments according to treatment assignments. Water containers were then placed on the left and right edges of the bunks. Cows were removed from the tie-stall and taken to the milking parlor approximately 1 h after water containers were removed. Therefore, cows were without drinking water for 2 h before water treatments were offered. During their absence, the remaining 50% of the ration, withheld during morning feeding, was added to the remaining feed in the bunks. Cows returned to their tie-stalls simultaneously, immediately after milking, to individual access to feed and two water containers. At this time (1600 h) the period of water intake measurement and manual recording of cow drinking behavior began.

Every 2 h containers were removed from all feed bunks at the same time, and behavior recording was paused. Each container was skimmed with a pool skimmer to remove as much feed as possible that was dropped into the containers by cows during the experimental period. Containers were weighed and the weight was recorded. If the weight was less than approximately 45 kg, the container was refilled in increments of 5 or 10 L, until the weight reached greater than that number. Containers were then replaced in each feed bunk at the same time and the behavior recording recommenced. Behavior recording ended 12 h after the experimental period began. Containers were weighed, refilled and replaced in the bunks.

Initially, some cows pushed containers from the bunk in order to access feed. If containers were pushed more than 20 cm from the bunk, they were pushed back into the bunk within cow's reach. If the cow routinely pushed the containers away, the containers were banded to the bunks with rubber straps. Straps did not interfere with drinking. If the containers contained less than approximately 10 L of water before the scheduled refilling time, containers were removed from all bunks, and the containers were refilled so that containers none were ever empty and cows were always able to drink from either container.

Containers were left in the bunks for cow's to access for 22 h. They were checked at 0900 h, weighed and refilled. Containers were removed and weighed at the end of the experimental period (1400 h).

Behavior Assessment. Drinking behaviors were recorded for the initial 12 h of the experimental period and categorized for analyses. Categories were lapping, drinking, and 'other'. Drinking was defined as a cow fully or partially submerging her muzzle in water to consume water. Lapping was defined as a cow consuming water without submerging her muzzle. The 'other' category included all behaviors associated with containers when cows were not actively

consuming water. Non-consumption behavior interaction examples included splashing, sniffing and pushing containers away. Frequency of interaction with water containers also was evaluated. Behavior measurements were recorded to the nearest second. The beginning of one behavior signified the end of the previous behavior, so only beginning time of behavior was recorded. If a behavior was associated with a water container, the designated treatment identifier was recorded. Other common behaviors recorded included eating, ruminating, urinating, defecating, standing up, lying down, resting, and standing.

Preparation of Treatments

The 2% sodium chloride treatment was prepared by adding 2 g of sodium chloride to 100 ml of water. Sodium chloride was pre-measured into containers that held 200 g of NaCl each, and were added to 10 L of water and mixed until NaCl dissolved into solution and then added to the water container. These 10 L aliquots were mixed until the water container was filled. Containers held approximately 50 L.

Water containers

Water was offered in two, 75 L capacity white Rubbermaid Brute™ (Rubbermaid Commercial Products, Winchester, VA) storage tote containers (W: 38.4 cm, x H: 44.1 cm x L: 70.8 cm) placed in each the left and right sides of individual cow's feed bunks with feed situated between the containers. There was a vacant tie-stall between cows to avoid cow social interactions that potentially might affect water intake.

Training

Cows were blocked from access to water in the milking parlor lane, so the only source of water was the source provided in the tie-stall throughout the pre-experimental and experimental periods. Nine d prior to the beginning of each experiment, cows were offered water from the on-site tap water source in the experimental water containers for 48 h to ensure that all cows became accustomed to the containers. Containers were removed from the bunks, weighed and refilled every 6 h during training. Every 24 h containers were removed, emptied, scrubbed with detergent, rinsed well and refilled. There were no issues with cows refusing to drink from containers. Following the initial training period cows were placed in tie-stalls with individual automatic water cups, as in normal settings, and water intake was measured with in-line water flow meters for 5 d to establish baseline water intake. Forty-eight h immediately prior to actual data collection cows were moved to stalls without automatic water cups, and water was accessible only in the experimental water containers to re-accustom the cows to the containers and establish baseline intake from water containers. All cows were exposed to water containers for the same length of time during pre-experimental acclimation periods and always located with an unoccupied stall on each side.

Common Feeding, Water and Milk Data Collection

A basal diet formulated to meet or exceed NRC (2001) recommendations for all nutrients was fed *ad libitum* throughout each experiment (Table 5.2). Cows were fed once daily in their individual tie-stalls, and milked twice daily. Milk yield and water and feed intake were recorded daily for the entire experimental period, including adjustment periods. Pre-experimental feed intake, water intake, and milk yield and composition are presented in Table 5.1 for characterization of experimental animals.

Feed and water intakes (in-line water flow meters) were measured daily throughout the experiment. Individual feed ingredients were sampled the week before each collection period began, and again between experimental periods. Each individual feed sample was weighed and dried in a forced-air oven at 55°C for 72 h until completely dry to determine DM percentage. Dried samples were ground through a 2 mm screen in a Wiley mill (Arthur H. Thomas, Philadelphia, PA). Similar feed ingredient samples were pooled by experiment and sent for analysis by Dairy One Laboratories (Ithaca, NY). Milk samples were taken from one AM and PM milking and sent for composition analysis at Michigan DHIA Universal Laboratory Services (Lansing, MI). Control water was sampled daily through the experiment and pooled into one composite sample. Raw water sample was sent to Midwest Laboratories (Omaha, NE) for Direct metals water analysis (EPA 200.7 method (1994)). A second sample was acidified (pH < 2.0) with 1 mL nitric acid, and sent to the same laboratory for acid digestion and metals analyses (Total recoverable metals analysis using EPA 200.7 method (1994)). Water analyses are presented in Table 5.3. Internal water reference samples containing either 0, 1, 4 and 8 mg of Fe/L from ferrous lactate were placed as unknowns within the set of samples in triplicate for analysis. The intra-assay %CV was 4.0%.

Statistical Analyses.

Data were analyzed by method of least-squares ANOVA using the Mixed Models procedure of SAS (Version 9.2, SAS Institute Inc.). Data were analyzed as completely randomized design. Differences in water intake were compared between treatments. Data of

hourly measurements were analyzed using the REPEATED statement, and the covariance structure that resulted in the lowest Bayesian information criterion (**BIC**) was used. Fixed effects in the model included treatment, hour, and treatment by hour interaction. Random effects included cow and period. If the interaction term was non-significant ($P > 0.15$), intake was analyzed separately by hour. Residual distributions were tested for normality using the Shapiro-Wilk test, and outliers were identified using Cook's D statistic. During evaluation of the full model, including interaction terms, intake data were transformed by taking the square root to accommodate the assumptions of normality. During analyses of individual hour water intake responses and total behavior responses, if required, data also were transformed to square root, $\log_{10}$ or natural log if required to accommodate the assumptions of normality. Results were back transformed for presentation. Differences between treatments were identified using the PDIFF option with Tukey's adjustment. Least-squares means are presented. Significance for main effects was declared at $P < 0.05$, and a trend toward significance at $P < 0.10$. Significance for interaction effects was declared at $P < 0.10$, and a trend toward significance at $P < 0.15$.

RESULTS

There was a treatment by hour interaction on water intake (P <0.0001). This effect was heavily influenced by intake responses in period 1, as cows greatly preferred NaCl treatment water initially (Figure 5.1). When all periods were included in analyses, there was no difference between Control and NaCl treatments after 2 h (P = 0.70). From h 4 through the end of the experimental period, water intake of the Control treatment was greater than NaCl (P < 0.001; Table 5.4). The drinking duration and drinking frequency of cows consuming Control water was greater than cows consuming the NaCl treatment (P = 0.0006; P = 0.0007; Table 5.5). Cows spent more total time lapping (P < 0.0001) and lapped more frequently (P < 0.0001) from the NaCl treatment water than the Control water. Total duration (P = 0.91) and frequency of behavioral interactions (P = 0.85) with containers, but not consuming water, was not different between treatments.

DISCUSSION

We were initially concerned that cows would not choose between water treatments, but instead immediately drink from the first container they encountered and not test both treatments. For this reason we decided to offer cows treatments that had drastically different tastes; tap water and 2% NaCl water. Additionally, cows respond to NaCl in water (Goatcher and Church, 1970b).

Cows initially preferred the NaCl treatment to the Control treatment in the first period (Figure 1). After 15 min, cows already had consumed so much NaCl that the experimental period had to be prematurely paused so that water containers could be refilled. This was unexpected as Goatcher and Church (1970b) noted that cattle offered pairs of treatments with varying concentrations of NaCl (0.2% to 1.25%) rejected water with NaCl concentrations greater than 0.32%. After the first 2 h of the initial period, cows exhibited the laxative effect characteristic of NaCl toxicity (Jaster et al., 1978). They also began drinking large amounts of the Control water. When all periods were included in the analyses water intakes at 2 h were not different between treatments (Table 5.4). By h 6 Control water intake was greater than intake of NaCl treatment ($P = 0.0012$). Intake of the Control treatment remained greater through the end of the experimental period ($P < 0.0001$).

For this reason, we decided that experimental periods should be extended beyond the 12 h period initially planned. Cow preference did not change beyond h 6. It also was not altered in the two additional periods (Figure 2). We determined that experimental periods should be 22 h in length, and 1 period (the initial period) was enough for cows to make a choice between two treatments.

Cardot et al. (2008) determined that water intake was least during the evening hours. This was a concern, as the initial 12 h of the experimental period took place from 1600 – 0400 h. We were unsure if afternoon milking and a second feeding would be enough to induce water consumption and allow us to find differences in treatment water intake. This was before we decided to extend the experimental period beyond 12 h. The 2-h period necessary for preparation of water containers and water treatments, and in combination with milking, was apparently a long enough period without water intake to induce drinking.

We developed a procedure to manually measure drinking behavior. One of the difficulties of recording data manually was that multiple behaviors took place within a short period of time, which made recording accurate data difficult, if both a beginning and end time had to be recorded. Recording continuously shortened the information that needed to be recorded. We realized that it was easier and lead to more consistent data recording among observers to record all behavior. Cows spent more time drinking Control water than NaCl water, and more time lapping from the NaCl treatment water than Control water (Table 5.5). We were able to detect treatment differences for drinking behavior duration, and frequencies as expected.

CONCLUSIONS

We determined that 22 h was adequate for cows to establish preference for a water treatment. The 2-h period required to prepare water container and treatments in combination with milking was long enough to drive thirst in concert with milking and feeding. Cows had adequate thirst drive to establish preference through water intake, and cows tested both treatments. Behavior was recorded manually continuously. Drinking behaviors were categorized for analysis and differences between treatments for lapping and drinking durations and frequencies were detected successfully.

TABLES

Table 5.1. Feed dry matter intake, milk yield, and water intake of cows during the pre-experimental period

	Mean	±SD[1]
DMI, kg DM	23	1.3
Water intake, L	114.9	9.93
Milk yield, kg	52.8	6.72

[1]SD = standard deviation.

Table 5.2. Ingredient and nutrient composition of diets fed

Ingredient	----------Percent of dietary DM----------
Chopped alfalfa hay	3.72
Corn silage	35.32
Whole cottonseed	5.95
Energy booster	0.40
Grass hay	5.02
Ground corn	9.53
Alfalfa haylage	9.51
High moisture corn	7.14
Soybean meal, 48% CP	14.72
Soyhulls	5.35
Mineral-vitamin mix[1]	3.34
Nutrient Composition	
DM, %	49.0
	----------Percent of dietary DM----------
Neutral detergent fiber	33.9
Acid detergent fiber	22.4
Crude protein	17.58
Ca	1.01
P	0.37
K	1.38
Na	0.34
S	0.24
Mg	0.25
	----------mg/kg----------
Co	0.17
Cu	10.2
Fe	253
Mo	2.3
Mn	44
Zn	41

[1]Mineral-vitamin mix contained 47.5% limestone, 22.5% sodium bicarbonate, 10.1% urea (45%N), 8.2% magnesium sulfate, 7.5% sodium chloride, 1.6% biotin premix (1.4 g/kg), 1.3% trace mineral premix, 30 KIU/kg vitamin A, 8 KIU/kg vitamin D, 56 KIU/kg vitamin E and 0.3% Se-yeast.

Table 5.3. Water quality constituent analyses of Control drinking water

	Control	Caution level[1]
Quality Constituent	----------mg/L----------	
TDS[2]	460	1,000
Ca	114	150
Cl	24	500
Cu	ND[3]	0.3
Fe (direct)[4]	0.05	0.3
Fe (total recoverable)[5]	0.28	-
Mg	35.3	80
Mn[5]	0.22	-
NO_3-N	ND	25
Na	9.3	150
SO_4	97	300
Zn[5]	0.31	-
Conductivity, mmhos/cm	0.71	1.5
pH	7.7	6.5-9

[1]Caution levels from Midwest Laboratories (Omaha, NE)

[2]TDS = total dissolved solids

[3]ND = not detected

[4]Direct metals analysis of raw (without acid digestion) water by Livestock Water Analysis using EPA 200.7 method (1994) by Midwest Laboratories (Omaha, NE).

[5]Total recoverable metals analysis of acidified water after acid digestion using EPA 200.7 method (1994) by Midwest Laboratories (Omaha, NE).

Table 5.4. Cumulative water intake (L) of cows pooled for all 3 periods

	Treatments[1]			
	Control	NaCl	SEM[2]	*P*-value
Hour				
2[3]	6.8	5.3	1.79	0.70
6	52.3	26.9	5.07	0.0012
12	85.4	35.0	5.69	<0.0001
17	102.1	39.2	7.55	<0.0001
22	136.6	45.0	9.75	<0.0001

[1]Treatments: Control = tap water; NaCl = 2% sodium chloride.

[2]SEM = standard error of the mean.

[3]Hour 2 data were natural log transformed for analysis, and LS means and SEM were back-transformed for presentation in this table.

Table 5.5. Cumulative drinking behavior durations and frequencies of cows

	Treatments[1]			
	Control	NaCl	SEM[2]	*P*-value
Behavior Duration (s)				
Drinking[3]	693	202	85.0	0.0006
Lapping	295	1292	142.2	<0.0001
Other[4,5]	3	3	1.1	0.91
Behavior Frequency				
Drinking	18.4	6.2	3.20	0.0007
Lapping	10.6	28.1	4.48	<0.0001
Other[4]	5.8	5.3	1.90	0.85

[1]Treatments: Control = tap water; NaCl = 2% sodium chloride.

[2]SEM = standard error of the mean.

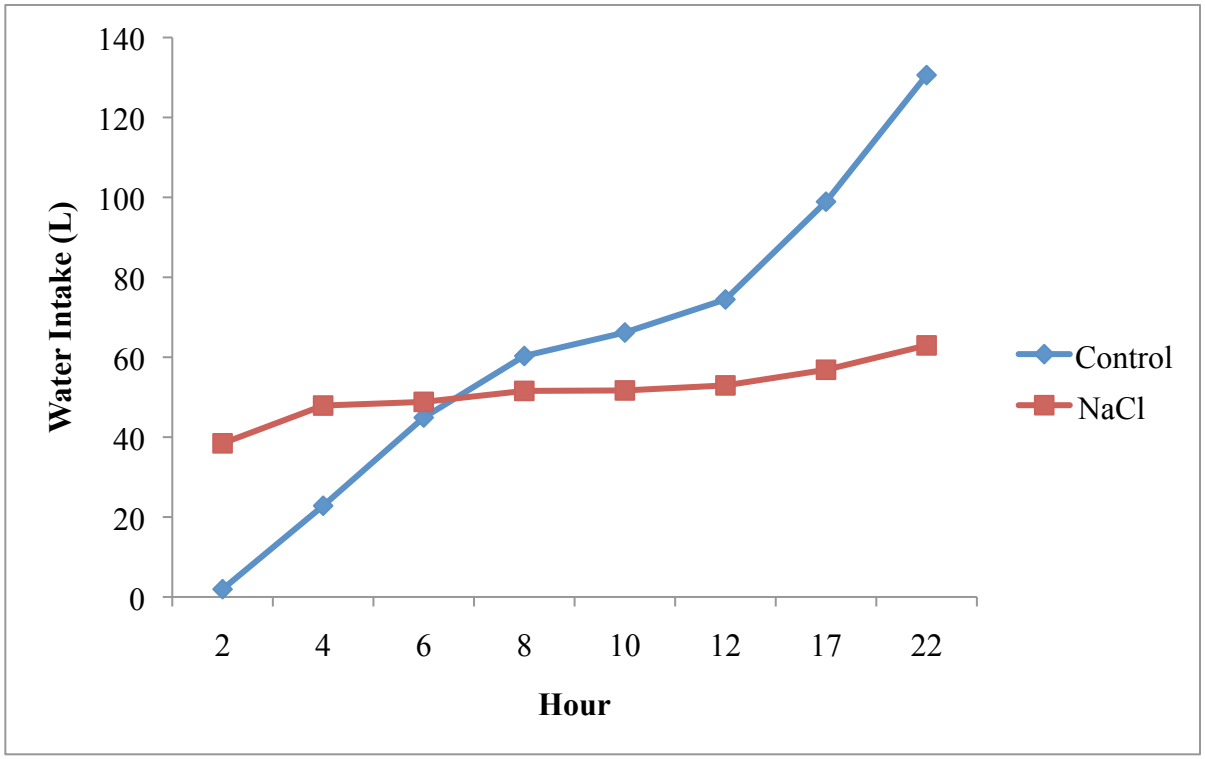
[3]Data were $\log_{10}$ transformed for analysis and LS means and SEM were back-transformed for presentation in this table.

[4]Other = interacting with water container but not consuming water.

[5]Data were square root transformed for analysis and LS means and SEM were back-transformed for presentation in this table.

FIGURES

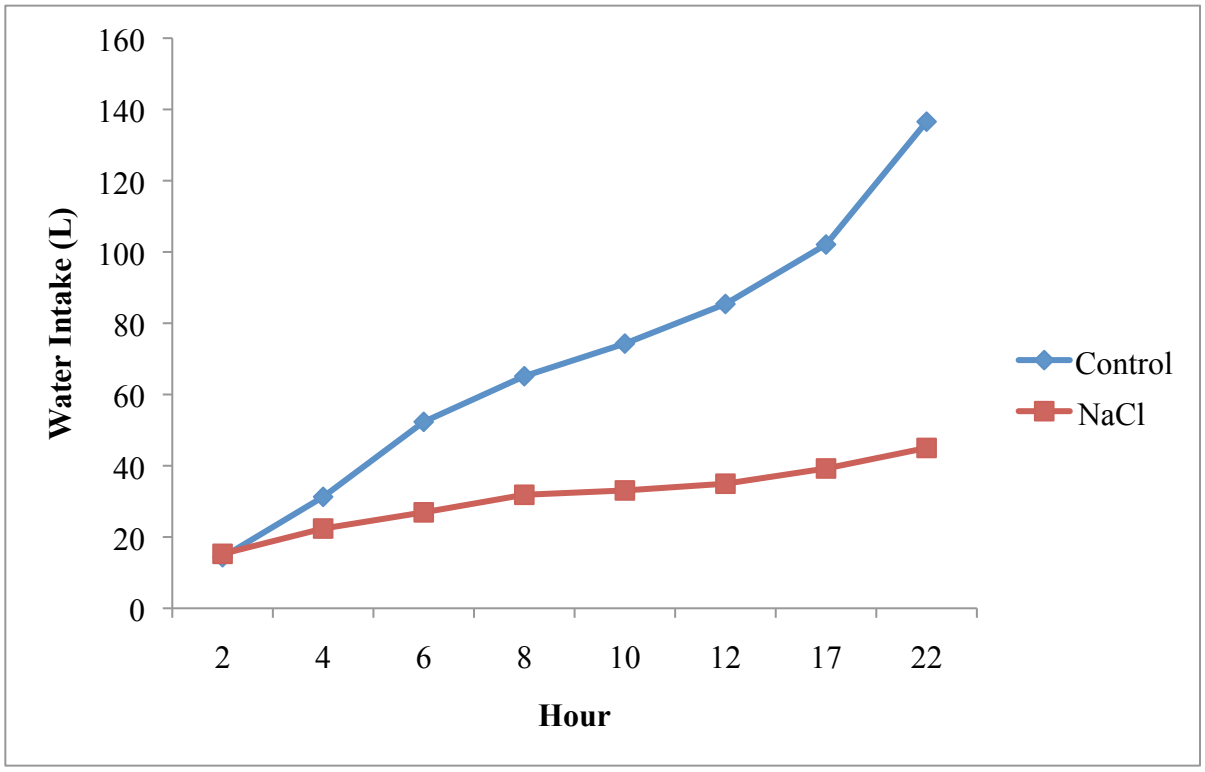
Figure 5.1. Treatment[1] by hour (SEM[2] = 8.03; $P < 0.0001$) response of water intake in period 1



[1]Treatments: Control = tap water; NaCl = 2% sodium chloride.

[2]SEM = standard error of the mean.

Figure 5.2. Treatment[1] by hour (SEM[2] = 6.68; $P < 0.0001$) response of water intake across all periods



[1]Treatments: Control = tap water; NaCl = 2% sodium chloride.

[2]SEM = standard error of the mean.

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