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# Diet-induced obesity decreases liver iron stores in mice fed iron deficient, adequate, or excessive diets

Brett J. Healy  
*Utah State University*

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DIET-INDUCED OBESITY DECREASES LIVER IRON STORES IN MICE FED

IRON DEFICIENT, ADEQUATE, OR EXCESSIVE DIETS

by

Brett J. Healy

A thesis submitted in partial fulfillment  
of the requirements for the degree

of

MASTER OF SCIENCE

in

Nutrition and Food Sciences

Approved:

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Korry J. Hintze  
Major Professor

---

Michael Lefevre  
Committee Member

---

Robert E. Ward  
Committee Member

---

Mark R. McLellan  
Vice President for Research and  
Dean of the School of Graduate Studies

UTAH STATE UNIVERSITY  
Logan, Utah

2013

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## ABSTRACT

Diet-induced Obesity Decreases Liver Iron Stores in Mice Fed Iron Deficient,  
Adequate, or Excessive Diets

by

Brett Healy, Master of Science  
Utah State University, 2013

Major Professor: Dr. Korry J. Hintze  
Department: Nutrition, Dietetics and Food Sciences

Epidemiological and observational evidence suggests that obesity is related to poor Fe status. To determine interactions between obesity, dietary Fe intake and Fe status; male, weanling C57BL/6J mice were fed either high fat diets to induce obesity or a standard diet for 16 weeks. Fe concentrations of both the high fat or control diet (4.5 vs 3.8 kcal/g) were set at: 5, 50 or 500 mg Fe/kg diet. Mice fed the high fat diets had significantly higher percentage body fat (17.9%) compared to mice fed control diets (5.3%,  $P < 0.001$ ). Among obese mice, dietary Fe levels did not significantly influence body composition. Conversely among lean mice, mice fed the iron excessive diet had significantly less fat mass when compared to mice fed the iron deficient diet ( $P < 0.05$ ). Obesity and/or dietary Fe concentration did not significantly affect plasma Fe levels. ANOVA analysis showed significant effects of diet-induced obesity, dietary Fe and an interaction between both factors on liver Fe levels ( $P < 0.05$ ).

Obese mice had significantly lowered liver Fe levels compared to lean cohorts fed the same amount of dietary Fe ( $P < 0.05$  for all comparisons). Moreover, lean mice fed the Fe deficient diet (5 mg Fe/kg diet) had similar liver Fe levels (127 mg Fe/kg  $\pm$  0.04) compared to obese mice fed the 50 mg Fe/kg diet (132 mg Fe/kg  $\pm$  0.05). These data suggest that obesity, independent of dietary Fe intake, influences liver Fe stores.

(31 pages)

## PUBLIC ABSTRACT

Evidence from numerous studies indicates a relationship between obesity and low iron deficiency. To determine interactions between obesity, dietary iron intake and iron status; mice were fed either high fat diets to induce obesity or a standard diet for 16 weeks. Iron concentrations of both diets were set at 5, 50 or 500 mg iron/kg diet. Mice fed the high fat diets had a higher percentage of body fat when compared to mice fed standard diets. Among obese mice, dietary iron levels did not significantly influence body composition. Conversely among lean mice, mice fed the iron excessive diet had significantly less fat mass when compared to mice fed the iron deficient diet. Neither obesity nor iron concentration significantly affected plasma iron levels. Statistical analysis showed significant effects of diet-induced obesity, dietary iron and an interaction between both factors on liver iron levels. Obese mice had significant lower liver iron levels compared to lean cohorts fed the same amount of dietary iron. Further, lean mice fed the iron deficient diet had similar liver iron levels compared to obese mice fed the iron-sufficient diet. The study suggests that obesity, independent of dietary iron intake, influences liver iron stores.

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Brett Healy

## CONTENTS

	Page
ABSTRACT.....	iii
PUBLIC ABSTRACT.....	v
ACKNOWLEDGMENTS .....	vi
LIST OF TABLES .....	viii
LIST OF FIGURES .....	ix
CHAPTER	
I. INTRODUCTION .....	1
II. MATERIALS AND METHODS.....	12
III. RESULTS AND DISCUSSION .....	17
IV. SUMMARY AND CONCLUSIONS .....	24
REFERENCES .....	26



## LIST OF TABLES

Table	Page
1 Six diets differing in total fat and iron content .....	13

## LIST OF FIGURES

Figure		Page
1	Effect of diet on food intake, weight gain, and fat mass .....	15
2	Effect of diet on liver iron status, liver copper status, and liver zinc status .....	18
3	Effect of diet on whole blood iron status .....	19
4	Effect of diet on relative liver hepcidin expression .....	19
5	Overall correlation between liver iron status and relative liver hepcidin expression .....	20
6	Effect of diet on relative liver IL-6 expression, relative adipocyte hepcidin expression, and relative adipocyte IL-6 expression .....	21

# CHAPTER I

## INTRODUCTION

### **Iron Absorption, Transport, and Storage**

Iron is an absolute requirement for life. It is involved in many important biochemical reactions ranging from cellular respiration to DNA synthesis. A healthy human body contains between 2 and 4 grams of iron with over 65% found in hemoglobin. Dietary iron is available in two forms, heme and nonheme. Heme iron is typically found in animal products while nonheme iron is found in plant products (1). Heme iron is absorbed at the brush border membrane of small intestinal cells by heme carrier protein 1 (hcp1) (1). Once inside the cell, iron is liberated from heme by heme-oxygenase. Once released from food components, nonheme iron is found as either ferric ( $\text{Fe}^{3+}$ ) or ferrous ( $\text{Fe}^{2+}$ ) iron. Ferrous iron is absorbed across the brush border membrane by the divalent metal transporter 1 (DMT1) while ferric iron is reduced by duodenal cytochrome b reductase then transported by DMT1 (2).

Iron absorption is regulated by the protein hepcidin, which is released when iron stores are adequate or high (3). Hepcidin works by binding and degrading the protein ferroportin (3). Ferroportin is a basolateral membrane protein found in the intestine responsible for the transport of iron from the intestine into circulation (2). Thus, with the loss of ferroportin iron can no longer be transported into circulation from intestinal cells. Once absorbed, iron may either be transported out of the intestinal cells and into the bloodstream for use in body tissues, stored for future use, or used by the intestinal cell (4). Cellular iron

is stored primarily inside the protein ferritin, which is typically produced in liver, spleen, bone marrow, and intestinal cells (4). Ferritin has a sphere-like structure and may store thousands of iron atoms (4). Iron is stored in ferritin to prevent cellular damage and limit availability to bacteria (5). Unbound iron can lead to production of harmful free radicals that are very reactive and incur damage to cells (4). The main storage site for iron in the body is the liver, though a significant amount is also stored in the bone marrow and spleen. Once transported to these tissues by transferrin, iron is stored bound to ferritin (2).

### **Iron Deficiency and Toxicity**

Although necessary for sustaining life, iron's chemical properties can also catalyze the formation of reactive oxygen species, which are hypothesized to be involved in the etiology of most common chronic diseases. Diseases attributable to iron toxicity are among the most common worldwide. With over 300,000 born each year with severe hemoglobin disorders as estimated by the World Health Organization (WHO) (*WHO Sickle-Cell Disease and Other Hemoglobin Disorders Fact Sheet*), sickle-cell anemia and thalassemia are among the most common genetically-inherited diseases. Treatment of thalassemia with regular blood transfusions leads to severe iron overload and toxicity. Hemochromatosis, a disease characterized by severe over-absorption of iron potentially leading to organ failure, is also one of the most commonly inherited diseases, affecting approximately 50 out of every 10,000 Americans. Unlike other essential metals, there is not a specific excretion mechanism. Therefore, iron can accumulate in tissues and result in toxicity.

Conversely, affecting approximately 2 billion people worldwide, iron deficiency is the most common mineral deficiency and affects not only the health of individuals, but may also decrease work capacity in populations having major effects on the economy and country development (*WHO Nutrition Deficiencies Fact Sheet*). The WHO also estimates that 50% of pregnant women and 40% of preschool children are suffering from iron deficiency (*WHO Nutrition Deficiencies Fact Sheet*). Furthermore, studies have shown a link between iron deficiency and slow cognitive development (6-10). For example, a review conducted by McCann and Ames (7) showed that although test performance was poor in iron deficient children over the age of two test performance improved significantly after iron treatment. Coupled together, the potential social and economic impact of iron deficiency is great and stresses the need to understand the mechanisms of iron metabolism and absorption from the diet.

### **Obesity**

In developed countries obesity has quickly become an epidemic and for the first time in human history over-nutrition is a larger problem than under-nutrition. WHO estimated that in 2008 more than 1.4 billion adults were overweight and at least 500 million adults were obese. WHO projects that by 2015, 2.3 billion adults will be overweight and 700 million will be obese. In addition, WHO estimates that overweight and obesity are the fifth leading risk for death worldwide with 44% of diabetes, 23% of ischemic heart disease and at least 7% of cancer being attributable to overweight and obesity (*WHO Obesity and Overweight Fact Sheet, May 2012*).

## Iron Status and Obesity

Obesity has long been known to be associated with poor iron status. Studies conducted as early as 1962 and 1963 showed obesity's association with low iron status in adolescents (11, 12). A study conducted in 2004 by Nead et al. (13) demonstrated the association of obesity and poor iron status. Iron status was defined by three factors: transferrin saturation, free erythrocyte protoporphyrin levels and serum ferritin levels. The adolescent was considered iron deficient if two of the three values were abnormal for age and gender. Overweight adolescents were defined as being in the 95<sup>th</sup> percentile and above, at risk adolescents were between the 85<sup>th</sup> and 94<sup>th</sup> percentile and normal adolescents were below the 85<sup>th</sup> percentile for age and gender. The results of this study showed increasing prevalence of iron deficiency as weight status increased. Further, LeCube et al. (14) conducted a study exploring iron deficiency in obese postmenopausal women. Obesity in this study was defined as having a BMI of at least 30. Menopausal status was determined by self-report of discontinuation of menstruation or hysterectomy. Iron status was determined by measuring soluble transferrin receptor (sTfR) in the blood where high concentrations of sTfR indicate low iron concentrations. Results of this study showed that as BMI increased sTfR also increased, indicating poor iron status in obese post-menopausal women. Other studies have shown this association in children and adolescents (15, 16) and adult men and women (17-20) and is an independent factor contributing to poor iron status (14, 16, 17, 19). The association shown in postmenopausal women and adult men is particularly compelling

because these are two populations not typically at risk for low iron status. Obesity is increasingly being recognized as an inflammatory condition and adipose tissue as an endocrine organ. Obesity is characterized by adipocyte hypoxia resulting in the increased expression of hormones like interleukin-6 (IL-6), TNF- $\alpha$  and leptin in adipose tissue, which are then secreted into the bloodstream (21). Due to the nature of obesity and the growing epidemics that are obesity and low iron status, it is crucial to understand the mechanisms underlying obesity-induced iron deficiency.

### **Hepcidin**

Hepcidin is often referred to as the master regulator of iron homeostasis and its discovery is the most significant recent finding for our understanding of dietary iron regulation. In an effort to investigate the effects of disrupting upstream regulatory factor 2 (USF2) on glucose-dependent gene regulation in the liver, researchers Nicolas et al. (22) discovered that USF2 knockout mice developed severe iron overload in the liver and pancreas. Though USF2 plays no role in iron metabolism, hepcidin (a gene adjacent to USF2) expression was unintentionally disrupted and therefore implicated as a causative factor of the iron overload. Since then, hepcidin has been shown to play a key role in regulating organismal iron metabolism. Increases in hepcidin expression have been shown to deplete serum iron levels (23). A study conducted by Rivera et al. (23) showed after an injection of 50  $\mu$ g of synthetic hepcidin serum iron levels in mice dropped more than 400% within one hour after injection and remained depressed for 48 hours. Hepcidin has also been shown to prevent iron efflux from enterocytes and

macrophages (3). Hepcidin targets the cellular iron exporter ferroportin leading to its internalization and degradation, ultimately preventing iron export from hepatocytes, macrophages and enterocytes thus dropping systemic iron levels (3). A study conducted by Yamiji et al further showed hepcidin to decrease expression of the apical iron importer DMT1 (24). In the study, Caco-2 human intestinal cells were exposed to hepcidin for 24 hours, and iron transport proteins were measured (24). DMT1 mRNA decreased while ferroportin mRNA went unchanged, showing that hepcidin also plays a role in luminal iron absorption from the diet (24). It appears that hepcidin is the key signal for decreasing plasma iron as a host defense mechanism by limiting iron for pathogens as well as limiting iron efflux from enterocytes in response to dietary iron overload.

Hepcidin is synthesized in the liver and gene expression is increased by iron overload and inflammation. To determine the effects of dietary iron on hepcidin expression, Nemeth et al. (25) conducted a study in which participants collected their first morning urine over the course of 9 days. The morning of days 3, 4, and 5 each participant consumed a large 65 mg dose of iron (recommended intake for males is 9 mg/day and for women 18 mg/day). The study showed a dramatic increase in hepcidin expression 24 hours after the first dose of iron. Hepcidin expression has also been shown to decrease under conditions of hypoxia and anemia (3). Accordingly, hepcidin is the iron/inflammation/oxygen sensor that signals for decreased dietary iron uptake after iron overload, decreased plasma iron after inflammation and/or infection, and increased iron uptake during hypoxia.



A study conducted by Wrighting et al. found that IL-6 appears to play a key role in increasing hepcidin expression in response to inflammation (23). HepG2 cells were treated with cycloheximide, a protein translation inhibitor, in the presence or absence of IL-6 to determine if IL-6 would directly induce hepcidin transcription (26). Hepcidin expression was 5.7-fold higher in cells treated with IL-6 compared to the untreated control cells. Cycloheximide-free cells showed a 3.5-fold difference in hepcidin expression in cells treated with IL-6 compared to cells not treated with IL-6. The results of the study showed that IL-6 directly induces hepcidin transcription (26). Further, in a study conducted by Nemeth et al. (25) hepatocytes were treated for 24 hours with 100 ng/ml lipopolysaccharide (LPS) to induce an inflammatory response or 100 ng/ml LPS and IL-6 antibodies. Hepcidin expression increased 12-fold in the LPS treated cells while expression fell below baseline levels when cells were treated with the IL-6 antibody. IL-6 mediates hepcidin induction by increasing the binding of the transcription factor STAT3 to hepcidin promoter (26,27). This appears to explain how hepcidin may be signaled by inflammation and infection.

### **Obesity, Inflammation, and Adipocyte Hypoxia**

Chronic inflammation is one of the hallmarks of obesity and has been implicated in many obesity-related problems like insulin resistance. The inflammatory cytokines TNF- $\alpha$  and IL-6 are known to reduce insulin sensitivity. Kahn and Flier (28) showed that chronic secretion of these cytokines is thought to contribute to the development of type II diabetes. A study conducted by Hotamisligil et al. (21) demonstrated that TNF- $\alpha$  mRNA was expressed 2.5-fold

more in obese premenopausal women when compared to lean controls. They also observed a positive correlation between TNF- $\alpha$  mRNA expression levels in fat tissue and the level of hyperinsulemia, an indirect measure of insulin resistance (21). Furthermore, weight reduction in these subjects improved insulin sensitivity and exhibited decreased TNF- $\alpha$  mRNA in fat tissue (21). Harkins et al. showed that obese mice (ob/ob line) have significantly higher plasma IL-6 levels than their lean counterparts (29). Since IL-6 expression in the liver and spleen were unchanged between obese and control, the difference was attributed to increased IL-6 expression in adipose tissue (29). These data suggest that adipose tissue itself plays an important role in determining plasma TNF- $\alpha$  and IL-6 levels.

Comparing adipose tissue in obese mice with that of lean counterparts, Ye et al. (30) showed that adipose tissue in obese mice is considerably more hypoxic than adipose tissue from lean mice. Hypoxia was measured using three different methods. First, partial pressure of oxygen ( $P_{O_2}$ ) was measured in the adipose tissue using an oxygen meter and probe (30).  $P_{O_2}$  in lean mice was considerably higher, suggesting hypoxia in the adipose tissue (30). Second, a chemical probe was injected into the mice and measured using Western blotting (30). The probe signal was eight times greater in the obese mice, again showing greater hypoxia in adipose tissue from obese mice (30). Third, researchers measured expression of genes known to be induced by hypoxia: hypoxia-inducible factor 1a (HIF-1a), glucose transporter 1 (GLUT-1), heme oxygenase (Hemox), pyruvate dehydrogenase kinase 1 (PDK1) and vascular endothelial

growth factor (VEGF) (30). In the obese mice, adipose tissue had increased expression in HIF-1a, GLUT-1, Hemox, and PDK1 compared to lean cohorts (30). The increases, however, were only shown in adipose tissue (30). Further, hypoxia increased expression of the inflammatory cytokines TNF- $\alpha$ , IL-1 and IL-6. Increased expression of cytokines was also demonstrated in 3T3-L1 cells cultured under hypoxic conditions (1% O<sub>2</sub>) suggesting that this system is an ideal model to study the effects of hypoxia on adipocyte cytokine gene expression. The data from Ye et al. (30) suggests that the phenomenon of obesity-induced inflammation may result from increased secretion of inflammatory cytokines from hypoxic adipocytes found in deep fat pockets.

Another consequence of increased adiposity and adipocyte hypoxia is macrophage infiltration of adipose tissue. Low partial pressure of O<sub>2</sub> in human adipose tissue is correlated to macrophage infiltration (31,32). Several studies have demonstrated that obesity in humans increases markers of adipose macrophage content (32,33). Macrophage infiltration of adipose tissue increases expression of the inflammatory cytokines TNF- $\alpha$  and macrophage inflammatory protein-1 $\alpha$ . Macrophage infiltration of adipose tissue is increasingly being recognized as part of the etiology of central obesity and a chronic inflammatory state.

A study conducted by Hintze et al. (34) explored the effects of adipocyte hypoxia on hepatocyte hepcidin expression. Adipocytes were cultured for 24 hours under hypoxic conditions (1% O<sub>2</sub>) or normal culture conditions (19% O<sub>2</sub>) and half of each were treated with LPS. Media from the adipocytes was then

transferred and used to treat hepatocytes. Hypoxic adipocytes had significantly higher IL-6 expression, similar to in-vivo findings. Results from this study showed significantly increased hepcidin expression in hepatocytes treated with either hypoxic or LPS media. Further, the STAT3 binding site, which is known to mediate hepcidin response to IL-6 was mutated and hepcidin promoter activity was tested in reporter constructs. When the STAT3 site was mutated, hepcidin promoter activation was significantly lower than in the non-mutated constructs when treated with conditioned media from hypoxic adipocytes. The data from this study suggests adipocyte hypoxia may increase hepcidin expression via IL-6 and the STAT3 promoter element and likely plays a role in obesity and poor iron status.

## **Conclusion**

The necessity for iron to sustain life cannot be understated. However, the very properties that make iron such an important building block of life also contribute to some of the most common chronic diseases plaguing society today. The growing obesity epidemic is likely contributing to growing numbers of cases of poor iron status and the literature has long shown this association. Adipose tissue is considered by many to function as an endocrine organ, secreting peptide hormones and inflammatory cytokines like IL-6, leptin, and TNF- $\alpha$  (28). Since adipocyte hypoxia and inflammation have been shown to be so closely related to obesity, a compelling case can be made that inflammation-induced increases in hepcidin may ultimately be a primary cause of poor iron status in obesity.

There are no studies to date comparing obese mice to lean controls fed deficient, adequate, or excess dietary iron and very few have examined the effects of obesity on iron metabolism. Ye et al. showed that pockets of adipose tissue from obese mice are prone to hypoxia and macrophage infiltration, inducing the release of inflammatory cytokines such as IL-6 in the body (30). Further, Nemeth et al. showed that IL-6 expression in human liver cell cultures, mice and human volunteers induced the expression of hepcidin (25). Moreover, Hintze et al. demonstrated that cultured cells treated with conditioned media from hypoxic adipocytes had increased hepcidin expression and this increase is mediated through IL-6 (34). Therefore, it is likely that diet-induced obesity and subsequent increases in inflammation induce hepcidin expression and inhibit dietary iron absorption. Because obesity results in chronic inflammation including increased IL-6 expression, a known inducer of hepcidin, we hypothesize that obesity will decrease iron status in mice compared to lean controls fed the same amount of dietary iron. The objective of this study was to determine the effects of diet-induced obesity on hepcidin expression and iron status. Mice were fed either iron deficient (5 mg Fe/kg feed), adequate (50 mg Fe/kg feed), or excessive (500 mg/kg feed) diets. Upon completion of the feeding trial, tissues were analyzed to determine hepatic iron concentration, hepatic hepcidin expression, hepatic IL-6 expression, adipocyte hepcidin expression, and adipocyte IL-6 expression.

## CHAPTER II

### MATERIALS AND METHODS

#### **Diet Formulation**

In order to determine the relationship between diet-induced obesity and iron status, the C57BL/6 mouse diet-induced obesity model was used incorporating iron deficient, adequate, or excess in the diet. Studies conducted by West et al. (35) and Collins et al. (36) have shown the C57BL/6 strain to be susceptible to diet-induced obesity and insulin resistance. A further study conducted by Surwit et al. (37) showed the C57BL/6 strain increased weight gain and inguinal and mesentery fat pad weight when fed high fat diets for 16 weeks compared to mice fed control diets. Following the Surwit et al. protocol male, weanling, C57BL/6 mice were fed either a high fat (45% energy from fat) or control diet (10% energy from fat). Diets were iron (FeSO<sub>4</sub>) deficient (5 mg Fe/kg feed), adequate (50 mg Fe/kg feed), or excessive (500 mg/kg feed). Similar iron concentrations (1 mg/kg, 35 mg/kg and 500 mg/kg) were used in a study conducted by Mazur et al. (38) to model differences in hepcidin gene expression. Feed intake was monitored twice weekly in order to calculate total intake and the mice were weighed once a week to track weight gain. Feed was monitored by cage, each cage containing 3-4 mice. The design was a 3X2 factorial with 10-11 mice per treatment (Table 1). After 16 weeks exposure to their respective treatments, mice were euthanized by CO<sub>2</sub> asphyxiation. Whole blood, serum, plasma, liver, and perigonadal white adipose tissue samples were

**Table 1.** Six diets differing in total fat (10% or 45%) and iron content (5, 50, or 500 mg Fe/kg diet) were formulated and fed to male C57BL/6 mice for 16 weeks.

Besides Fe, diets were identical for all micronutrients including Cu and Zn.

	Fe Deficient Diet <i>5 mg Fe/kg Feed</i>	Fe Adequate Diet <i>50 mg Fe/kg Feed</i>	Fe Overload Diet <i>500 mg Fe/kg Feed</i>
Control Non-Obese <i>10% Dietary Fat</i>	n=11	n=10	n=11
Diet Induced Obesity <i>45% Dietary Fat</i>	n=11	n=11	n=11

collected from the mice and flash frozen in liquid nitrogen and then stored at -80°C until time for tissue analysis.

### Body Composition

Body composition, including fat and lean mass, was determined by MRI after 16 weeks of feeding.

### Gene Expression

**RNA Extraction.** RNA was extracted from liver tissue using a TRIzol RNA isolation method. Approximately 100 mg tissue was homogenized in 1 ml TRIzol reagent and then left to incubate for 5 minutes to allow for the complete disassociation of the nucleoprotein complexes. 0.2 ml chloroform was then added to the solution, shaken vigorously then centrifuged at 12,000 x g for 15 minutes at 4°C. After centrifugation, the mixture separated into a lower red-colored organic phase and a clear, upper aqueous phase. The aqueous phase

was transferred to a new tube using a Pasteur pipette. The RNA was then precipitated by mixing the aqueous phase with 0.5 ml isopropanol. After 10 minutes of incubation, samples were centrifuged for 10 minutes at 12,000 x g and 4°C. The supernatant was carefully removed and the pellet was washed with 70% ethanol. The sample was again centrifuged for 2 minutes at 12,000 x g and 4°C. The remaining ethanol was carefully removed and the pellet was left to dry for approximately 5 minutes. The sample was dissolved in 0.2 ml DEPC-treated water. Sample purity and concentration was determined by spectroscopy. RNA was isolated from adipose tissue using the RNeasy Minikit (Qiagen), giving more than adequate amounts of RNA. Concentration was determined by spectroscopy.

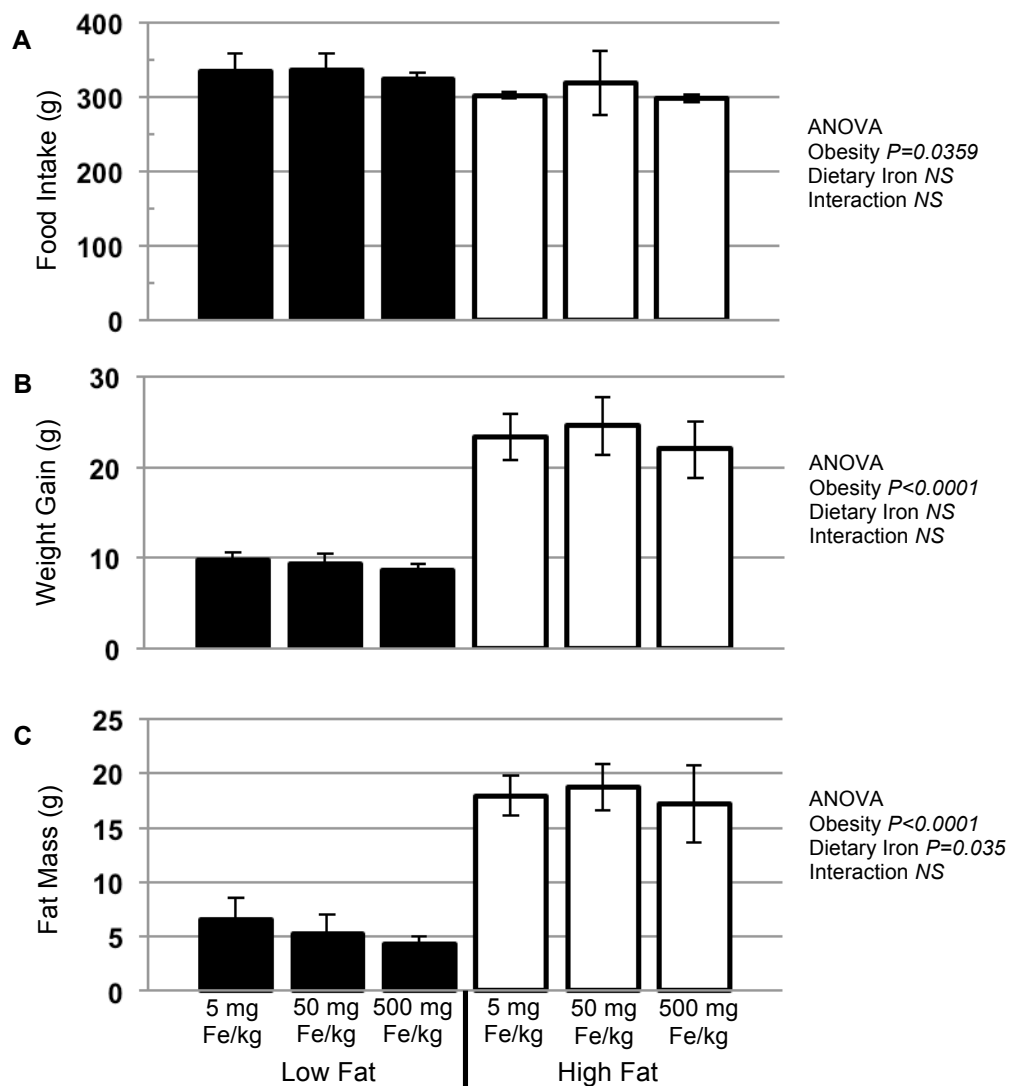
***cDNA Synthesis.*** RNA from liver and adipose tissue was converted into cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems) following manufacturer's specifications. Concentrations were determined by spectroscopy.

***mRNA Quantification.*** mRNA concentrations of IL-6 and hepcidin was assessed using the TaqMan Gene Expression Assay Kit (Applied Biosystems) and a DNA Engine Opticon® 2 Two-Color Real-Time PCR Detection System (Biorad) following manufacturer's instructions. mRNA concentrations were quantified using the cycles to fluorescence midpoint cycle threshold calculation ( $2^{-(\Delta Ct_{\text{experimental gene}} - \Delta Ct_{\text{housekeeping gene}})}$ ), using  $\beta$ -actin as the housekeeping gene.

### **Iron Status**

Whole-blood hemoglobin concentration was determined by Drabkin's Reagent (Sigma-Aldrich) using the method of Drabkin et al. (39). Liver and





**Figure 1.** Effect of diet on (A) food intake, (B) weight gain, and (C) fat mass.

Food intake values are cage means ( $n=3$ )  $\pm$  standard deviation. Weight gain and fat mass are mean ( $n=10$  or  $11$ )  $\pm$  standard deviation. Significant differences were determined by ANOVA and Tukey's HSD test ( $P<0.05$ ).

plasma concentrations of iron, copper, and zinc were determined by inductively coupled plasma atomic-emission spectrometry (ICP).

### **Statistical Analysis**

Differences in feed intake, weight gain, body composition, liver mineral status, hemoglobin and hepcidin and IL-6 expression were determined by ANOVA and Tukey's HSD test.

## CHAPTER III

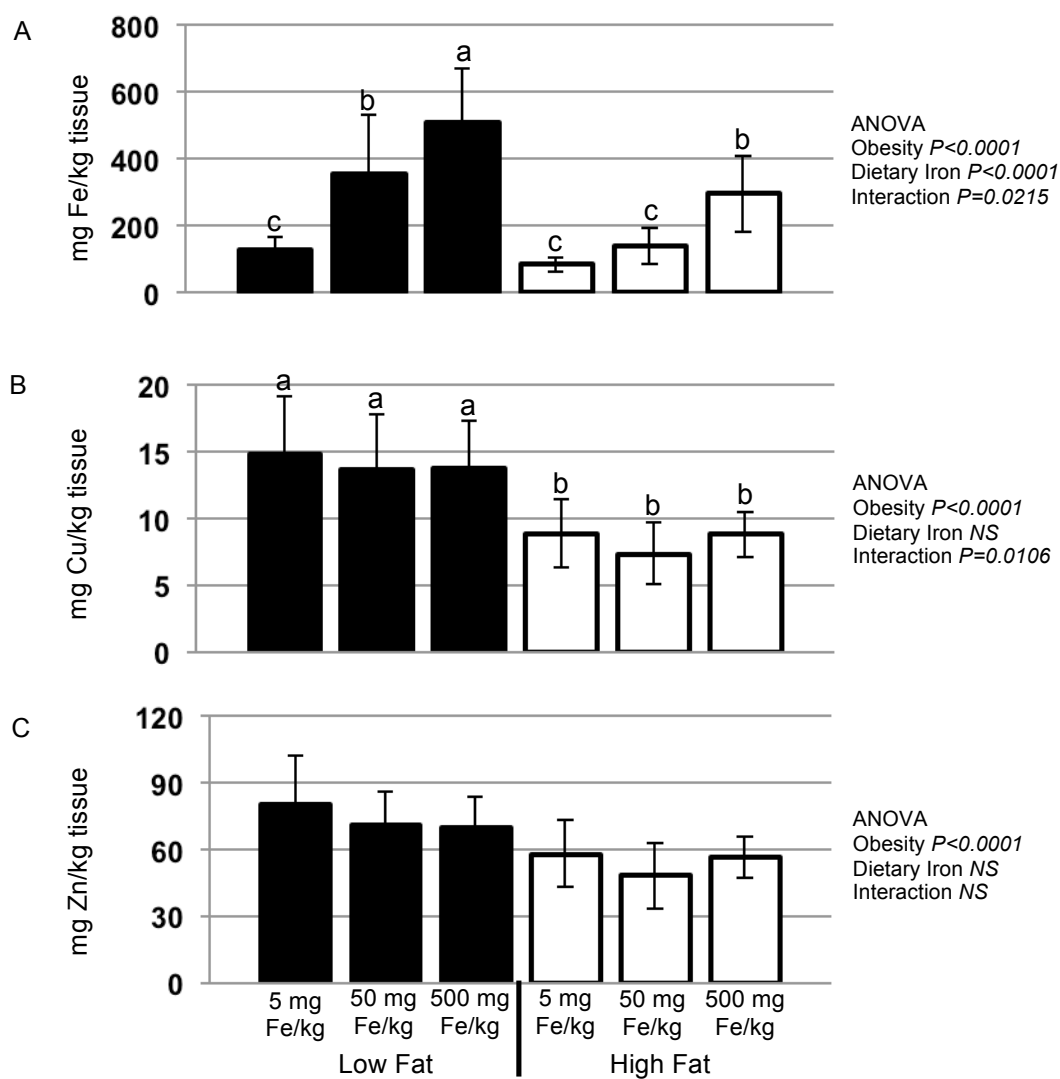
### RESULTS AND DISCUSSION

#### **Diet, Weight Gain, Fat Mass**

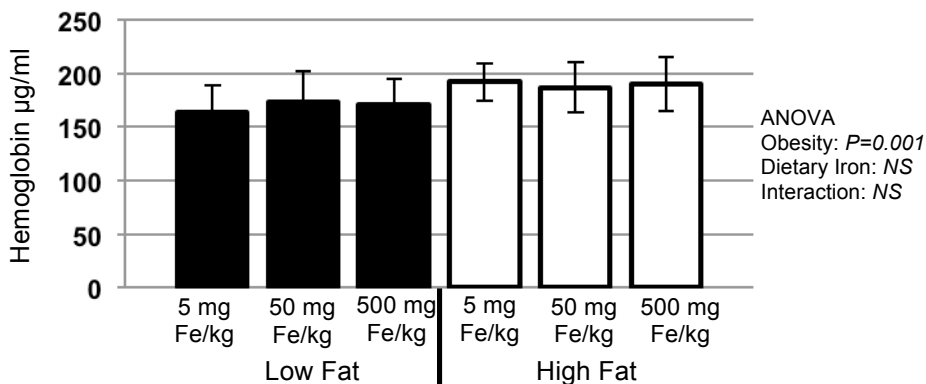
Mice consuming the low fat diet ultimately consumed significantly more feed than their obese ( $P=0.0359$ ). However, consumption was not significantly affected by dietary iron content (Figure 1A). Further, mice consuming the high-fat diet gained significantly more weight than their lean counterparts ( $P<0.0001$ ). However, weight gain was but was not affected by dietary iron content (Figure 1B). MRI data showed mice consuming low fat diets had less fat mass when compared to their obese cohorts ( $P<0.0001$ ). Furthermore, mice fed diets with lower iron content had less fat mass than their high iron counterparts ( $P=0.035$ ). However, there was no significant interaction effect (Figure 1C).

#### **Liver Iron, Zinc, Copper Concentration**

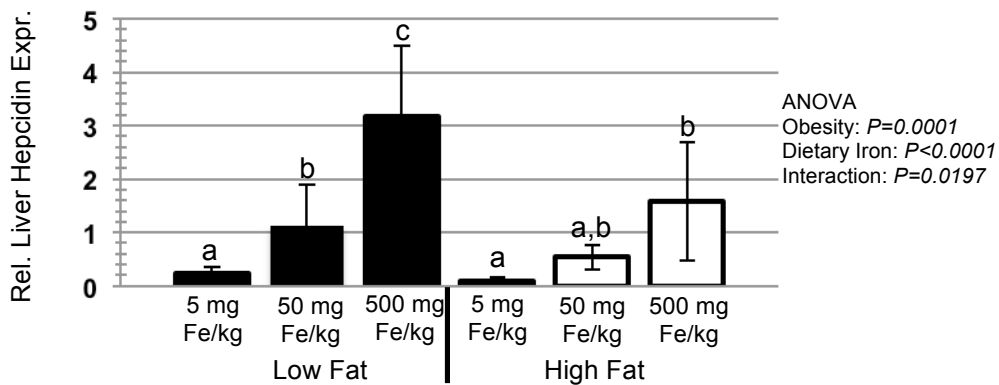
ICP analysis of liver tissue showed increased hepatic iron concentrations in mice consuming the low fat diet when compared to their obese cohorts ( $P<0.0001$ ). As expected, increased hepatic iron concentration was also influenced by higher dietary iron ( $P<0.0001$ ). Analysis further revealed a significant interaction between treatments ( $P=0.0215$ ) (Figure 2A). Mice consuming high fat diets had higher hepatic copper concentrations than their obese cohorts ( $P<0.0001$ ) while there was no significant effect of dietary iron on hepatic copper concentration. However, there was a significant interaction between treatments ( $P=0.0106$ ) (Figure 2B). Obesity had a significant effect on



**Figure 2.** Effect of diet on (A) liver iron status, (B) liver copper status, and (C) liver zinc status. Values are means ( $n=10$  or  $11$ )  $\pm$  standard deviation. Differently labeled columns are significantly different. Significant differences were determined by Student's T-test ( $P < 0.05$ ).



**Figure 3.** Effect of diet on whole blood iron status. Values are means ( $n=10$  or  $11$ )  $\pm$  standard deviation. Main effects of obesity and iron concentration were determined by ANOVA ( $P<0.05$ ). Post-hoc testing was determined by Tukey's HSD test.



**Figure 4.** Effect of diet on relative liver hepcidin expression. Values are means ( $n=10$  or  $11$ )  $\pm$  standard deviation. Differently labeled columns are significantly different. Main effects of obesity and iron concentration were determined by ANOVA ( $P<0.05$ ). Post-hoc testing was determined by Tukey's HSD test.

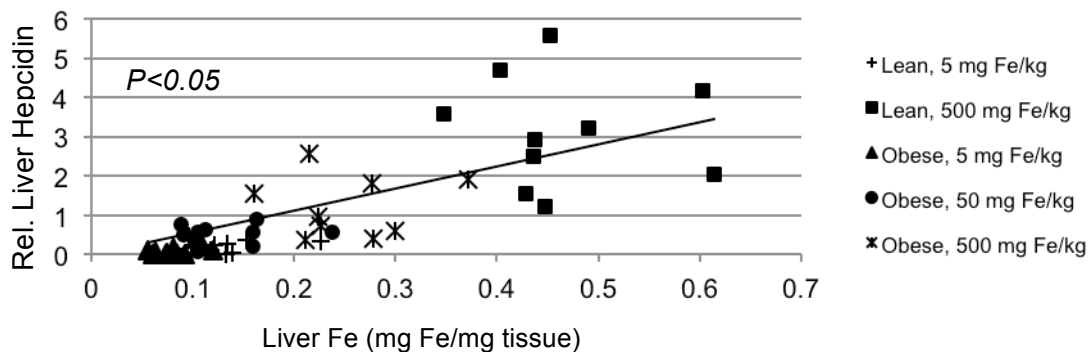
concentrations than their obese cohorts ( $P<0.0001$ ). Dietary iron had no effect on hepatic zinc concentration and there was no significant interaction between the treatments (Figure 2C).

## Hemoglobin

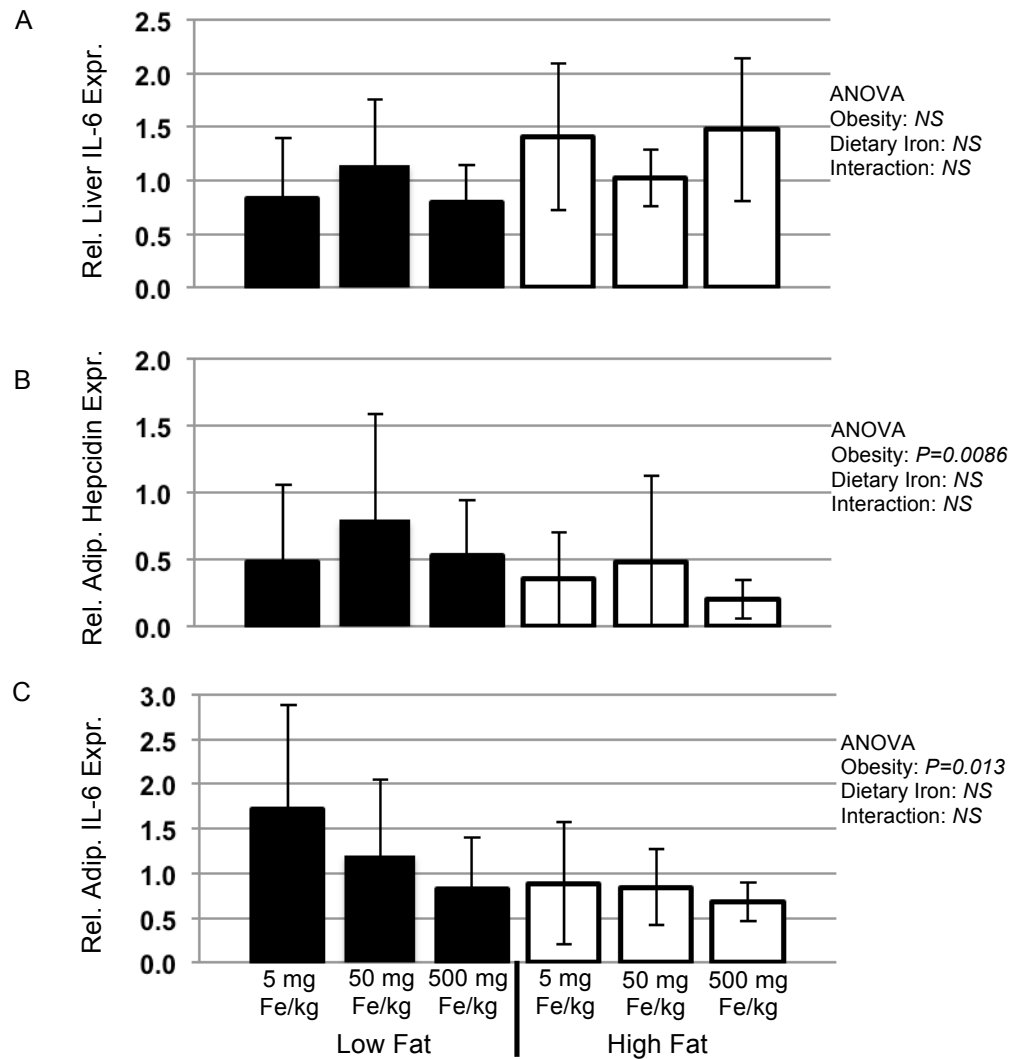
Analysis of whole blood samples showed lower concentrations of hemoglobin among mice consuming the low fat diet when compared to their counterparts fed the same amount of dietary iron ( $P<0.0001$ ) (Figure 3). Dietary iron had no significant effect on whole blood iron status and there were no significant interaction effects between treatments.

## Liver Hepcidin

Dietary fat had a significant effect on liver hepcidin expression ( $P=0.0001$ ). Mice consuming low fat diets had increased hepatic hepcidin expression than their lean cohorts (Figure 4). In addition, dietary iron had a significant effect on liver hepcidin expression ( $P<0.0001$ ). Mice consuming higher amounts of iron had increased liver hepcidin expression than those



**Figure 5.** Overall correlation between liver iron status and relative liver hepcidin expression.



**Figure 6.** Effect of diet on (A) relative liver IL-6 expression, (B) relative adipose hepcidin expression, and (C) relative adipose IL-6 expression. Values are means (n=10 or 11)  $\pm$  standard deviation. Main effects of obesity and iron concentration were determined by ANOVA ( $P < 0.05$ ). Post-hoc testing was determined by Tukey's HSD test.

consuming smaller amounts. Furthermore, there was a significant interaction effect between treatments ( $P=0.0197$ ). For example, lean mice consuming higher amounts of iron had increased hepcidin expression when compared to obese mice consuming all amounts of iron. Further, lean mice consuming high amounts of iron showed increased hepcidin expression when compared to other lean mice. Moreover, Figure 5 shows a tight correlation between increased liver hepcidin expression and increased liver iron status ( $P<0.05$ ). Taken together the data suggests mice may adapt under chronic inflammatory conditions and that liver iron stores are a more important determinant of hepcidin expression than obesity induced inflammation.

### **Liver IL-6**

Neither obesity nor dietary iron had an effect on liver IL-6 expression (Figure 6A).

### **Adipose Hepcidin**

Dietary fat content had a significant effect on adipose hepcidin expression ( $P=0.0086$ ) (Figure 6B). Mice consuming the low fat diet had higher adipose hepcidin expression than their obese cohorts, consistent with liver hepcidin expression. However dietary iron consumption did not significantly affect adipose hepcidin expression and there were no interaction effects.

### **Adipose IL-6**

Obesity significantly affected adipose IL-6 expression as mice consuming low fat diets exhibited increased IL-6 expression than their obese counterparts. Mice fed low fat, iron deficient diets had increased IL-6 expression when



compared to mice fed the low fat, iron overload diet and high fat, iron adequate or overload diets ( $P < 0.05$ ) (Figure 6C). Further, mice fed the low fat, iron adequate diet had increased IL-6 expression when compared to mice fed the high fat, iron overload diet ( $P < 0.05$ ). There were no other significant differences between other treatments.

## CHAPTER IV

### SUMMARY AND CONCLUSIONS

Iron regulation is a complex process. Various diverse proteins and hormones contribute to the process, illustrating the importance of iron metabolism in biology. The discovery of hepcidin and its role in iron regulation is vital to our understanding of the metabolic pathways of iron. Since obesity has become the great epidemic of western society and iron deficiency is common among the obese, it is important to understand the relationship between obesity and iron metabolism. Understanding how this relationship works may help with understanding how to best help those plagued with iron deficiency and increase overall worldwide health. No other studies to date have systematically investigated the interplay between diet induced obesity, dietary iron and effects on iron metabolism.

The results of this experiment support previous findings that mice with high liver iron stores also have high hepatic hepcidin expression (3). Conversely, the data does not support the hypothesis that inflammation-induced increases in hepcidin expression are a primary cause of poor iron status in obesity. Considering the pathway for the regulation of dietary iron, low iron stores would suggest high hepcidin expression in connection with increased amount of inflammatory cytokines. However, the data shows obese mice had lower hepatic hepcidin expression than lean mice fed the same amount of dietary iron in spite of increased IL-6 expression. The data was collected after an extended period of time on treatment and may thus show a metabolic adaptation to chronically low

iron stores. The data also shows that diet-induced obesity resulted in decreased hepatic iron stores when the mice were fed iron deficient, adequate or excessive diets compared to their lean counterparts fed the same amount of dietary iron, supporting the association shown in many different populations. Taken together, the data suggests inflammatory stress may be less important in long-term iron regulation and liver iron stores may be a more important indicator of hepcidin expression and when obesity decreases liver iron stores, hepcidin expression also decreases.

This study has unveiled important questions in regards to obesity, inflammation and iron regulation that further research could help answer. To demonstrate the adaptation to chronic inflammation, further studies could include the same endpoints being measured over the same amount of time but at shorter intervals. This type of study would be a great indicator of any immediate effects and give a broader picture of obesity's influence on iron regulation over time. Further, to examine how inflammation affects iron regulation IL-6 knockout mice could be included in the aforementioned study. Doing so would provide a more mechanistic understanding of obesity induced inflammation and its effects on iron regulation. Moreover, a human study could be carried out to see if similar results occur. A longitudinal study involving obese and lean participants and measuring amounts of IL-6, circulating hepcidin and iron status, though expensive, would provide excellent insight on how this regulatory process works in humans.

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