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Dietary Iron Supplementation Increases Severity of Salmonella Typhimurium Infection

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DIETARY IRON SUPPLEMENTATION INCREASES SEVERITY OF *SALMONELLA* TYPHIMURIUM INFECTION

By

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A Thesis submitted to the
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I dedicate my thesis work to my friends and family, who have supported me profusely throughout my time at Florida State University. I would like to give a special acknowledgement to my parents, for their endless words of encouragement and love. I would like to honor the memory of my grandmother Frances Ippolito, who would be so proud to see where I am today. I thank my grandparents, aunts, uncles, and cousins for their support as I have matured. I thank all of my lifelong friends for keeping me company and being there for me when I needed it most. The love I have received from those closest to me has been my lifelong encouragement.

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ABSTRACT

Background: Less than 20% of non-heme iron is absorbed in the small intestine and the remaining iron travels to the lower intestine for excretion. *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) and other enteric pathogens may use unabsorbed iron in the lower intestine as a nutrient source, potentially leading to a more severe infection.

Objective: The objective of this study was to determine whether unabsorbed iron in the lower intestine of mice increases the severity of *S.* Typhimurium infection.

Method: Twenty-one-day-old female C57BL/6 mice were fed diets containing 300 ppm iron (iron supplemented, FeS) or <6 ppm iron (iron deficient, FeD) for 6 weeks (n=30/diet). After 6 weeks, mice were given an oral gavage of streptomycin 24 h prior to oral gavage of 10^8 CFU *S.* Typhimurium ($FeS^{S.Tm}$ or $FeD^{S.Tm}$, n=15/diet/treatment). The remaining mice in each group acted as control animals (FeS^{Ctrl} or FeD^{Ctrl}, n=15/diet/treatment). Mice were monitored for up to 1-week post-infection prior to euthanasia and tissue collection. Severity of infection was assessed by cecal weight, spleen index, and change in bodyweight post-infection. Data are presented as means ± SD.

Results: Hematocrits were greater in FeS^{Ctrl} (51.3 ± 0.8%) compared to FeD^{Ctrl} (43.8 ± 2.7%), $FeS^{S.Tm}$ (40.3 ± 3.8%) and $FeD^{S.Tm}$ (40.2 ± 7.3%, $P<0.01$ for all comparisons). FeS^{Ctrl} had higher cecal iron (331.3 ± 59.3 μg Fe/mg tissue) compared to FeD^{Ctrl} (5.7 ± 2.4 μg Fe/mg tissue), $FeS^{S.Tm}$ (64.6 ± 26.4 μg Fe/mg tissue) and $FeD^{S.Tm}$ (6.7 ± 3.3 μg Fe/mg tissue, $P<0.0001$ for all comparisons). Cecal weight was 40% lower in $FeS^{S.Tm}$ (0.3 ± 0.1 g) compared to FeD^{Ctrl} (0.5 ± 0.1 g), FeS^{Ctrl} (0.5 ± 0.2 g) and $FeD^{S.Tm}$ (0.5 ± 0.1 g, $P<0.05$ for all comparisons). Spleen index was greater in $FeS^{S.Tm}$ (15.3 ± 7.2 A.U.) compared to FeS^{Ctrl} (4.3 ± 0.5 A.U.), FeD (4.0 ± 0.5

A.U.) and $FeD^{S.Tm}$ (8.8 ± 5.2 A.U., P<0.01 for all comparisons). $FeS^{S.Tm}$ lost more bodyweight (-21.7 ± 5.4%) compared to $FeD^{S.Tm}$ (-14.5 ± 6.3%, P<0.01) post-infection.

Conclusions: Findings suggest that unabsorbed iron in the lower intestine of mice promotes a more severe *S.* Typhimurium infection. Alternative iron supplementation strategies should be considered in areas where *S.* Typhimurium infections are common.

CHAPTER 1

LITERATURE REVIEW

1.1 Biology and function of iron

Iron is one of the most abundant elements present in the Earth's crust and is biologically essential to almost all living organisms.[1,2] In 1932, the first widely accepted proof of iron's biological importance was established related to its essential role in synthesizing hemoglobin.[3] Since then, the biological function of iron has been further explored and it is well documented that iron is directly involved in the human circulatory, digestive, endocrine, immune, muscular, nervous, reproductive, respiratory, and hematopoietic systems. Iron is a top-row transition metal, meaning that its electrons are easily lost or gained (i.e., a transition metal). This is observed in nature as iron exposed to oxygen is readily oxidized ($Fe^{2+} \rightarrow Fe^{3+}$). The oxidation of iron is reversible, as iron can also be reduced ($Fe^{3+} \rightarrow Fe^{2+}$) by a number of reducing agents. This exchange of electrons is a fundamental reason why iron behaves as observed in biological systems.

1.1.1 Oxygen transport and storage

One of the most important functions of blood is to transport oxygen from the lungs to the peripheral tissues. At any given time, the majority of iron in the body (~75%) is being used for oxygen transport and storage.[2] In a normal, healthy individual, almost half of blood (~45%) is composed of red blood cells.[4] A primary component of red blood cells is the tetrameric molecule hemoglobin. Hemoglobin itself is composed of four globin proteins, which are referred to as the alpha- 1, 2 and beta- 1, 2 chains. Each globin chain binds to one heme molecule.[4] The heme molecule is composed of four pyrrole groups (termed a porphyrin ring)

which each contain a central-facing Nitrogen atom that binds to Fe^{2+}, holding the ion in the middle of the structure.[4,5] This central iron ion is able to bind to oxygen, which allows for a single red blood cell to transport up to one billion oxygen atoms.[5] This is why iron is an imperative nutrient required for oxygen transport. Thus, hemoglobin is responsible for carrying a majority (98.5%) of all oxygen found in the blood. Red blood cell synthesis occurs in the bone marrow, where mature deoxygenated red blood cells travel to the capillary bed of the alveoli in the lungs to pick up oxygen.[4] Once oxygenated, the red blood cells travel to peripheral tissues to deliver the oxygen. Red blood cells may also bind and transport the waste product CO_2 from peripheral tissues back to the lungs.

While hemoglobin is the primary molecule used for oxygen transport, myoglobin is the primary molecule used for oxygen storage.[6] Myoglobin is found in striated muscle cells, which includes skeletal and cardiac muscle.[6,7] In contrast to the tetramer hemoglobin which contains four oxygen binding sites, myoglobin is made of a single peptide chain consisting of a backbone and one oxygen binding site.[7] Myoglobin acts as a reserve of oxygen (O_2) when oxygen delivery from the lungs is insufficient or has stopped. This may include periods of hypoxia or increased oxygen need, such as aerobic exercise.[7] Similar to hemoglobin, the myoglobin molecule contains an oxygen binding site of four pyrrole rings with a central-facing Nitrogen atom bound to iron, allowing for iron to bind to oxygen.[7] When myocytes require oxygen, the O_2 is released from the myoglobin and diffused to the mitochondria for cellular respiration and thus, energy production to the working muscle.[6]

1.1.2 Electron transport and energy metabolism

Within cells containing mitochondria, iron is important for the function of the electron transport chain, which produces adenosine triphosphate (ATP) for energy production. Iron can be found within Complex I of the electron transport chain, as part of iron-sulfur centers which carry electrons through the protein.[8] Complex II also involves iron: electrons from succinate enter Complex II and transfer to flavin adenine dinucleotide (FAD) to create FADH, which then donates electrons to iron-sulfur groups.[8] Complex III contains a 2-iron ferredoxin, which is a protein that mediates electron transfer by way of reducing or oxidating iron.[8] Cytochrome C is a protein which contains a heme group that is used to transfer electrons from complex III to Complex IV in the electron transport chain.[8] The final protein of the electron transport chain is Complex IV, which contains two heme molecules within its structure.[8] The electron transport chain, therefore, creates substrates that feed into the tricarboxylic acid cycle, which can also be used to generate ATP.

1.1.3 Other functions

Iron is a vital component of immune system function. The immune system is comprised of lymphocytes and granulocytes which rely on iron for growth and differentiation.[9] Enzymes that generate peroxide and nitrous oxide, which are necessary for immune cell function, also require iron for proper function.[9] An example is myeloperoxidase, which contains heme within its structure, and is found in neutrophils, a type of white blood cell.[5] These peroxidase enzymes act to catalyze reactions which produce oxidizing agents that destroy bacteria.[5] Lymphocytes in iron-deficient individuals have shown reduced production of interleukin-2, a messenger cytokine

necessary for proper immune function.[9] Thus, iron deficiency can decrease proper immune system function.

1.2 Absorption and regulation of iron status

For humans, ingestion of iron via diet is the primary acquisition method. In contrast to other micronutrients, iron homeostasis exists as a closed system, meaning that no physiological mechanism to excrete iron exists. That is, once iron is absorbed, it is stored, utilized, and recycled, and cannot be excreted. The only ways to remove iron from the body after absorption are via bleeding, menstruation, and sloughing cells from skin and mucosal surfaces such as the gastrointestinal tract. Therefore, iron homeostasis is regulated at the point of absorption within the small intestine.

1.2.1 Dietary sources

Omnivorous vertebrates such as humans consuming both animal and plant-based foods consume two main dietary forms of iron: heme and non-heme iron.[10] While heme iron is only available from animal sources, nonheme iron may be obtained from both animal and plant-based sources. Heme iron refers to any iron which is bound to the heme molecule found in hemoglobin and myoglobin. Sources of heme iron in the diet include animal-based products (with the exception of dairy), such as red meat, eggs, poultry, and shellfish. Of the total iron content of these foods, approximately 50% is heme iron, while the rest is non-heme iron.[5] For an individual consuming a typical Western diet, about 10-15% of total dietary iron intake is in the form of heme iron.[11]

Non-heme iron exists associated with macromolecules within the food matrix. Non-heme iron is found in animal and plant-based foods. Some plant-based foods high in iron include dark leafy greens, tofu, legumes, and pumpkin seeds.[5] Refined flour, such as that made from wheat, has its natural iron content stripped during processing. Thus, in the United States and other countries, refined flours are fortified with iron to increase nutritional value. Fortified iron sources include, but are not limited to, ferric citrate, ferrous sulfate, ferric pyrophosphate, and ferric chloride, all of which are non-heme iron.[5]

1.2.2 Digestion

Digestion of iron is a critical component of homeostasis, as iron absorption may only occur if digestion of the bolus has occurred. Heme iron digestion starts in the stomach, where globular portions of hemoglobin are removed via protease enzymes.[5] The remaining product is heme-bound iron. This product may continue to the duodenum for absorption. Nonheme iron is typically found bound to molecules within the food matrix. In the stomach, both hydrochloric acid and protease enzymes act to free the iron from the food matrix, typically as Fe^{3+}.[5] This iron can then continue to the duodenum for absorption.

1.2.3 Absorption

The absorption of heme iron is relatively high (15-35%) while non-heme iron has a low absorption (2-20%).[2] This is due to a number of factors which modify the bioavailability of iron within the digestive tract, along with the physiology of the intestinal transporters involved in iron absorption. Starting in the lumen of the duodenum, dietary iron must be first absorbed into the enterocyte to eventually enter the bloodstream for utilization.

Less is understood about heme iron absorption compared to non-heme iron absorption. The current understanding is that a membrane-bound protein, heme carrier protein-1, shuttles heme iron across the apical membrane into the enterocyte via active transport.[5,12–14] Once in the enterocyte, heme oxygenase-1 mediates the release of Fe^{2+} from heme, and degrades the heme components into carbon monoxide and biliverdin (a precursor to bilirubin).[10,15] The iron is now contained within the enterocyte and will be stored, exported into the bloodstream, or utilized for biochemical processes within the cell.

Non-heme iron absorption is more complex than heme iron absorption, but much better characterized. Non-heme iron in the lumen of the duodenum exists either in the ferric (Fe^{3+}) or ferrous (Fe^{2+}) form. The only way for an enterocyte to absorb the iron is if it is in the Fe^{2+} form, meaning that all Fe^{3+} that does not get reduced to Fe^{2+} is not absorbed, and instead continues through the gastrointestinal system. The need to reduce non-heme iron prior to absorption is a major reason why non-heme iron absorption is relatively lower than heme iron absorption. Many reducing agents have the capability to reduce Fe^{3+} to Fe^{2+}, including endogenous hydrochloric acid from the stomach, and ascorbic acid (vitamin C) from the diet. The apical membrane of enterocytes also contains reductases including duodenal cytochrome B, cytochrome b ferric cupric reductase, and others.[5,10,16] Once the iron has been reduced to Fe^{2+}, divalent mineral transporter-1 (DMT-1), found on the apical membrane of enterocytes, transports iron into the enterocyte.[5] DMT-1 also transports other minerals, including Zn^{2+}, Cu^{2+}, Mg^{2+}, and Ca^{2+}, and consuming those minerals simultaneously with Fe^{2+} may lead to decreased iron absorption.[5]

1.2.4 Storage, excretion, transport

Once absorbed into the enterocyte, ferrous iron exists within the cytosol bound to other molecules for stabilization.[5] There are three main fates for the iron at this point: storage within the enterocyte, use in a functional role within the enterocyte, or export out of the enterocyte into the bloodstream. If the iron is to be stored within the enterocyte, it will be bound to ferritin. One protein has been identified, poly (rC)-binding protein-1, which will capture three iron atoms per molecule and transfer them to ferritin.[5] Now bound to ferritin, the iron will remain until it is needed for functional use (whether that is in the enterocyte itself or another cell in the body). The lifespan of an enterocyte is relatively short compared to most other cell types, with enterocytes being sloughed off from the gastrointestinal tract after three days. If iron is not used within the three days of the enterocyte's existence, then it will also be sloughed off and excreted in feces. Thus, not all of the iron absorbed into the enterocyte will actually enter the systemic circulation.

Iron which does need to be used by the body must first be exported from the enterocyte into blood for travel throughout the body. The only known exporter of iron is the membrane-bound protein ferroportin.[10] When iron binds to ferroportin on the cytoplasmic face, a conformational change of the transmembrane protein occurs which moves the iron to the extracellular side, allowing for release of iron into the blood. Once exported from the enterocyte, hephaestin, a copper-dependent oxidase, almost instantaneously oxidizes ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) so it can bind to transferrin.[10] Two iron atoms are bound to each transferrin molecule. The transferrin complex then travels through the bloodstream to peripheral tissues. Iron-bound transferrin may distribute to the liver, where most of body iron stores are located. Another portion of iron is used by macrophages, which recycle 20-25 mg of iron per day from

senescent red blood cells.[17] Recycled iron can then re-enter the circulation for physiological use. Most of this recycled iron will be used in the bone marrow for erythropoiesis.

1.2.5 Regulation

Regulation of iron absorption is primarily mediated by the liver-derived hormone hepcidin.[18] Binding of hepcidin to ferroportin causes a conformational change in ferroportin, which exposes the ubiquitination sites of ferroportin, triggering for the internalization and lysosomal degradation of the protein.[18–20]

Hepcidin transcription is triggered by two known molecular messengers: bone morphogenetic protein 6 (BMP-6) and interleukin 6 (IL-6).[21] IL-6 signals for hepcidin transcription via the Janus Kinase/Signal Transducer and Activator of Transcription protein (JAK/STAT) pathway while BMP-6 signals via the Small Mothers Against Decapentaplegic (SMAD) signaling cascade. IL-6 signaling can be the result of inflammation, which may be due to injury, infection, and/or disease states. Elevated levels of systemic iron induce BMP-6 signaling to initiate the SMAD signaling cascade for hepcidin transcription.[21] Thus, inflammation is expected to decrease iron absorption, which may lead to iron deficiency.

1.3 Assessment of iron status

Proper iron status assessment requires the use of multiple relevant clinical indicators. There are three main functional categories which may be used to group these indicators: iron stores, transport iron, and functional iron.[22] Iron stores are found throughout the body, primarily within hepatocytes, bone marrow, and macrophages.[5,10] Iron stores can be used as a “bank” from which to withdraw iron when needs are increased, absorption is low, or dietary intake is lacking.

A clinical indicator reflecting iron stores is serum ferritin. Transport iron refers to iron which is being circulated systemically. This is iron which is typically protein-bound and traveling through the blood to target tissues. Indicators reflecting transport iron include transferrin saturation and soluble transferrin receptor. Functional iron refers to iron that is being used to synthesize red blood cells. An indicator reflecting functional iron is serum iron. Markers of anemia, such as hemoglobin, hematocrit, mean corpuscular volume, and red blood cell distribution width, may also be useful, but do not give direct information on assessing iron status.[23]

1.3.1 Serum ferritin

Serum ferritin is taken as a measure of total body iron stores. While ferritin in blood does not account for most of the ferritin found in the body, the concentration of ferritin in the blood reflects the overall stores of iron in the body.[22] A majority of ferritin is found in hepatocytes and macrophages, however, feasibility of measurement in those cells would be difficult. As iron status declines, it is expected that serum ferritin will decline as well. Thus, serum ferritin is typically the earliest marker that will change with the onset of iron deficiency.[23] Serum ferritin poses a potential problem, though, as ferritin is an acute-phase protein and increases with inflammation.[23] Therefore, if ferritin is measured, it is important to also measure inflammatory markers in the blood such as C-Reactive Protein (CRP) or Alpha(1)-acid GlycoProtein (AGP).[24] The increase in inflammation could cause serum ferritin to artificially increase, meanwhile body iron stores may not be adequate.

1.3.2 Transferrin saturation and soluble transferrin receptor

Transferrin is the primary protein that functions to carry two iron ions simultaneously throughout the bloodstream such that delivery to peripheral tissues is possible. Transferrin may only accept iron in the Fe^{3+} form, meaning that once Fe^{2+} exits the enterocyte via ferroportin, the iron must be oxidized to Fe^{3+} before it may associate with transferrin. Hephaestin is the primary oxidase enzyme which will cause this to occur, and this enzyme requires copper to function. Therefore, an individual with a copper deficiency may develop iron deficiency due to the inability to transport iron to tissues.[25–27] Transferrin saturation refers to the percent of total binding sites on transferrin within the body that are actually bound to iron.[22] Thus, a higher transferrin saturation would indicate that more iron is being circulated, and a lower transferrin saturation would indicate that less iron is being circulated. Transferrin saturation is not a direct measurement, that is, it is calculated as the ratio of serum iron to the total iron binding capacity.[22] As in most cases, it is important for iron to be bound to a molecule such that it does not create reactive oxygen species in the body. Therefore, it is estimated that <1% of total plasma iron is not bound to transferrin, referred to as non-transferrin-bound iron (NTBI).[22] NTBI will increase in conditions resulting in iron overload, such as hemochromatosis.[27]

Soluble transferrin receptor is another measurement taken in blood used to assess iron status. Transferrin receptors are found on the surface of a majority of cells, but expression is highest in developing erythrocytes, hepatocytes, and rapidly dividing cells (such as cancerous cells).[28,29] Transferrin receptors are important because they are the proteins expressed on the outer membranes of cells to which transferrin will bind for iron delivery to the cell. Once bound, the transferrin-transferrin receptor complex is internalized in clathrin-mediated endocytosis.[16,30] Once an endosome is formed in the cell, a number of factors including endosome acidification,

transferrin conformation change, and enzymatic reduction of Fe^{3+} cause the release of iron from transferrin as Fe^{2+}.[16] At this point, DMT-1, present on the endosomal membrane, will now function as previously described to allow Fe^{2+} to enter the cytosol and be used by the cell.[16,30] Soluble transferrin receptor is a fragment of transferrin receptor found circulating in the bloodstream bound to transferrin.[22] Transferrin receptor is upregulated when cells require more iron, meaning this measurement will increase with iron deficiency.[31,32]

1.3.3 Hemoglobin and hematocrit

It is important to begin by clarifying that hemoglobin and hematocrit are not direct measurements of iron status. Instead, these measurements indicate the presence of anemia, which is defined by reduced and/or impaired red blood cell production. Anemia can be caused by severe iron deficiency (iron-deficiency anemia) due to the inability to properly form red blood cells with hemoglobin. Hemoglobin, as previously described, is a protein and primary component of red blood cells which requires iron for normal function. If iron is not present when red blood cell synthesis occurs, then less red blood cells will be generated, and the ones that are, will be microcytic and hypoferremic.[29] If hemoglobin is low, this indicates that erythropoiesis is impaired, suggesting iron deficiency.

Hematocrit is defined as the percentage of whole blood which is composed of red blood cells. As previously noted, anemia is defined by low presence of red blood cells. Therefore, measuring hematocrit gives a direct indication of the amount of red blood cell production occurring. Thus, in an iron-deficient anemic individual, hematocrit is expected to decrease.

1.4 Iron deficiency and iron deficiency anemia

Symptoms of iron deficiency include fatigue, difficulty concentrating, and low work productivity.[33,34] Iron deficiency adversely affects both physical labor capacity along with ability to focus, thus, iron deficiency poses a potential problem to all populations, regardless of occupation or activity status. Iron deficiency is recognized as the most common nutrient deficiency worldwide.[23,35] Iron deficiency in the absence of anemia is important to diagnose, because anemia is considered a late-stage manifestation of iron deficiency, whereas early identification of iron deficiency could have been addressed before anemia develops. Iron deficiency without anemia is likely under-diagnosed and therefore under-reported. Johnson et al. described that, of a sample of 114 menstruating women aged 9-19 years attending a hematology clinic for complaints not related to iron deficiency, 50% had ferritin levels indicative of iron deficiency, without the presence of anemia.[34]

Anemia is one of the most common adverse health conditions affecting people worldwide. [36,37] Anemia may develop due to a number of reasons, including genetic disorders, deficiencies of several nutrients, and disease states.[37] All of these reasons have a common trait, which is that red blood cell synthesis is impaired in some way. This leads to poor quality of life and, if severe and left untreated, death.

1.4.1 Prevalence

The World Health Organization (WHO) has reported that 33% of the world's population is anemic, and iron deficiency is the most common cause of anemia worldwide.[36,37] Data from the National Health and Nutrition Examination Survey (NHANES) suggests that 5.6% of the overall U.S. population is anemic.[38] Epidemiologic data for other countries is limited to the high

risk groups of preschool age children (<5 years old), and both pregnant and non-pregnant women.[36] However, anemia is much more prevalent in low- and middle-income countries, especially among women of reproductive age, such as areas of Africa where 40-60% of this population may be anemic.[39] Moreover, 68% of preschool age children in Africa are anemic, while rates in South-East Asia and the Eastern Mediterranean are approximately 66% and 47%, respectively.[39]

1.4.2 Populations at risk

Populations at risk for iron deficiency and anemia include children <5 years old, women of reproductive age (ages 15-49), and elderly adults (aged 65+).[37] The demographic groups with the most severe clinical outcomes from iron deficiency anemia are typically pregnant women and preschool age children.[37] Pregnant women have increased iron needs during gestation to support the growth of the fetus.[37] Iron deficiency is typically more prevalent later in pregnancy, with 27.5% of third trimester women being classified as iron deficient, compared to women in their first or second trimester (12.7% and 5.3%, respectively).[40] Pregnant women with anemia have higher rates of maternal depression, premature births, low birth weight, and maternal, perinatal and neonatal death rates compared to non-iron deficient women.[37,41] Young children have high iron requirements as part of rapid growth and development during this time in life. Anemic children present with irreversible cognitive and motor development.[37] Non-pregnant women of reproductive age are vulnerable to iron deficiency anemia due to blood loss through menstruation and low dietary iron intake.[42] Older adults may be at risk of iron deficiency due to low dietary iron intake and decreased absorption due to chronic inflammation or chronic kidney disease.[37]

Anemia in the elderly population is linked with higher rates of hospitalizations, higher rates of dementia, decreased muscle strength, and increased risk of death.

1.4.3 Strategies to combat

Iron supplements are a primary method for treating iron deficiency in the United States. The most common iron supplement is ferrous sulfate, but other forms, such as ferrous fumarate, ferrous gluconate, and polysaccharide-iron complexes are also available. Iron supplements typically provide 65-200 mg of iron per dose, with dosing strategies varying at an individual level. Recent research indicates more efficient absorption in alternate-day versus everyday dosing, so future dosing strategies may vary.[43] In countries where iron-deficiency anemia affects >5% of the population, the WHO recommends mass fortification of staple food items.[37] This can be done by adding minerals to the food items during production, or via biofortification. Biofortified crops are nutritionally enhanced via biotechnology practices. Both iron supplements and biofortification of crops have been shown to improve iron status in a target population of reproductive-age females.[44–47] It is important to note that since iron supplements and biofortified crops are a source of non-heme iron, absorption is low, estimated at 0-15%.[48]

The most recent 2012 NHANES data shows that 19% of U.S. adults use an iron-containing supplement.[49] This is a 12% decrease in use as 31% of adults reported iron-containing supplement use in the 1999-2000 report. As previously mentioned, women of reproductive age are at higher risk of iron deficiency anemia and may be instructed by a healthcare practitioner to use an iron-containing supplement. Women aged 20-44 had a 6% decrease in reported iron-containing supplement usage from 35% to 29% in the 1999-2000 and 2011-2012 reports, respectively. An analysis of the 2003-2006 NHANES report found the age group with the

highest percent consumption of iron-containing supplements (20%) was children 4-8 years old.[50] Children 1-3 years old, who are highly vulnerable to iron deficiency and iron deficiency anemia, were not far behind, with 16% using an iron-containing supplement. There is adequate reasoning for why an iron supplement may be needed. Iron consumption is often low because typical consumption of dietary sources of iron may not provide adequate amounts of iron for some individuals.[51] An analysis of NHANES data reported that 13% of U.S. individuals ≥2 years of age consumed below their respective estimated average requirement (EAR) for iron from dietary sources alone (excluding enriched/fortified products).

Although NHANES reports strong data indicating frequency of dietary supplement use, only recent surveys included information on why participants were taking dietary supplements.[52] Data collected from 2007-2010 indicate that 67% of users taking an iron-containing supplement report using it for anemia or low iron. Thus, 33% of iron-containing supplement users report using it for reasons other than anemia or low iron, which can be of concern, since this may lead to unintended negative side effects, such as gastrointestinal distress.

1.5 Nutritional immunity

Nutritional immunity is a term coined in 1975 by Dr. Eugene Weinberg.[53] This term refers to the process by which an organism (in a case of infection, both a host and an invading pathogen) can scavenge and sequester trace minerals such that they are withdrawn from the opposing organism. While this term does include other trace minerals such as zinc and manganese, for the purpose of this thesis, only iron will be covered. Nutritional immunity is an important defense mechanism for a host against pathogenic invasion of tissue by making nutrients unavailable to the invading pathogen. Since almost all living organisms require iron to

survive, by scavenging and sequestering available iron in the body, the host is able to prevent an invading pathogen from obtaining the essential nutrient. However, in response, pathogens have also developed mechanisms in an attempt to sequester those nutrients.

1.5.1 Host mechanisms

The host employs a number of different mechanisms to sequester iron from pathogens. First, iron's excretion from the enterocyte via ferroportin is blocked in times of inflammation or infection. This is due to hepcidin-mediated declines in ferroportin, which decrease circulating concentrations of iron and the amount of iron available for blood-borne pathogens. It is important to note that iron also exists largely in hepatocytes and macrophages, both of which also may have their ferroportin expression regulated by hepcidin.[54] In addition, when iron travels through the blood, it is bound to large proteins (such as heme or transferrin) which acts as another mechanism preventing iron from being accessible to invading pathogens. Neutrophils secrete lactoferrin during infection, which acts in a similar way to transferrin to bind iron and prevent its accessibility to pathogens.

1.5.2 Pathogenic mechanisms

Once a pathogen has invaded a host, it must take advantage of the environment it is in to obtain nutrients like iron and continue its lifespan. In order to do this, a pathogen may secrete iron-chelating molecules called siderophores.[54] Siderophores will bind to iron at a higher affinity than a host's transferrin, meaning that siderophores are more likely to take up available iron than host transferrin.[2,54] Then, receptors on the outer membrane of the pathogen can identify those siderophores bound to iron. The iron can then be reduced via ferric reductase enzymes to a

biologically active form that can then be taken up by the pathogen. The host may combat pathogenic mechanisms of iron sequestering. In response to iron-bound siderophores, a host may produce lipocalin-2.[54] This protein can bind to iron-bound siderophores, rendering them unavailable to pathogens. Lipocalin-2, therefore, is seen as a surrogate marker for assessing severity of infection. In addition to siderophores, pathogens also have receptors for transferrin, lactoferrin, and hemoglobin.[54] Thus, although these proteins provide protection of iron, they can also be taken up by the bacteria.[55]

1.6 Non-typhoidal *Salmonella enterica*

Non-typhoidal *Salmonella enterica* species are gram-negative, rod shaped pathogenic bacteria estimated to cause more human deaths worldwide than any other bacterial diarrheal disease agent.[56] In fact, *Salmonella* infection is the most common bacterial foodborne illness experienced worldwide.[57] Infection with non-typhoidal *Salmonella enterica* is referred to as salmonellosis, which typically causes gastroenteritis resulting in nausea, vomiting, and diarrhea.[58] Over 2,600 serovars of non-typhoidal *Salmonella enterica* exist, with each having different characteristics including varying levels of virulence for specific hosts (such as humans, cattle, or poultry).[57,58] *Salmonella* is the second most commonly identified diarrheal-disease causing pathogen confirmed by laboratory diagnosis in the United States, but causes more deaths annually than any other bacteria.[59,60] In 2016, the CDC reported that *Salmonella enterica* serovar *Typhimurium* (*S.* Typhimurium) was the third-most commonly identified serotype in the United States.[61]

1.6.1 Infection of the host

The most common exposure to *Salmonella* in humans is via consumption of contaminated food or water. The bacterial load of foods increases rapidly at temperatures ranging from 4 – 60ºC. *Salmonella* infection cases peak in the Summer months due to increased temperatures favoring bacterial growth.[58] Both animal and plant-based foods can be contaminated with *Salmonella* and cause salmonellosis, but infection is most commonly linked to consumption of improperly prepared eggs, poultry, ground beef, and dairy products.[58] In low and middle-income countries lacking public-health-focused water treatments, *Salmonella* infection rates are especially high due to fecal contamination and lack of sanitation practices.[62] A minority (<5% of documented cases) of *Salmonella* infection occurs via nonfood sources, such as host contact with animals, reptiles, or insects.[58,60] For example, one may touch a surface (such as a turtle shell) contaminated with *Salmonella* and then touch their mouth, which provides a route for ingestion of the bacteria.

Following oral ingestion of *Salmonella*, bacteria continue through the gastrointestinal tract, where the bacteria must survive the acidic conditions of the stomach and avoid contact with duodenal bile salts which may lyse the bacterial cells.[58] *S.* Typhimurium expresses an acid tolerance response in the stomach which combats the low pH of the stomach by increasing intracellular pH.[63] Successfully avoiding initial host defense mechanisms, the bacteria attach to the distal ileum and large intestine where they colonize in humans.[64] Following colonization, *Salmonella* may invade enterocytes, M cells, phagocytes, and dendritic cells of the intestine using a type III secretion system, known as T3SS1.[65] Most of the Salmonella will invade M cells, part of Peyer's patches in the intestinal epithelial barrier.[63] The T3SS1 involves coordinated expression of specific proteins (including SipA, SipB, and SipC) to create a needle-like complex

which injects effector proteins into host cells (such as intestinal cells) and causes host cell cytoskeleton rearrangement, creating membrane ruffles that allow the bacteria to enter the host cell.[65–67] T3SS1 effectors can also modify epithelial tight junction proteins including occludins and claudins.[68] Once inside the host cell, the bacteria exists within a *Salmonella*-containing vacuole where it may replicate.[65] The Salmonella-containing vacuole moves towards the nucleus and Golgi apparatus, which is believed to allow for convenient uptake of nutrients and fragments of organic molecules, that the bacteria uses for fuel.[63] Within the *Salmonella*-containing vacuole, proteins such as SpvB and SlrP are expressed which induce apoptosis of epithelial cells. *Salmonella*-containing vacuoles may then transcytose the basolateral membrane, thereby successfully crossing the epithelium of the large intestine. Then, phagocytes including neutrophils, inflammatory monocytes, and dendritic cells engulf the *Salmonella* in an effort to eliminate the foreign matter from the body. Interestingly, Peyer's patches and mesenteric lymph node knockout mice have shown that these tissues are not necessary for infection, suggesting that enterocyte transcytosis alone can lead to clinical manifestation of salmonellosis.[69] Invading lymphoid tissue provides a route to the systemic circulation. Once in the bloodstream, *Salmonella* may spread to other organs, causing a systemic infection. This systemic infection may cause death due to infection of vital organs, such as the liver.[69]

An important element in *Salmonella* infection is the microbial composition of the host's gut. A microbiome which contains commensal bacteria may prevent *Salmonella* from colonizing – this is termed "colonization resistance" of the host.[67] Commensal bacterial growth in the large intestine is increased with host consumption of fiber. Specific fibers have been shown to preferentially increase growth of certain commensal bacteria – for example, galacto-oligosaccharides increase growth of *Bifidobacteriaceae* and *Lactobacillaceae.*[70] Increased

presence of these bacteria due to fiber supplementation has been associated with decreased respiratory tract infections in infants.[71] This is thought to be due to modulation of immune system functioning, and thus, this protection could extend to protection from enteric infections. Administration of other commensal bacteria, like *Escherichia coli* strain Nissle 1917, has been shown to decrease *S.* Typhimurium in a mouse model.[72] In contrast, the mouse model of *S.* Typhimurium infection used for the experiment in this thesis involves administration of a broad-spectrum antibiotic before introduction of *S.* Typhimurium.[69] Therefore, the antibiotic presumably destroys the bacteria in the large intestine, which allows for *S.* Typhimurium to successfully colonize and cause infection.

1.6.2 Symptoms and complications of infection

Infection with non-typhoidal *S.* Typhimurium causes mild to severe gastroenteritis; it may also spread and cause life-threatening complications. Symptoms of gastroenteritis caused by *Salmonella* include diarrhea, abdominal cramps, fever, nausea, and vomiting.[64] These symptoms typically appear within 24-48 hours after ingestion of the bacteria and may last anywhere from 2-7 days, on average. If systemic infection occurs, the symptoms may be more severe and possibly life-threatening. The vomiting and diarrhea involved in salmonellosis result in dehydration. This may be life-threatening for both the elderly (65+ years) and young children (<5 years). Most people will experience salmonellosis and manage symptoms at home/without assistance, but in some cases, hospitalization is required. Hospitalization will typically involve rehydrating the body via intravenous saline infusion.

1.6.3 Prevalence and populations vulnerable to infection

Despite having one of the safest food supplies in the world, there are over 1.2 million reported cases of *Salmonella* infection every year in the United States alone.[60] Of those, 23,000 end in hospitalization, and 450 result in death.[60] Worldwide, there are an estimated 95.1 million cases yearly which lead to over 50,000 deaths.[73] These numbers are likely underestimated due to a lack of reporting of foodborne illness and a lack of infected adults seeking medical care.

Forty percent of yearly documented cases of foodborne illness are in children under age 5.[56] Although children are a population highly at risk from foodborne illness, this number should be interpreted with caution, since many adults with foodborne illness do not seek medical care, artificially creating a higher documented percentage of children experiencing foodborne illness. Gastric acid typically has a pH $\leq$2, which has been demonstrated to kill *S.* Typhimurium along with other foodborne pathogens.[74] Thus, elderly individuals and those on commonly prescribed medications such as proton pump inhibitors or H_2 antagonists are at an increased risk of infection.[74,75] Pregnant women are another group of individuals who are of higher concern in terms of infections, including those that are foodborne.[76] Thus, the infection may be life-threatening, although this is typically not a common occurrence. Because the fetus is genetically different from the mother, the mother's immune system must be slightly downregulated to allow for the growth and support of the fetus, rather than the rejection of it. With decreased immune function comes more susceptibility of both mom and her fetus to infection.

A weakened immune system may be due to many different factors, including malnutrition.[77] Many nutrients are involved in immune system function, and specifically iron is a well-documented regulator of T lymphocytes.[78] Iron deficiency has been shown to impair neutrophils and natural killer (NK) cell activity. Therefore, iron deficiency may predispose one

to increased risk of infection, however this may not be apparent without anemia. Areas of Sub-Saharan Africa are documented to have more cases of non-typhoidal *Salmonella* infection per capita than any other region in the world.[73] These areas also have widespread iron supplementation programs in place designed to address malnutrition.

1.7 Relationship between *Salmonella enterica* and iron

Iron is an essential nutrient for growth of all gram-negative bacteria, including *S.* Typhimurium. Well-controlled investigations specifically related to *S.* Typhimurium and iron are limited, but the host-bacterial competition for iron has been previously described and is a well-established proof of the nutrient requirement.[54] Iron may also act as a regulator of protein expression in bacteria. For example, about 7% of the genome of *S.* Typhimurium is controlled, either directly or indirectly, by the presence of iron.[79] Recent work by Karash and Kwon has identified iron-dependent genes specific to *S.* Typhimurium that are essential for growth and homeostasis of the bacterium.[80] Testing the relative importance of iron *in vivo* is difficult and costly, because the minimum estimated requirement of iron for pathogens is about 10^{-18} M, which is close to zero.[81] However, rather than focus on minimizing iron availability, the supplementation of iron is a practical approach which has provided interesting insights to the relationship of iron with growth and virulence of pathogens *in vivo*.

1.7.1 Uptake and utilization of iron in *Salmonella enterica*

Presence of iron in the environment alone is not enough to affect *S.* Typhimurium behavior. In order for the pathogen to benefit from the nutrient, it must first be taken up and then utilized. This thesis will focus on *Salmonella* use of iron found in the large intestine. As

previously mentioned, dietary iron consumed by the host may exist as heme or nonheme iron. Since less than 40% of all consumed iron is absorbed, the unabsorbed iron is what will travel through the gastrointestinal system to the large intestine. Thus, the large intestine may be an environment rich in both heme and non-heme iron.

As previously mentioned, *Salmonella* secrete siderophores, iron-chelating molecules which scavenge for iron in the environment. The pathogen can then take up these iron-siderophore complexes via surface receptors to engulf the nutrient. *S.* Typhimurium has a unique gene, iroN, which controls expression of proteins that act as surface receptors for siderphores.[82] However, there needs to be an initial biochemical signal for the pathogen to scavenge for iron. The ferric uptake regulator (Fur) is a negative transcriptional regulator responsible for several iron-responsive genes.[83] When Fe^{2+} is present in the environment of the pathogen, the Fur protein is activated which prevents expression of the proteins which scavenge and uptake iron.[83] Due to the Fur regulator's role in preventing iron uptake, it is believed that this is to control intracellular iron concentration to prevent toxicity. Fur is involved, directly or indirectly, in the production of a number of proteins including ferritin, T3SS-1 proteins, nitrate/nitrite respiration, and acid tolerance response.[84] Some of these traits are directly related to virulence, while all of them can contribute to infection of the host and replication of the bacteria. Free iron can diffuse through the outer layer of gram-negative bacteria like *Salmonella* which then allows for use of the Fur-dependent FeoABC system to actively transport Fe^{2+} into the cytoplasm.[84,85] FeoABC consists of membrane bound protein FeoB, along with two cystolic proteins, FeoA and FeoC.[85] FeoB utilizes active transport to facilitate Fe^{2+} transport across the plasma membrane. FeoC binds to intracellular iron and causes transcriptional changes.[85] FeoA has an unknown role in the

process of iron transport.[85] Once iron is transported into the cytoplasm, it may now be available for various intracellular functions to support the life and virulence of the pathogen.

1.7.2 Function of iron in *Salmonella enterica*

Iron has similar functions in *Salmonella enterica* as it does in human cells. In the cytoplasm, iron may act as a cofactor for enzymes performing biochemical reactions related to electron transport, glycolysis, DNA synthesis, and defense against reactive oxygen species.[86] Genes specific to *S.* Typhimurium identified to require iron include those related to synthesis of RNA, DNA, proteins, and cell walls.[80] There are also iron-dependent genes related to DNA replication, Coenzyme A function, fatty acid metabolism, glutamine metabolism, protein transport, transcription, and translation.[80] Within the iron dependent genes of the *S.* Typhimurium genome, 37.8% are related to metabolic pathways, while 15.1% have unknown function, 14.3% are related to ribosome function, and the remaining have various roles.[80] Therefore, a majority of *S.* Typhimurium consumed iron may be used for basic metabolism to support the growth of the pathogen.

This is a logical explanation as to why *S.* Typhimurium exposed to increasing iron concentrations has been associated with increased growth. Kortman et al. cultured *S.* Typhimurium in iron-free media spiked with increasing levels of ferric citrate (0, 0.01, 0.2, 50, 1000 µmol/L) and found a dose-dependent increase in the growth of *S.* Typhimurium with increasing concentrations of ferric citrate.[87] In line with these results, Parmanand et al. utilized a cellular model to assess *S.* Typhimurium growth in the presence of differing concentrations of iron chelators. In this model, *S.* Typhimurium was incubated with 0, 10, 20, and 50 µM of an iron chelator and growth was assessed after 24 hours. An inverse relationship was observed with

increased growth of *S.* Typhimurium with decreasing concentrations of the iron chelator. This information further emphasizes the point that iron is required for growth of *S.* Typhimurium.

1.7.3 Iron and virulence

Virulence of *S.* Typhimurium in relation to iron has been tested in several *in vitro* studies. It is known that several virulence genes of *S.* Typhimurium are dependent on iron.[1,80,88] Kortman et al. assessed the virulence of *S.* Typhimurium at the intestinal epithelial interface with differing levels of ferric citrate.[87] To perform this experiment, Caco-2 cells were grown under standard conditions and allowed to differentiate. *S.* Typhimurium was grown in media containing increasing concentrations of ferric citrate as described above. The *S.* Typhimurium was then pelleted and resuspended in iron-free media before being applied to the Caco-2 cell monolayers for 2 hours. Adhesion of *S.* Typhimurium the Caco-2 cells increased with increasing ferric citrate concentrations. Similarly, the ability of *S.* Typhimurium to invade Caco-2 cells increased with increasing ferric citrate concentrations. Finally, using Transwell inserts the researchers assessed the ability of *S.* Typhimurium to translocate from the apical chamber to the basolateral chamber and again found a dose-dependent response with increased translocation with increasing levels of ferric citrate. Taken together, findings from this *in vitro* study indicate that increased availability of iron to *S.* Typhimurium leads to increased virulence, which potentially means an increased severity of infection.

Therefore, an important relationship between *S.* Typhimurium and iron is documented in a cellular model, but there are currently no studies testing this relationship *in vivo*. In an *in vivo* model, *Salmonella* colonize in the gut, and if there is more iron available in the gut, the *Salmonella* may be able to grow, attach, invade, and translocate the epithelial barrier more

efficiently. In addition, the populations of interest in both the high-risk group of iron deficiency and foodborne infection have a clear overlap. To review, iron deficiency is a high-risk situation with children, the elderly, and women of reproductive age (both pregnant and not pregnant). Similarly, those at particular risk of foodborne illness include children, the elderly, pregnant women, and immunocompromised individuals.

One such area with high incidence of both iron deficiency anemia and non-typhoidal *Salmonella* infection is Sub-Saharan Africa. Non-typhoidal *Salmonella* infection rates are high in this area due to a lack of public health standards (like water treatment). To address malnutrition in this area, iron-containing multivitamin/mineral supplements are used, which are poorly absorbed. This may create an iron-rich environment within the large intestine that a potential pathogen may utilize when infecting a host. Thus, iron supplements may contribute to the increased severity of enteric infections. This thesis sets out to test if dietary iron supplementation is associated with increased severity of *S.* Typhimurium infection *in vivo*.

CHAPTER 2

INTRODUCTION

Iron deficiency is a common nutrient deficiency in both low- and high-income countries.[39] Left untreated iron deficiency may lead to microcytic anemia, which is a major public health concern due to increased rates of mortality and declines in cognitive and functional outcomes.[39] The WHO and other international organizations recommend supplementing with oral iron, such as pills or micronutrient powders, as a cost-effective strategy to prevent iron deficiency.[89] Although this is an effective strategy to correct or prevent iron deficiency, only ~10-15% of iron from these sources is absorbed, and the remaining iron travels to the large intestine.[90] Thus, iron supplementation creates an iron-rich environment in the large intestine, which may promote the growth of enteric pathogens.[48]

Salmonella are a common enteric bacterial pathogen and leading cause of morbidity and mortality in humans.[91] Following ingestion of contaminated food and/or water, *Salmonella* may colonize the lower intestine (ileum and large intestine), invade the intestinal epithelium, and proliferate. The infection, called salmonellosis, causes an inflammatory response that results in gastrointestinal pain, fever, nausea, vomiting, and diarrhea. Symptoms typically occur 6-72 hours after ingestion of *Salmonella* and last approximately 4-7 days. Most individuals recover without treatment, especially in high-income countries; however, in some cases infection can spread from the intestines to the lymph nodes and blood and to other locations in the body, which can lead to death. Children, the elderly, and people with weakened immune systems typically have more severe infections.[73]

Kortman et al. documented a dose-dependent increase in growth when *Salmonella* enterica subsp. enterica serovar Typhimurium (*S.* Typhimurium), one of the most common

serovars of *Salmonella* known to cause salmonellosis in humans, were grown in media containing increasing concentrations of ferric citrate.[92,87] Using Caco-2 cells as an *in vitro* model of the intestinal epithelium, the study found increased adherence, invasion, and translocation of *S.* Typhimurium exposed to increasing concentrations of ferric citrate. These findings suggest that *S.* Typhimurium may be more virulent when exposed to iron, leading to a more severe infection. The objective of this study was to determine if the severity of *S.* Typhimurium infection is increased in an iron-supplemented host, compared to an iron-deficient host.

CHAPTER 3

METHODS

3.1 Animals and diets

All animal procedures were approved by the Florida State University Animal Care and Use Committee (Protocol #2019-00005) (**Appendix A**). Female weanling C57BL/6 mice (n=60, Envigo, Madison, WI) obtained at 21 d of age were individually housed and randomly assigned to 1 of 2 diets (n=30/diet): iron supplemented or iron deficient. Diets were based on AIN-76A formulation and differed only in the amount of iron in the diet (**Appendix B: Table 1**). The iron supplemented diet contained 300 ppm iron as ferrous sulfate (TD.190434, Envigo) and the iron deficient diet contained 2-6 ppm iron (TD.190433, Envigo) (**Appendix C: Table 2**). An iron content of 300 ppm was chosen to represent the amount of iron consumed from an iron supplement, which typically provides 60 mg iron per day. Mice were fed their respective diets for 6 weeks to create an iron-deplete or iron-rich environment in the lower intestine and continued on their diets for the remaining study period. Animal bodyweight, feed, and water intake were monitored every-other-day.

3.2 Streptomycin-induced mouse model of *S.* Typhimurium infection

This study used the streptomycin-induced mouse model of *S.* Typhimurium infection.[69] After 6 weeks of feeding, mice were administered 100 µL streptomycin (200 mg/mL; S7739, PhytoTech Labs) by oral gavage. Twenty-four hours later, mice were infected with 10^8 colony forming units of *S.* Typhimurium (ATCC 14028) or saline placebo by oral gavage (n=15/diet). Feed was removed for 3 h prior to streptomycin and *S.* typhimurium treatments. Thus, four

experimental groups exist: iron supplemented control (FeS^{Ctrl}), iron deficient control (FeD^{Ctrl}), iron supplemented infected ($FeS^{S.Tm}$), and iron deficient infected ($FeD^{S.Tm}$).

3.3 Preparation of *S.* Typhimurium

A sterile loop was used to transfer *S.* Typhimurium into 9 mL of tryptic soy broth (TSB) and cultured at 35ºC overnight. The next day, a sterile loop was used to transfer inoculated TSB into a new tube of TSB. This process was repeated for 5 days in total. On the morning of infection (day 6), cultured TSB was poured into a 15 mL conical tube and centrifuged at 7000 *x g* for 10 minutes at 20ºC. The supernatant was poured off, leaving behind the concentrated *S.* Typhimurium pellet. The pellet was washed with phosphate buffered saline (PBS) and centrifuged at 7000 *x g* for 10 minutes at 4ºC. The PBS supernatant was then poured off and the pellet was resuspended in PBS. To confirm the *S.* Typhimurium dose, a serial dilution of the *S.* Typhimurium was made and twice plated at 10^{-8} and 10^{-9} concentrations on nutrient agar. These plates were cultured overnight at 35ºC and colonies were counted the next day.

3.4 Infection monitoring and early endpoint scoring

Mice were monitored daily after administration of streptomycin and assessed using an early endpoint scoring system (**Appendix D**) to monitor change in bodyweight, feed intake, water intake, body condition score, and physical appearance. Body condition score was based on the scoring system by Ullman-Culleré and Foltz (**Appendix E**).[93] Mice were euthanized early if they lost >20% of bodyweight pre-streptomycin, did not eat or drink water for consecutive days, had a body condition score of 1, or a physical appearance score of 3.

3.5 Tissue collection

Euthanasia was performed by CO_2 asphyxiation, followed by immediate cardiac puncture and collection of blood into lithium-heparinized tubes. Tissue was collected immediately post-euthanasia, weighed, and kept on dry ice before storage at -80ºC. Cecum, liver, and spleen were collected. Spleen index was calculated by dividing the weight of the spleen (in milligrams) by the bodyweight of the mouse (in grams) to produce a number with arbitrary units (A.U.). Whole blood was used to obtain hematocrits and then centrifuged to separate into plasma and red blood cells.

3.6 Tissue iron

Serum and tissue iron concentrations were measured using a colorimetric kit (Stanbio Iron and TIBC Kit). Transferrin saturation was calculated by dividing serum iron by total iron binding capacity (TIBC). To determine liver and spleen iron concentrations, samples were homogenized in PBS using a handheld sonicator device. The homogenized solution was added to protein precipitation solution, vortexed, and then placed in a 95ºC heat block for 1 hour. Heated mixtures were then centrifuged at 12,000 *x g* for 20 minutes at 20ºC and the supernatant was transferred to a new tube. The iron concentration of the supernatant was determined using the Stanbio Iron and TIBC Kit. Briefly, a buffer was added to the supernatant and absorbance measured at 560 nm. After addition of a color reagent, the samples were incubated for 10 minutes and absorbance was measured at 560 nm. These values were used to calculate the iron content of the samples using the equation: $[(A2-A1)/(A2_{Standard}-A1_{Standard})]*500$.

3.7 RNA isolation and real time quantitative PCR (RT-qPCR)

RNA was isolated using Trizol as described by the manufacturer (Invitrogen). Complementary DNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher). TaqMan gene expression assays were used to assess liver hepcidin mRNA (Mm04231240_s1) by RT-qPCR. *Actb* (Mm02619580_g1) was used as the housekeeper gene. Fold change was calculated using the ΔΔCT method.

3.8 Statistical Methods

Data are reported as means ± standard deviations. To assess differences in iron and food consumption pre-infection between FeD and FeS groups, independent t-test was used. To assess iron status and infection severity outcomes post-infection, 2-way analysis of variance was used and effects of diet, infection, and their interaction were analyzed. Post-hoc comparisons were made using Tukey's test. P-values ≤ 0.05 were considered statistically significant. Data were analyzed using GraphPad Prism version 8.4.1.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Iron status and tissue iron

FeS and FeD consumed similar amounts of feed (FeS, 2.6 ± 0.2 g/d; FeD, 2.6 ± 0.2 g/d, P=1.0) and bodyweights did not differ (FeS, 21.2 ± 2.1 g; FeD, 21.1 ± 1.8 g, P=0.86) during the 6-week feeding period. By design, FeS consumed more iron (171.9 ± 17.0 mg iron/day) compared to FeD (3.5 ± 0.3 mg iron/day, P<0.001).

There was a significant diet-by-infection interaction (P=0.009) for hematocrit. Hematocrits were 16% lower in FeD^{Ctrl} mice compared to FeS^{Ctrl} (P=0.005). Infection reduced hematocrits in $FeD^{S.Tm}$ and $FeS^{S.Tm}$ compared to FeS^{Ctrl} (P<0.05 for both) (**Appendix F: Figure 1A**).There was a main effect of diet (P<0.01) but no effect of infection (P=0.79) or interaction (P=0.57) for transferrin saturation. Transferrin saturation increased by 18% in FeS compared to FeD mice (**Appendix F: Figure 1B**). Liver hepcidin mRNA increased at least 2-fold with a high iron diet, but not infection (data not shown).

There was a significant effect of diet (P<0.0001), but not infection (P=0.10) or interaction (P=0.62) for spleen iron content. Spleen iron was significantly higher in FeS compared to FeD groups (**Appendix G: Figure 2A**). Similarly, there was a significant effect of diet (P<0.0001), but not infection (P=0.44) or interaction (P=0.39) for liver iron content. Liver iron was significantly higher in FeS compared to FeD groups (**Appendix G: Figure 2B**).

Effects of diet (P<0.001), infection (P<0.001), and interaction (P<0.001) were observed for cecal content iron concentrations. FeS^{Ctrl} had 58 times greater iron in the cecum compared to FeD^{Ctrl} (P<0.001). Cecal iron concentrations were also greater in $FeS^{S.Tm}$ compared to $FeD^{S.Tm}$;

however, $FeS^{S.Tm}$ had 80% less iron in the cecum compared to FeS^{Ctrl} ($P<0.001$) (**Appendix G: Figure 2C**).

4.2 Severity of Infection

There was a significant diet-by-infection interaction for cecal weights ($P<0.02$). Cecal weights were 40% lower in $FeS^{S.Tm}$ compared to $FeD^{S.Tm}$ ($P=0.02$) (**Appendix H: Figure 3A**).

A significant diet-by-infection interaction was observed for spleen index ($P=0.05$). Both $FeD^{S.Tm}$ and $FeS^{S.Tm}$ had higher spleen index values than FeD^{Ctrl} and FeS^{Ctrl}. Spleen index was 75% greater in $FeS^{S.Tm}$ compared to $FeD^{S.Tm}$ ($P=0.005$) (**Appendix H: Figure 3B**).

There was a significant diet-by-infection interaction for bodyweight loss ($P=0.05$). Both control groups gained bodyweight over the infection period (FeD^{Ctrl}, 9.8 ± 4.8% and FeS^{Ctrl}, 9.5 ± 3.6%, $P=0.99$). Both infected groups lost bodyweight post-infection ($FeD^{S.Tm}$, -14.51 ± 6.33% and $FeS^{S.Tm}$, -21.73 ± 5.44%, $P<0.001$). $FeS^{S.Tm}$ lost significantly more bodyweight than $FeD^{S.Tm}$ by euthanasia ($P=0.004$) (**Appendix H: Figure 3C**).

4.3 Discussion

To our knowledge, this is the first study to assess severity of *S.* Typhimurium infection in C57BL/6 mice consuming an iron supplemented diet (at 300 ppm iron) compared to an iron deficient diet (2-6 ppm iron). The major finding was that mice consuming an iron supplemented diet had a more severe infection with *S.* Typhimurium compared to mice consuming an iron deficient diet. These findings suggest that unabsorbed iron may be a nutrient source for *S.* Typhimurium leading to a more severe enteric infection.

Previous studies have assessed severity of infection in iron supplemented animals with other enteric pathogens, such as *Citrobacter* Rodentium.[94] Importantly, animals in the present study were provided iron via the diet, whereas other studies have provided iron via peritoneal injection, which bypasses iron absorption in the small intestine. This was necessary for the present study because unabsorbed luminal iron is the suggested nutrient source for *S.* Typhimurium. The iron supplemented diet used in the current study contained 300 ppm iron, which was intended to mimic a human taking a typical (60 mg) iron supplement. This is important to note as some standard chow diets contain upwards of 200-300 ppm iron without the intention of being iron supplemented.[94] For example, the widely used NIH-07 diet contains 350 ppm iron.[95] Thus, 300 ppm iron is a relatively low amount compared to other studies that have examined the relationship between iron and infection. For example, Zhang et al. fed mice either a low (2-6 ppm iron) or high (1600 ppm iron) iron diet for 3 or more months and examined risk of malaria infection.[96]

It is also important to note the many different sources of non-heme iron in the diet, all of which may have varying bioavailability. To date, most iron supplementation strategies include ferrous sulfate ($FeSO_4$) due to its relatively high bioavailability compared to other forms of iron.[97] In the context of enteric infection, high bioavailability may be advantageous to the host as more of the nutrient will be absorbed, leading to less unabsorbed iron in the digestive tract and potentially available to an enteric pathogen. The bioavailability of the unabsorbed iron is therefore important to enteric pathogens like *S.* Typhimurium. Costa et al. compared the growth of *S.* Typhimurium grown in nutrient broth (NB) or NB containing 40 µM $FeSO_4$, 40 µM $FeCl_3$, or the iron chelator dypiridyl for 24 hours.[98] The growth of S. Typhimurium was similar among the NB, NB+$FeSO_4$, and NB+$FeCl_3$ groups, while significantly less growth was observed in the

iron chelator group. These results suggest that, at least when comparing two types of iron salts, the source of iron may not be as important as the absolute presence or absence of iron in the environment for *S*. Typhimurium growth. Therefore, to combat this growth, minimizing the amount of iron in the lumen of the digestive tract is important.

Typical exposure to *Salmonella* occurs via consumption of contaminated food or water. After ingestion, the pathogen then continues through the digestive tract, leading to its colonization in the large intestine. Similar to other enteric pathogens, *Salmonella* species utilize iron and cannot thrive if the essential nutrient is not available.[99] The current study demonstrates that there is increased iron in the lumen of the large intestine of mice fed diets containing higher levels of iron compared to an iron deficient diet, and that an iron-rich environment in the intestine increases the virulence of *Salmonella*. Within the large intestine, there is a competition between the host and pathogen over essential nutrients, with one of the most well-documented being iron.[55] Hosts have developed mechanisms to withhold iron from invading pathogens, while pathogens have developed mechanisms to steal iron from the host. For the host, iron is obtained via the diet and absorbed into circulation via the small intestine. In response to infection and inflammation, the liver-derived protein hepcidin inhibits iron absorption by degrading ferroportin.[54] Thus, a higher amount of iron remains in the lumen, which may be utilized by an enteric pathogen. This is consistent with findings from the current study that showed increased liver hepcidin mRNA with a high iron diet compared to a low iron diet. Interestingly, hepcidin was not further increased with infection, suggesting that diet had a more significant effect on hepcidin mRNA transcription. Iron that has been absorbed into an enterocyte is stored associated with the protein ferritin, which is a further mechanism for withholding the iron from pathogens in the lumen.[10] A majority (80%) of the body's iron is found in hemoglobin molecules in the

blood. One way to measure this is via hematocrit, as an expression of packed red blood cell percentage. In the present study, there was a diet-by-infection interaction for hematocrit; infection decreased hematocrit concentrations to levels observed in mice fed the iron deficient diet compared to the iron supplemented diet, suggesting that less iron-containing hemoglobin molecules were being synthesized.

Pathogens have developed effective mechanisms to acquire iron.[54] Pathogens scavenge for iron through siderophores, which are iron-chelating molecules that bind to iron at higher affinity than transferrin.[55] Once bound to iron, pathogens utilize surface receptors to identify siderophore-iron complexes and subsequently release the bound iron via ferric reductase enzymes.[54] In response to siderophores, host neutrophils release lipocalin-2, which sequesters siderophores, making them unable to benefit the pathogen.[55] Once *S.* Typhimurium has access to reduced iron, the ATP-dependent transport protein FeoB imports the nutrient into the cytoplasm.[99,100] In the cytoplasm, iron is used for various biochemical reactions that support the life, and ultimately virulence, of the pathogen. For example, reactions regarding cellular respiration, metabolism, and repair of DNA all require iron as a cofactor.[101] Iron also plays a role in *Salmonella* regulatory proteins including Fur, Fnr, NorR, SoxR, IscR, and NsrR.[101] By limiting the iron entering the gastrointestinal system, viability of *Salmonella* was likely limited, which is a potential explanation for iron deficient animals experiencing a less severe infection.

$FeS^{S.Tm}$ lost significantly more bodyweight during the course of the infection compared to $FeD^{S.Tm}$. The bodyweight loss observed in the current study is in line with previous studies. For example, Ren et al. documented a bodyweight loss of 20% after 4 days of infection with *S.* Typhimurium.[102] This study used doses ranging from 1×10^6 to 3.4×10^8 CFU and observed similar bodyweight loss results across doses and varying ages (4-24 months old) consuming an

unspecified diet which likely contained high iron content.[102] In enteric infections, a majority of bodyweight loss is due to diarrhea complications, while a smaller portion may be related to decreased food intake. Diarrhea may be a life-threatening complication, especially for those prone to dehydration, such as infants and the elderly. Iron fortification has been shown to induce diarrhea, especially in infants.[103] It is unlikely that this was the primary source of bodyweight loss in the current study, since the FeS^{Ctrl} mice did not lose bodyweight during the infection. Therefore, the bodyweight loss in this study is likely due to a more severe infection occurring in the iron-supplemented animals, leading to increased water loss. Fluid loss associated with diarrhea leads to increased mortality in humans and mice.[104,105] Therefore, if the infection had continued beyond 20% bodyweight loss (which was part of the early endpoint scoring system criteria in the present study), there is reason to believe that those mice may have died without intervention. These results are in line with previous studies that have shown a >20% bodyweight loss 6 days after infection of 10^9 CFU *S.* Typhimurium in mice fed 500 ppm $FeSO_4$ diets, while mice fed a 250 ppm $FeSO_4$ diet lost less than 20% bodyweight; however, differences between groups were not statistically significant.[106] Interestingly, Kortman et al. found that mice infected with *Citrobacter* Rodentium and fed an iron deficient (<6 ppm iron) diet lost similar amounts of bodyweight as both mice fed iron supplemented (225 ppm iron) and normal (45 ppm iron) iron diets, however the iron deficient group recovered their bodyweight while the other two groups did not.[94] This may have occurred because the animals consuming the iron deficient diet likely absorbed a majority of the virtually nonexistent iron in the chow, while animals consuming the other diets did not absorb all of the iron from the chow. The unabsorbed iron could have acted as a nutrient source for the bacteria, therefore hindering the mouse ability to recover from the

infection. This also highlights that increased infection severity from dietary iron content is not limited to *Salmonella* genus.

The weight of the cecum decreased significantly in $FeS^{S.Tm}$ compared to all other groups. The streptomycin-induced mouse model of *S.* Typhimurium infection used in this study has shown that mice infected with *S.* Typhimurium have significantly lower cecum weights compared to controls.[69] It is documented that decreased cecum weight is associated with *S.* Typhimurium infection.[69,107] Cecum weight may have decreased due to increased diarrheal disease, as previously mentioned. Consistent with our results, Grassl et al. documented a >30% decrease in cecum weight in mice infected with 3×10^6 CFU *S.* Typhimurium after seven days of infection.[108] Barman et al. assessed cecum weight in mice infected with 10^8 CFU *S.* Typhimurium and found a significant decrease in bodyweight compared to controls by day 3 and bodyweight continued to decline until day 7.[109] It has previously been shown that mice infected with *Salmonella* Enteritidis grown in egg yolk had increased severity of infection, as noted by a greater decrease in cecal weight (0.6 g in 48 hours) compared to groups losing <0.4 g cecal weight.[110]

In the current study, spleen index of the $FeS^{S.Tm}$ animals was higher than the spleen index of the $FeD^{S.Tm}$ animals. The spleen is an important organ of the host immune system. In response to infection, the spleen weight increases in size and weight as part of the immune response.[111] Spleen weight is known to increase with *S.* Typhimurium infection, and mouse strains resistant to *S.* Typhimurium tend to have smaller spleens after infection than mouse strains not resistant to infection.[107] Betz et al. administered 10^9 CFU S. Typhimurium via oral gavage to C57BL/6 mice and reported spleen weights almost double those of uninfected control mice.[112] Chen et al.

assessed spleen weight in a model of *Escherichia coli* infection, reporting significantly increased spleen weight in infected mice compared to control mice.[113]

While iron supplementation may have associated complications, it is still important to provide iron supplements to individuals whose diets lack the nutrient. In 2019, Stanaway et al. conducted a systematic review and calculated the incidence of non-typhoidal *Salmonella* infections, which are typically associated with diarrheal disease.[73] In a 27-year period starting in 1990, sub-Saharan Africa had the highest incidence of infection for all years compared to every other geographical location worldwide.[73] In 2008, the WHO published a systematic review that calculated the incidence of anemia worldwide between the years 1993-2005.[36] This report indicates that, throughout the world, within preschool-age children, pregnant women, and non-pregnant women of reproductive age, Africa has the highest incidence of anemia out of all other geographical locations.[36] This report also indicates that iron deficiency is responsible for more cases of anemia than any other cause, and that iron deficiency should be corrected with iron-containing supplements.[36] Therefore, an overlap exists in sub-Saharan Africa where there are high rates of infection coincide with high rates of iron supplementation. Considering the results of this study, an area with high rates of non-typhoidal *Salmonella* infections needs both a safe and effective way of correcting iron deficiency. Alternative iron supplementation strategies or forms of iron may be required to correct iron deficiency while preventing increased severity of infection.[114,115] This may include developing novel forms of iron, or developing alternative supplementation stategies.[115,116] For example, Bries et al. fortified the *Aspergillus* Oryzae fungus, which has the capability of taking up a large amount of iron and found increased absorption of the iron compared to traditional ferrous sulfate.[115] In addition, Uyoga et al. compared iron dosing schedules, and found that alternative-day supplementation increased iron absorption compared to

consecutive-day supplementation.[116] These strategies may increase absorption of iron, therefore decreasing the iron content of the lower intestine, and reducing the potential virulence of enteric pathogens.

4.3.3 Conclusions

The current study suggests that dietary iron supplementation creates an iron-rich environment in the lower intestine of mice, which is associated increased severity of *S.* Typhimurium infection. These findings provide evidence for the need to create improved iron supplementation programs, especially in areas where infection may be common.

APPENDIX A

IACUC APPROVAL ASSURANCE LETTER

FLORIDA STATE UNIVERSITY

ANIMAL CARE AND USE COMMITTEE [ACUC]
101 BIOMEDICAL RESEARCH FACILITY
TALLAHASSEE, FL 32306-4341
TELEPHONE: 644-4262 FAX: 644-5570
MAIL CODE: 4341

October 15, 2020

The Graduate School
Florida State University

To Whom It May Concern:

Concerning the thesis/dissertation submitted to the Graduate School by:

Graduate Student:	**James Ippolito**
Thesis/Dissertation Title:	**Dietary Iron Supplementation Increases Severity of Salmonella**
Department:	**Nutrition, Food, and Exercise Sciences**
Major Professor:	**Dr. Stephen Hennigar**

The graduate student named above has provided assurance to the FSU Animal Care and Use Committee that all animal procedures utilized in work resulting in this thesis/dissertation are described in FSU ACUC Protocol(s):

Protocol Number	Title	Date ACUC Approval
201900005	Host iron status and the pathogenic potential of *Salmonella typhimurium*	05/13/2019

The Animal Care and Use Committee has confirmed that this student was included as a project member during the period covering their thesis/dissertation work. This institution has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare. The Assurance Number is D16-00491 (A3854-01).

Sincerely,

ACUC Veterinarian
FSU Animal Care and Use Committee

KMH/kjj

cc: James Ippolito
Dr. Stephen Hennigar

APPENDIX B

TABLE 1

Table 1. Ingredient information for the iron supplemented and deficient diets.

Ingredient	**Iron Deficient Diet, TD.190433 (g/kg)**	**Iron Supplemented Diet, TD.190434 (g/kg)**
Casein	200	200
DL-Methionine	3	3
Sucrose	549.89	548.39
Corn starch	150	150
Corn oil	50	50
Vitamin mix (76A)	10	10
Mineral mix, Fe def	35	35
Ferrous sulfate	0	1.5
Choline bitartrate	2	2
Ethoxyquin	0.01	0.01

APPENDIX C

TABLE 2

Table 2. Nutrition information for the iron supplemented and deficient diets.

Nutrient	**Iron Deficient Diet, TD.190433**	**Iron Supplemented Diet, TD.190434**
Carbohydrate, % kcal	70.4	70.3
Fat, % kcal	11.8	11.8
Protein, % kcal	17.8	17.9
Iron, ppm	<6	300
Energy, kcal/gram	4	4

APPENDIX D

EARLY ENDPOINT SCORING SYSTEM

[1] Study days -1 and 0 are day of streptomycin and *S.* Typhimurium or placebo treatment, respectively.
[2] Δ Body weight >20% of day -1 indicates early endpoint.
[3] Ventral surface temperature (VST) ≥ 23.5ºC indicates early endpoint.
[4] Consecutive days of not eating or drinking may indicate early endpoint.
[5] Body condition score (see Figure 3) ≤1 indicates early endpoint.
[6] Physical appearance: 0, normal; 1, lack of grooming; 2, rough coat, nasal/ocular discharge; 3, very rough coat, abnormal posture, reduced mobility. A score of 3 indicates early endpoint.

	Study Day[1]								
	-1	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Body weight (g)									
Δ Body weight[2]									
VST (ºC)[3]									
Feed intake (g)[4]									
Water intake (mL)[4]									
Body Condition Score[5]									
Physical Appearance[6]									

APPENDIX E

BODY CONDITION SCORING CHART

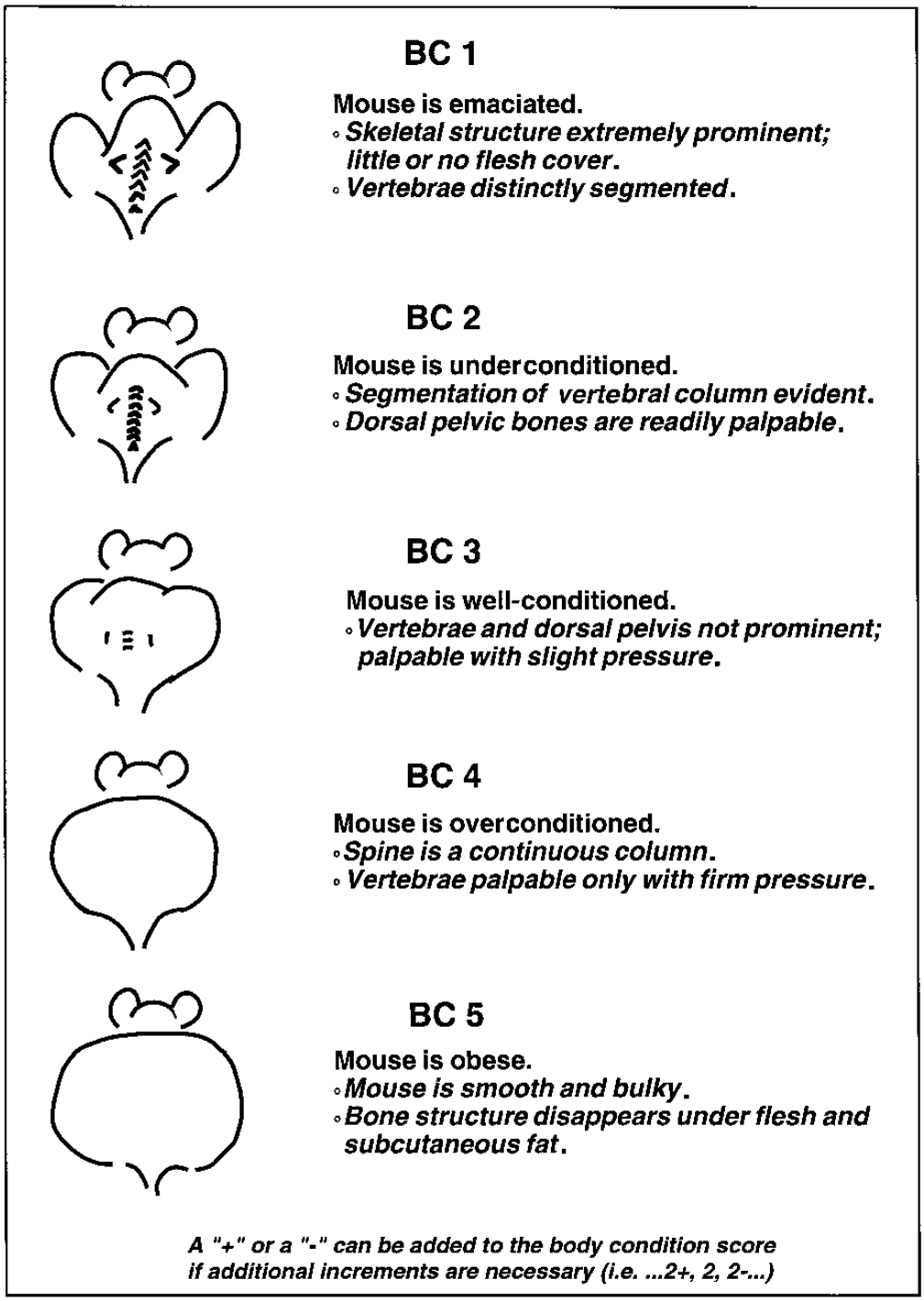

Chart from Ullman-Culleré and Foltz.[93]

APPENDIX F

FIGURE 1

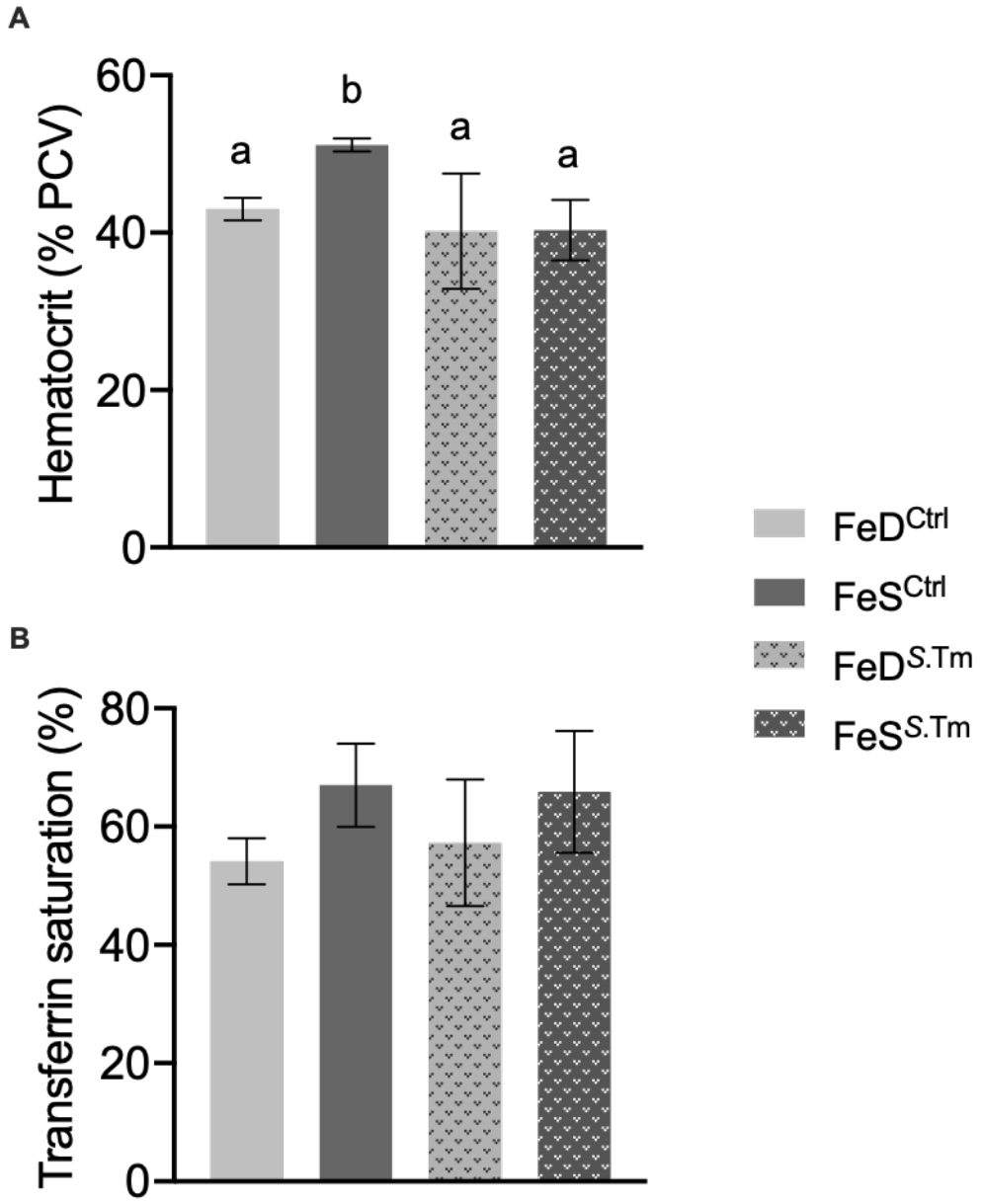

Figure 1. Markers of iron status in mice fed iron deficient or iron supplemented diets for 6 weeks. Three-week-old female C57BL/6 mice were fed AIN-76A diets containing either <6 ppm iron or 300 ppm iron for 6 weeks. Mice were euthanized and indicators of anemia and iron status were measured. **(A)** Hematocrit (main effects: $P_{Diet}<0.01$, $P_{Infection}<0.0001$, $P_{Interaction}<0.01$); **(B)** transferrin saturation (main effects: $P_{Diet}<0.01$, $P_{Infection}=0.79$, $P_{Interaction}=0.57$). Different letters indicate significant difference ($P<0.05$). Data are means ± standard deviations. FeD^{Ctrl}, iron deficient control; FeS^{Ctrl}, iron supplemented control; $FeD^{S.Tm}$, iron deficient *Salmonella* Typhimurium infected; $FeS^{S.Tm}$, iron supplemented *Salmonella* Typhimurium infected.

APPENDIX G

FIGURE 2

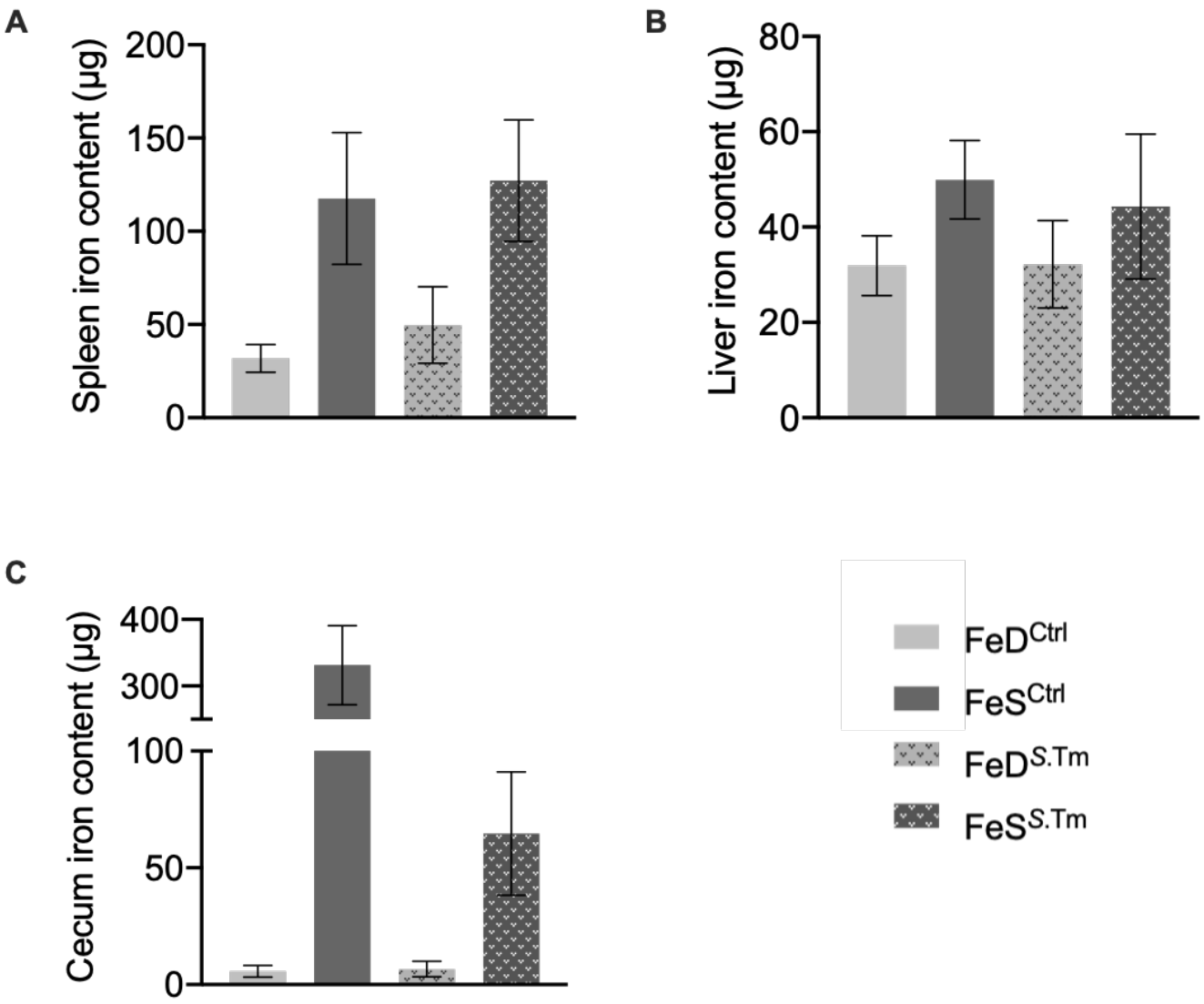

Figure 2. Tissue iron in mice fed iron deficient or iron supplemented diets for 6 weeks. Three-week-old female C57BL/6 mice were fed AIN-76A diets containing either <6 ppm iron or 300 ppm iron for 6 weeks. Mice were euthanized and indicators of anemia and iron status were measured. Concentrations of iron in (**A**) spleen (main effects: $P_{Diet}<0.0001$, $P_{Infection}=0.10$, $P_{Interaction}=0.62$); (**B**) liver (main effects: $P_{Diet}<0.0001$, $P_{Infection}=0.44$, $P_{Interaction}=0.39$); (**C**) cecum content (main effects: $P_{Diet}<0.0001$, $P_{Infection}<0.0001$, $P_{Interaction}<0.0001$). Different letters indicate groups significant difference ($P<0.05$). Data are means ± standard deviations. FeD^{Ctrl}, iron deficient control; FeS^{Ctrl}, iron supplemented control; $FeD^{S.Tm}$, iron deficient *Salmonella* Typhimurium infected; $FeS^{S.Tm}$, iron supplemented *Salmonella* Typhimurium infected.

APPENDIX H

FIGURE 3

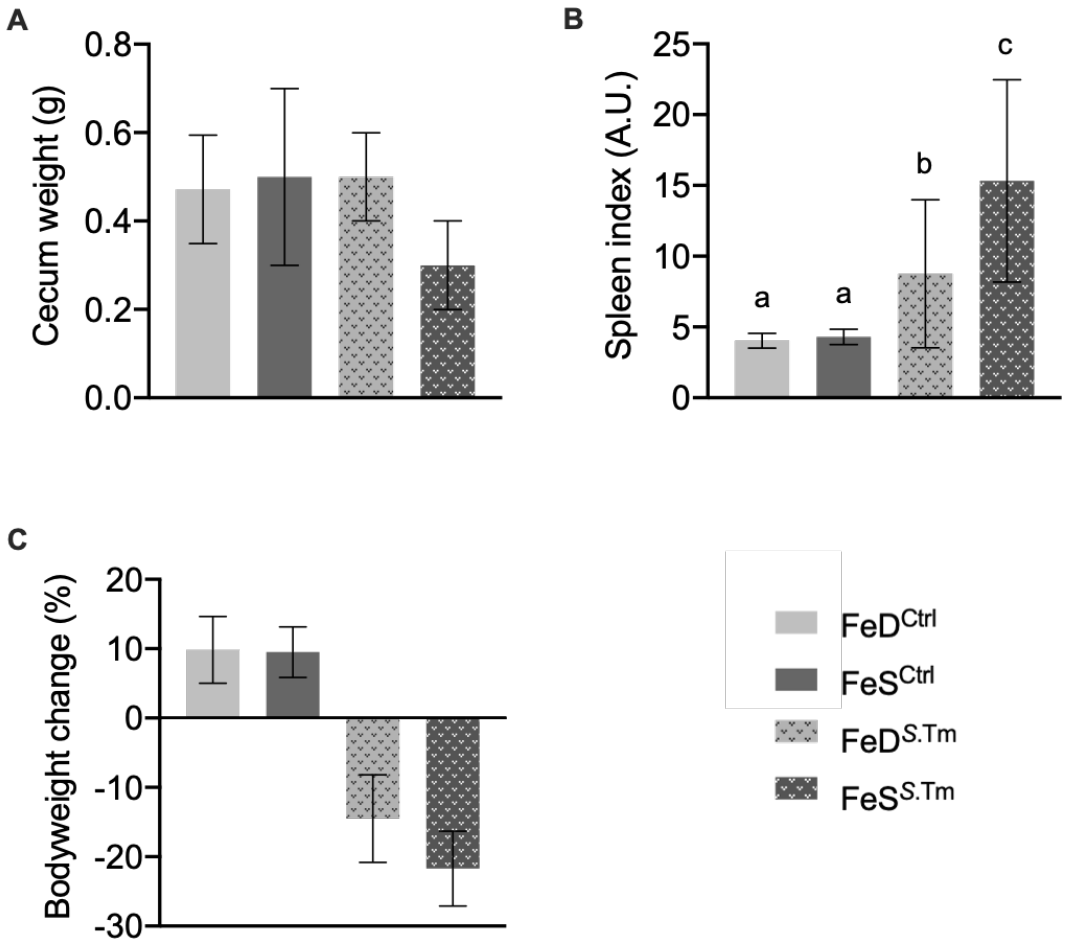

Figure 3. Severity of infection in mice infected with *Salmonella* Typhimurium and fed iron deficient or iron supplemented diets for 6 weeks. Three-week-old female C57BL/6 mice were fed AIN-76A diets containing either <6 ppm iron or 300 ppm iron for 6 weeks. Half of mice in each diet group were either infected with *S.* Typhimurium or received sham oral gavage. Bodyweights were recorded daily. Mice were euthanized and indicators infection severity were measured. **(A)** Weight of the cecum at euthanasia (main effects: P_{Diet}=0.08, $P_{Infection}$=0.08, $P_{Interaction}$=0.02). **(B)** Spleen index at euthanasia (main effects: P_{Diet}=0.04, $P_{Infection}$<0.0001, $P_{Interaction}$=0.05). **(C)** Change in bodyweight at euthanasia compared to pre-infection (main effects: P_{Diet}=0.03, $P_{Infection}$<0.0001, $P_{Interaction}$=0.05). Different letters indicate significant difference (P<0.05). Data are means ± standard deviations. FeD^{Ctrl}, iron deficient control; FeS^{Ctrl}, iron supplemented control; $FeD^{S.Tm}$, iron deficient *Salmonella* Typhimurium infected; $FeS^{S.Tm}$, iron supplemented *Salmonella* Typhimurium infected.

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BIOGRAPHICAL SKETCH

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EDUCATION

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PROFESSIONAL EXPERIENCE

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2016-2016 Undergraduate Research Assistant
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PEER-REVIEWED PUBLICATIONS

Manuscripts in review

James R. Ippolito, David E. Barney, Jamel Ali, Prashant Singh, Stephen R. Hennigar. Dietary iron supplementation increases severity of *Salmonella* Typhimurium infection.

Published abstracts

David E. Barney, **James R. Ippolito**, Claire E. Berryman, Stephen R. Hennigar. Plasma hepcidin increases with prolonged running in male, but not female, collegiate distance runners with low iron stores. *Current Developments in Nutrition*, 4 (supplement 2): 1777, 2020.

James R. Ippolito, David E. Barney, Prashant Singh, Stephen R. Hennigar. Oral iron supplementation increases severity of Salmonella Typhimurium infection. *Current Developments in Nutrition*, 4 (supplement 2): 1812, 2020.

James R. Ippolito, Jesse S. Morrell. Relationship between multivitamin/mineral supplement use and presence of metabolic syndrome biomarkers among college students. *Current Developments in Nutrition*, 2 (issue 11), 2018.

RESEARCH PRESENTATIONS

Oral presentations
Graduate School Master's in Four Competition, Florida State University, Tallahassee, FL (2020): Can iron supplementation increase the severity of *Salmonella* infection?

College of Human Sciences Research Showcase, Florida State University, Tallahassee, FL (2020): Oral Iron Supplementation Increases Severity of *Salmonella* enterica serovar Typhimurium Infection.

Poster presentations

Nutrition 2020, Online: Oral Iron Supplementation Increases Severity of Salmonella Typhimurium Infection

Nutrition 2018, Boston, MA (2018): Relationship between multivitamin/mineral supplement use and presence of metabolic syndrome biomarkers among college students.

Undergraduate Research Conference, Durham, NH (2018): Relationship between multivitamin/mineral supplement use and presence of metabolic syndrome biomarkers among college students.

MEMBERSHIP & SERVICE

Membership

- American Society for Nutrition (ASN)
- ASN Early Career Nutrition Interest Group
- ASN Vitamins & Minerals Research Interest Section
- Institute of Food Technologists (IFT)
- IFT Student Interest Group
- Academy of Nutrition and Dietetics (AND)
- Massachusetts Academy of Nutrition and Dietetics

Service

- Abstract Reviewer
    - Nutrition 2020
    - Nutrition 2019

HONORS & AWARDS

Recognition

- Florida State University Student Star, October 2020
- Florida State University College of Human Sciences Anne Marie Erdman Endowed Scholarship Fund in Honor of Betty M. Watts
- University of New Hampshire Nutrition Program Undergraduate Research Award, 2018

Assistantships & fellowships

- Florida State University Teaching Assistantship, 2019-

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