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Effects of Interleukin-6 and Hepcidin on Iron and Zinc Homeostasis in Mice

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EFFECTS OF INTERLEUKIN-6 AND HEPCIDIN ON IRON AND ZINC HOMEOSTASIS IN MICE

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ABSTRACT

The iron regulatory hormone hepcidin mediates the hypoferremia of inflammation by binding to, occluding, and signaling for the degradation of ferroportin, the only known cellular iron exporter. A regulator for the hypozincemia observed with inflammation and infection is not known; however, recent in vitro evidence indicates that hepcidin reduces intestinal zinc export by downregulating the zinc exporter ZnT1. The objective of the current study was to determine whether hepcidin attenuates molecular indicators of zinc status and intestinal zinc absorption in mice. Six-week-old male C57BL/6 mice were fed a modified AIN-93G diet containing 30 ppm zinc and 10 ppm iron for 2 weeks. After a 3 hour fast, mice were injected with 10, 50, or 100 μg/mouse hepcidin or 10, 25, or 50 μg/kg interleukin-6, an upstream regulator of hepcidin. Mice were then immediately given an oral gavage containing ^{67}Zn isotope and sacrificed 3, 6, or 24 hours later. One-way and two-way ANOVA with Tukey's post-hoc comparisons were used to assess the effects of treatment, time, and their interaction. Data are represented as means ± standard deviations. Plasma iron concentrations were decreased by up to 26% following IL-6 injections and 24% following hepcidin injections. IL-6 injections resulted in hypozincemia ($P = 0.035$), with 50 μg/kg IL-6 injections showing the greatest decline (1.05 ± 0.16) compared to control (1.18 ± 0.16). There were no treatment effects for plasma zinc for mice injected with hepcidin ($P= 0.366$). Liver and plasma appearance of ^{67}Zn did not differ with hepcidin or IL-6 injection. Collectively, these findings indicate that acute injection of IL-6, but not hepcidin, result in a hypozincemia in mice. Future studies should confirm these findings and perhaps examine the chronic effects of hepcidin on zinc homeostasis in mice.

CHAPTER 1

INTRODUCTION

Iron and zinc are essential minerals for most forms of life. Humans utilize these minerals for countless biological processes, such as oxidative phosphorylation and DNA production. Due to the importance of iron and zinc, humans have evolved complex regulatory systems of transporters and hormones to maintain homeostasis. Microorganisms, such as bacteria, also require iron and zinc for functions like DNA production and stabilizing structural proteins.

Bacteria have evolved mechanisms to obtain iron and zinc from their hosts and humans have evolved mechanisms to sequester iron and zinc from bacteria. The tools that humans employ and process of sequestering minerals is referred to as nutritional immunity[1,2]. One of the mechanisms of nutritional immunity includes a reduction in circulating concentrations of iron and zinc (i.e., hypoferremia and hypozincemia). The hypoferremia and hypozincemia of infection is a classic symptom of inflammation/infection and functions to starve blood-borne pathogens of iron and zinc.

The hypoferremia of infection is well studied and is the result of the 25-amino-acid peptide hormone, hepcidin. Hepcidin is upregulated in times of infection, notably by interleukin-6 (IL-6), and functions by binding to, occluding, and signaling for the degradation of ferroportin, the only known iron exporter[3]. Ferroportin is highly expressed in intestinal epithelial cells and splenic macrophages and thus, hepcidin-mediated declines in ferroportin inhibit intestinal absorption and the recycling of iron, lowering serum iron levels and causing hypoferremia.

In contrast to the hypoferremia of infection, the cause of the hypozincemia is not as well defined. IL-6 knockout mice have an attenuated hypozincemic response to infection[4] and recently, hepcidin was shown to inhibit zinc efflux *in vitro* by decreasing the expression of

ZnT1, the main exporter of zinc from enterocytes[5]. The primary objective of this study is to determine whether hepcidin regulates hypozincemia in an *in vivo* mouse model.

Iron and Zinc Metabolism in Humans

Iron and zinc are considered essential minerals because living organisms cannot synthesize them and therefore, they must be obtained from the diet. Following ingestion, iron and zinc are absorbed in the small intestine and transported through the blood to the body's tissues for storage or cellular function. Iron and zinc are involved in many processes such as amino acid metabolism and sexual maturation[6,7]. Iron is a necessary component of hemoglobin in humans, which carries oxygen from the lungs to the body's tissues[8]. Iron is required as a cofactor for many metalloproteins and enzymes. Zinc is a cofactor for approximately 10% of the human proteome[9]. Zinc is also crucial for the proper development of immune cells[10].

Conversely, too much iron and zinc can result in toxicity. Excess zinc intake in humans is associated with hypocupremia and deleterious effects on blood lipid profiles.[11]. Free iron is a highly reactive mineral that can generate hydroxyl radicals through the Fenton reaction[12,13]. For this reason, iron must be chaperoned throughout the body so that it does not create excess free radicals. Unbound iron in circulation is generally referred to as labile or non-transferrin-bound iron (NTBI), which is a hallmark of iron overload[14]. Although zinc may be labile in circulation, the movement of both of these crucial minerals throughout the body is tightly regulated by several transporters that maintain homeostasis, such as ferroportin for iron efflux and ZnT1 for zinc efflux.

Dietary Iron Absorption and Transport in Humans

Animal and plant sources of food both contain iron, but only animal sources contain heme iron. Heme iron is iron complexed with a heme molecule, which allows it to perform the oxygen transport function that makes it ubiquitous among animals. Heme iron is more readily absorbed and partially explains why those who do not consume meat in their diet are more likely to be iron deficient[15]. Non-heme iron may be complexed with anything besides a heme protein, including phytates and oxalates which inhibit absorption. Non-heme is the predominant form of iron from dietary animal sources and the only source of iron from plants. While heme iron is always in the ferrous (Fe^{2+}) oxidation state, non-heme can be found in the ferrous or ferric (Fe^{3+}) oxidation state.

After ingestion, non-heme iron is first reduced to the ferrous state before absorption, which may be carried out by duodenal cytochrome b or by concurrent ingestion of an antioxidant like vitamin C. Non-heme iron is absorbed across the apical membrane of the enterocyte by divalent metal transporter-1 (DMT-1), where it may be used by the enterocyte, stored in ferritin in the enterocyte, or exported across the basolateral membrane of the enterocyte by ferroportin to be utilized by other tissues. While the name suggests its function as an importer of divalent metals across the apical membrane of enterocytes, DMT-1 exhibits a strong preference for iron. Its absence has been shown to cause severe iron deficiency anemia in mice, but did not affect the status of other critical divalent metals such as zinc and manganese[16]. DMT-1 expression is also mediated by iron status. Hypoferremia causes expression of DMT-1 to be upregulated in duodenal enterocytes and downregulated in hepatocytes[17].

Ferritin (*FTH / FTL*) is an iron storage molecule comprised of both a heavy and light subunit that is capable of storing thousands of molecules of iron in the ferric form[18]. As well as

being expressed in the enterocyte, ferritin can be found in the liver, the spleen, and circulating in serum. Although serum ferritin is a small portion of total iron, it is used as a common indicator of iron status. However, accurate assessment may be confounded by inflammation as ferritin is an acute phase protein that increases in response to cytokine signaling associated with the inflammatory response. When intracellular iron is needed elsewhere, ferrireductases reduce iron to the ferrous form to be able to escape the ferritin before its cellular export via ferroportin.

Ferroportin (*SLC40A1*) is the only known exporter of iron. Ferroportin is mainly expressed by intestinal epithelial cells, hepatocytes, and splenic macrophages. These are major sites of absorption, storage, and recycling of iron, respectively. Hepcidin controls expression and function of ferroportin in all these tissues and others, which will be discussed in greater detail below. Embryonic knockout of *Slc40a1* in mice results in death, underlining the key role in life this transport protein plays[19].

Transferrin is a glycoprotein responsible for delivering ferric iron through the blood to various tissues and binds to iron as it exits cells via ferroportin. Transferrin can only bind iron in the ferric (Fe^{3+}) form, so iron must be oxidized to the ferric state by the copper-dependent enzyme hephaestin as it is exported by ferroportin. Transferrin is a negative acute phase protein that is downregulated in response to cytokines in times of infection. When bound with iron, it may be taken up by tissues to decrease circulating concentrations and sequester the iron from pathogens[20]. Apo-transferrin has two high-affinity binding sites for ferric iron, but may also bind other minerals such as manganese to aid in nutritional immunity[21].

The majority of iron in humans (~60-70%) is found in erythrocytes, mainly as part of the vital oxygen transporting heme molecule. Heme synthesis and incorporation into hemoglobin and erythrocytes is predominantly accomplished in the bone marrow. Holo-transferrin binds to

transferrin receptors on immature erythrocytes and the complex is then endocytosed. Iron is released from the complex and is incorporated into a protoporphyrin ring to form heme.

Animal foods humans consume also contain relatively highly bioavailable heme iron. Heme iron uptake into the enterocyte is less understood but is purported to be taken up by the enterocyte intact via heme carrier protein 1-mediated endocytosis[22]. The heme containing the iron is then degraded by heme oxygenase-2, releasing the iron which is transported into the cytoplasm via DMT1 and can then be used as described above[23].

Daily iron needs are primarily met by recycling iron from senescent red blood cells through splenic macrophages. While humans only absorb about 1-2 mg iron/d from the diet, they recycle about 20-21 mg/d per day from these macrophages, which are part of the reticuloendothelial system (RES)[24]. Iron acquisition for the RES most commonly occurs in the macrophage-rich red pulp of the spleen, followed by the liver and the bone marrow. In order to recycle the iron, senescent erythrocytes are first phagocytosed by macrophages, where each erythrocyte donates about a billion iron atoms to be recycled[25]. Proteolytic digestion in the phagolysosome result in heme being freed from the erythrocyte. Heme is then transported to endoplasmic reticulum (ER)-bound heme oxygenase proteins (HMOX) for further degradation, freeing iron to be stored by ferritin in the macrophage or released into circulation by ferroportin[26]. When ferroportin is prevented from performing this efflux of iron, much of our daily iron needs are left unmet. Iron available in serum is quickly taken up by tissues that need it, resulting in hypoferremia.

Dietary Zinc Absorption and Transport in Humans

Dietary zinc is found in the Zn^{2+} oxidation state and shares many similarities with iron. Although it is found in both plant and animal foods, the best sources of zinc are red meats and seafood. Similar to iron, zinc absorption is reduced if it is complexed with phytates or oxalates. Adequate plasma zinc levels range from 13.8 - 22.9 μmol/L although this value may not be reliable as a measure for deficiency[27]. Zinc status seems to be the main determinant of absorption efficiency. Fractional zinc absorption estimates in those of adequate status range from 20-50%[28–30]. However, just fifteen days of zinc deprivation caused the absorption rate to climb to 93% in some subjects[31].

Zinc absorption and transport centers around two protein families, the Zrt, irt-like protein family (ZIP1-14 encoded by *SCL39A1-14*) and the Zn Transporter family (ZnT1-10 encoded by *SLC30A1-10*). These families transport zinc into and out of the cytoplasm, respectively. The expression and localization of members of both families are both tissue and cell specific[32]. For example, ZIP4 (*SLC39A4*) is found on the apical membrane of enterocytes and is the primary transporter responsible for zinc uptake into enterocytes[33,34]. The inherent functional redundancy of having twenty four zinc transporters participating in the regulation of zinc homeostasis is thought to allow for compensation by one transporter when another is dysfunctional, or in response to changes in zinc availability[35,36].

After uptake, zinc may be utilized in the cell, stored free in vesicles or bound to metallothionein, or exported to other tissues via ZnT1. ZnT1 (*SLC30A1*) is found on the basolateral membrane of enterocytes and is the primary exporter of zinc into the portal blood[37]. ZnT1 was the first human zinc transporter identified and is critical for preventing intracellular zinc toxicity. Metallothionein-1 (*MT1*) is a metal storage protein expressed in enterocytes.

Metallothionein is predominantly composed of cysteine and is capable of binding 7 atoms of zinc per unit[38]. Two hours following a large oral zinc dose, metallothionein KO mice showed a more than doubling of serum zinc concentrations, while control mice had higher levels of intestinal zinc, showing that metallothionein maintains zinc homeostasis by storing zinc in the enterocyte[39]. Metallothionein is thought to work alongside ZnT1 via MTF-1 regulation in order to increase or decrease zinc absorption as needed[40].

The majority (99%) of plasma zinc is bound by transport proteins, such as albumin and transferrin, and circulate to various tissues[41]. Although plasma zinc may only constitute approximately 1% of total zinc in the body, it is an important and rapidly available pool of zinc for tissues in need. In accordance with zinc's necessity for proper bone growth and muscle recovery, over 80% of absorbed zinc can be found in skeletal muscle and bone[42]. During an infection, it is important that this zinc be sequestered from pathogens, which is accomplished through processes of nutritional immunity.

Nutritional Immunity

The process by which hosts withhold circulating minerals such as iron, zinc, and manganese from pathogens is referred to as nutritional immunity[2,43]. The body will limit absorption of minerals in the diet, sequester circulating minerals in the tissues, and downregulate methods of mineral resorption to hinder the virulence of the pathogen[1]. For example, infusion of humans with the endotoxin lipopolysaccharide (LPS) induces hypoferremia at 6 h and reduces serum iron levels by 57% at 22 h post-injection[44]. Importantly, an increase in urinary hepcidin excretion preceded this decline and followed shortly after peak IL-6 measurements. LPS injection has also been shown to result in a greater than 40% decline in serum zinc levels 6 h

after administration[45]. This decline was preceded by an increase in IL-6 and a decrease in urinary zinc excretion, indicating a intracellular method of sequestration mediated by cytokines. LPS injections have also been used to mimic the hypozincemic response to infection. Approximately 40% declines in serum zinc have been shown following LPS injections in humans and mice[4,45]. Serum albumin, a key zinc transport protein, remained largely unchanged following intravenous injection in humans[45]. However, Zip14 was shown to be highly upregulated following intraperitoneal (IP) LPS injection in mice, showing that there is a large intracellular zinc sequestration during infection[4]. Failure to resolve the infection could lead to prolonged hypozincemia, which could lead to a multitude of issues including a further compromised immune system[46].

If hypoferremia and hypozincemia are not induced by the body, infection duration and severity tend to increase. Higher serum iron concentrations have been associated with negative outcomes in severe infections. Sepsis patients (n=1,891) were categorized into quartiles based on serum iron levels, and for each increase in serum iron quartile the risk of death by day 90 significantly increased[47]. There are a multitude of pathogens whose growth is increased in high iron environments, and many reports of hemochromatosis, or iron overload disease, causing increased rates of infection as well as more severe infections in those with iron overload[48]. Having more zinc available in the environment allows pathogens such as *S.* aureus to form biofilms more easily, which contribute to their survival and antibiotic resistance[49].

Pathogenic Mechanisms to Obtain Iron and Zinc

All invading pathogens require iron for essential metabolic processes[50]. These necessary functions include electron transfer in electron-transport chains and metabolism of the iron-

containing form of superoxide dismutase[51]. These functions are so crucial that microbes synthesize and secrete compounds known as haemophores and siderophores to capture heme and non-heme iron, respectively. For example, gram-negative bacteria such as *S*. Typhimurium produce enterobactin, a siderophore that can acquire iron directly from host transferrin proteins, which make the action of inducing hypoferremia during times of infection all the more crucial[52]. Bacteria that produce more siderophores can be more virulent[53]. A hypervirulent strain of *K*. Pneumoniae was found to be able to drastically increase its growth and infectious potential both *in vitro* and *in vivo* simply by secreting more and novel iron-acquisition compounds compared to classical *K*. Pneumoniae[54].

Many of the tools that bacteria use to obtain iron from the host are controlled by ferric uptake regulators. The ferric uptake regulator family is a family of proteins identified in pathogens that play a role in mineral homeostasis in bacteria. Ferric uptake regulator (Fur) repressor and zinc uptake regulator (Zur) repressors are proteins that modulate several bacterial genes in response to a low-iron or low zinc environment, respectively. Fur is considered to be the master regulator of iron in bacteria. Fur proteins bind to ferrous iron when it is available in the environment. This Fur-Fe^{2+} complex then represses genes involved in iron acquisition and promotes antioxidant and iron metabolism genes[55]. Fur also modulates virulence factors like toxin-coregulated pilus in *V*. cholerae[56]. Zur is also a member of the ferric uptake regulator family of proteins in bacteria. Just as ~10% of the human genome requires zinc, ~6% of prokaryotic proteins require zinc to function properly[57]. Zinc is crucial to bacteria for biofilm formation, antibiotic resistance, and the virulence capacity of the pathogen[58]. Similar to Fur, Zur proteins have a high affinity for zinc and bind to DNA promoters when complexed with the mineral. This binding prevents transcription of proteins related to bacterial acquisition and

transport of zinc[59]. ZnuABC is a transporter protein-complex necessary to scavenge zinc from hosts that is repressed by Zur bound to its promoter[60]. In an enteric *S.* Typhimurium infection, ZnuABC has been shown to overcome calprotectin zinc-sequestration and even utilize calprotectin's method of excretion in the feces as a way to deliver more zinc to the pathogen[61]. When the host creates a hypozincemic environment, Zur proteins do not bind zinc as frequently and uptake genes will be more readily expressed.

Host Mechanisms to Sequester Iron and Zinc

The host has many tools at its disposal to engage in the war for minerals with pathogens. Natural resistance-associated macrophage protein 1 (NRAMP1) is an iron and manganese importer that is localized to the phagolysosomes of macrophages and neutrophils[62,63]. NRAMP1 is important for the recycling of iron present in senescent red blood cells, as it can transport iron out of the phagolysosome after it has been released from the heme molecule. During infection, NRAMP1 can be tasked with transporting these minerals into the cytoplasm to reduce the survival and virulence of pathogens that have been captured by the phagolysosomes[64,65].

Neutrophils contain several proteins that sequester minerals. Lactoferrin is secreted at the site of infection to bind to iron. This protein is remarkably similar to transferrin, down to the number of binding sites. The largest difference between the two is lactoferrin's much greater affinity for iron, highlighting its specialized use by these neutrophils during infection[66].

Calprotectin is an S100 protein that constitutes approximately half of the soluble protein content in the cytosol of neutrophils[67]. Calprotectin can bind to iron and manganese only in the presence of calcium, or to zinc regardless of calcium content. Stimulated by the inflammatory

cytokines IL-17 and IL-22 during infections, each calprotectin molecule can bind two divalent mineral atoms and inhibit their access by microbes[68,69].

Hepcidin (*HAMP*) is one of the most important components a host employs to sequester iron from pathogens. Primarily secreted by hepatocytes, this 25-amino acid peptide hormone seeks out and binds to ferroportin. Hepcidin's upregulation is key to inhibiting the transport of iron throughout the body as well as its absorption within the small intestine[3]. Hepcidin is a cationic amphipathic peptide with antimicrobial properties, but it is structurally and functionally distinct from other defensins. When murine hepcidin mRNA was found to be induced in iron-overloaded mouse livers, researchers began to question if it was the long-awaited master regulator of iron[70]. Since then, hepcidin has been shown to impact body iron homeostasis, being expressed primarily in hepatocytes, enterocytes, and macrophages. Its expression is modulated by iron stores, increased erythropoietic activity, and inflammatory cytokines such as IL-6[71].

IL-6 is considered a primary inflammatory stimulus and has multiple functions within the body[72]. Once released from the local leukocytes at the site of infection, IL-6 travels to the liver and signals for the release of acute phase proteins such as transferrin[73]. Acute phase proteins are proteins that significantly change their concentration in serum in response to inflammatory cytokines. When a pathogen invades a host, IL-6 is the primary signal for an increase in *HAMP* transcription. When IL-6 binds to the Interleukin-6 Receptor (IL-6R), the cell-surface receptor induces the Janus Kinases (JAK) to phosphorylate inside the cell and subsequently phosphorylate the intracellular signal transducer and activator of transcription (STAT) proteins, forming a dimer. In this case, the STAT3 dimer then migrates to the interior of the nucleus of the cell and to binds to DNA on the STAT3 response element and induces the HAMP promoter to transcribe preprohepcidin. These actions occur primarily in hepatocytes. Following enzymatic cleavage of

the 84-amino-acid preprohormone, 25-amino-acid hepcidin becomes active in the plasma. Hepcidin then travels through the plasma to tissues were the iron exporter ferroportin is expressed. Ferroportin can be treated as a receptor for hepcidin, which binds to the extracellular side of the iron-secreting protein and signals for ubiquitination. Lysosomes envelope the endocytosed ligand-receptor complex and proteosomes degrade both molecules within the lysosome[74].

In the seminal paper describing how IL-6 induced the expression of hepcidin, which resulted in hypoferremia, six humans were infused with IL-6 for 3 hours. There was a rapid 7.5-fold increase in urinary hepcidin excretion and a subsequent 33% decrease in serum iron and transferrin saturation[75]. *In vitro* efforts in this study showed IL-6 induced *HAMP,* the gene that encodes for hepcidin, when IL-6 antibodies completely negated the increase in *HAMP* expression from the LPS challenge. Creating an inflammatory response in IL-6 KO mice by turpentine injection which did not elicit a hypoferremic response also showcased IL-6's necessity for inflammation-related hepcidin upregulation. This paper showed that IL-6 upregulation of hepcidin is paramount to inducing hypoferremia during infection. Prolonged hypoferremia, however, can lead to iron-restricted erythropoiesis and anemia of inflammation[76]. Later studies utilizing LPS injections as an even further upstream regulator of hepcidin showed the same cascade of IL-6 and subsequent hepcidin increases followed by a rapid decline in serum iron[44].

Increasing IL-6 concentrations because of inflammation is not the only signal for the upregulation of hepcidin. Iron may be a nutrient vital to life, but it is also a very reactive transition metal that can propagate reactive oxygen species, which can be damaging to tissues. This issue is compounded by the fact that the human body does not have an efficient way of

excreting iron once it is absorbed. Getting too much iron in the diet may not signal for an inflammatory response, so hepcidin can also be induced by the presence of excess iron.

Hemojuvelin is a glycosylphosphatidylinositol (GPI)-anchored protein expressed by periportal hepatocytes that induces hepcidin expression in response to dietary iron intake[77]. Mutations in the gene encoding for hemojuvelin, *HFE2*, lead to early-onset juvenile hemochromatosis independent of *HAMP* mutation[78]. Initially believed to be a receptor for hepcidin, hemojuvelin was eventually shown to be a major protein involved in iron sensing and is protective in the event of excessive iron ingestion.

The body uses transferrin receptors, the human homeostatic iron regulator (HFE), and certain bone morphogenic proteins (BMP) to sense when iron levels are too high and call for increased transcription of hepcidin[79]. This is accomplished by BMP 2/6 binding to hemojuvelin and this complexing with BMP receptors on hepatocytes. The receptor then phosphorylates mothers against ddp (SMAD) complexes which enter the nucleus and demand *HAMP* upregulation.

Another interesting facet of the hemojuvelin-hepcidin axis is hemojuvelin's response to inflammatory signals. Following a stimulus for acute inflammation, the cytokines IL-6 and TNF-α not only upregulate the expression of hepcidin, but they signal for the downregulation of hemojuvelin as well[77]. This is necessary because the iron-sensing pathway that hemojuvelin is a prominent member of would otherwise sense the hypoferremia induced by the inflammatory pathway and counteract this necessary defense tool. An important takeaway from this is that even though hemojuvelin's role in iron homeostasis is inflammation-independent, the hypoferremia induced by inflammation is reduced in efficiency if the same cytokines that call for the upregulation of hepcidin do not also signal for the downregulation of hemojuvelin. This was

showcased by IL-6's attenuation by antibodies resulting in a below baseline expression of HAMP following LPS challenge[75].

Hepcidin Mediates the Hypoferremia of Infection

Hepcidin is the primary mediator of the hypoferremia that is associated with inflammation[3]. Starving these pathogens of minerals can be a viable defense strategy for the body. Hepcidin sequesters iron from bacteria by binding to ferroportin and occluding its iron-releasing function while simultaneously signaling for its degradation[80]. Hepcidin accomplishes this task in any tissue that contains ferroportin, namely the small intestine, liver, and splenic tissue rich in macrophages. Research has shown that macrophages may be more sensitive to hepcidin challenge than other cell types, which may be due to their role in iron recycling[81].

Hepcidin blocks the release of iron via ferroportin in multiple ways. When hepcidin binds to ferroportin, the conformation achieved occludes passage of iron from the transmembrane protein. This binding also calls for tyrosine phosphorylation of the hepcidin-ferroportin complex. This phosphorylation results in the endocytosis of the complex, after which the phosphates are removed and ferroportin is ubiquitinated, packaged in multivesicular bodies (MVB), and fused into lysosomes for degradation[82].

The time-course for multiple actions of hepcidin on iron homeostasis seem to be cell specific. Chaston et al. focused on the differences in effect of hepcidin challenge on macrophage and intestinal ferroportin both *in vitro* and *in vivo*[83]. Caco-2 cells were grown on Transwell inserts and THP-1 cells were seeded on Transwell filters for *in vitro* testing[83]. Caco-2 cells were originally derived from a human colon adenocarcinoma and can mimic many functions of the human enterocyte[84]. THP-1 cells are monocytic cells capable of differentiating into macrophage-

like cells when treated with phorbol myristate acetate[85]. Following differentiation, these cells were placed in medium containing 1 μmol/L synthetic hepcidin peptide or HuH7 cells that were stimulated by IL-6 to produce hepcidin, which has been shown to inhibit iron transport from THP-1 monocytes[86]. Co-culture of THP-1 with IL-6 stimulated HuH7 cells decreased macrophage ferroportin protein levels significantly after 4 h and remained at 24 h. Conversely, co-culture of HuH7 with Caco-2 cells did not produce the same decrease in ferroportin at either time point. As a positive control, Caco-2 and THP-1 cells were then incubated with synthetic hepcidin. The expression of ferroportin matched the co-culture with HuH7 for each cell type. For the *in vivo* testing, four-week-old male C57BL/6 mice were fed a 44 mg Fe/kg diet for three weeks prior to the experiment. The experiment involved a single IP injection of 10 μg hepcidin or saline followed by experimentation 4 h later. Spleen and duodenum were isolated from the mice 4 h following injection of hepcidin. Immunofluorescent staining and western blotting both showed a 60% reduction in ferroportin protein expression in the hepcidin-treated spleen compared to control. Immunofluorescence also revealed that ferroportin activity was concentrated in the macrophage-rich red pulp of the spleen. No significant change in ferroportin expression was seen 4 h post injection in the duodenum of the mice. Interestingly, ferroportin-expressing cells in the lamina propria that were likely macrophages were only present in the images associated with the control mice. As expected, a 60% decline in serum iron concentrations was observed following injections of hepcidin. However, duodenal iron transport determined by gamma counting was not altered following the treatment. Furthermore, they found no differences in ferroportin expression on liver membranes in mice injected with hepcidin when compared to control. These findings show *in vivo* evidence that there are cell-specific responses to hepcidin, with ferroportin on macrophages being the most sensitive to hepcidin challenge.

Continuing studies revealed more about the tissue-specific response of hepcidin[81]. In this study, mice were acclimated similarly to the previous work, followed by daily IP injections of 10 μg hepcidin daily for up to three days. THP-1 and Caco-2 cells were grown using the same procedure as the previous study. An important differentiation between this study and the last is that cellular ferritin levels were measured in the Caco-2 cells following exposure to hepcidin in order to see their role in duodenal iron transport. DMT-1 expression on the brush border of enterocytes was also observed via immunofluorescence. Hepcidin has been shown to act on DMT-1 in order to further limit iron efflux from enterocytes[87,88]. In contrast with the last study, a longer time following hepcidin injection resulted in reduced duodenal iron transport *in vivo.* 24 h following hepcidin challenge, the total amount of dietary iron absorbed was reduced by ~45%. Incredibly, duodenal iron transport was reduced by ~80% following 72 hours of daily IP hepcidin injections. Immunofluorescent staining of spleen and duodenum showed marked reduction of ferroportin and DMT-1 expression following *in vivo* hepcidin injection at both the 24 and 72 h timepoints. Only the ferroportin localized to the splenic macrophages showed evidence of recovery towards basal expression at 72 h, highlighting the tissue-specific differences in response to hepcidin. Differing from the *in vivo* results, Caco-2 ferroportin protein was unchanged 24 h following hepcidin challenge. However, DMT-1 protein levels decreased in step with increases to the concentration of hepcidin. Iron accumulation and ferritin expression was significantly upregulated in hepcidin-treated Caco-2 cells, while iron transport was inhibited. While ferroportin mRNA showed no changes by any hepcidin treatment, DMT-1 mRNA was unchanged *in vivo* but significantly downregulated in Caco-2 cells.

These studies provide *in vitro* and *in vivo* evidence that there are cell-specific responses to hepcidin, with ferroportin on macrophages being the most sensitive to post-translational

downregulation by hepcidin at the onset of infection. Hepcidin does inhibit iron efflux from enterocytes but may be targeting other transporters along ferroportin to accomplish this. The differing concentrations of iron may be causing the disparity in effects in different tissues, as hepcidin has an 80-fold affinity for ferroportin on iron-containing cells rather than those without[80]. Our study proposes to expand on these findings by describing the cell-specific response of mouse ferroportin when subjected to IP injection of both hepcidin and its upstream regulator, IL-6. Furthermore, we will show expression at 3, 6, and 24 h timepoints, more accurately defining the time-course of hepcidin's effect on different cell types.

Hepcidin as a Potential Mediator of the Hypozincemia of Infection

Hypozincemia of infection is not as well characterized as hypoferremia of inflammation and does not have a known master regulator as of yet; however, limited evidence suggests that hepcidin may play a role. For example, other divalent minerals besides iron have been found to upregulate hepcidin in high concentrations *in vitro*, with zinc being the most efficient due to its interaction with a metal response element on the hepcidin promoter[89].

Xenopus oocytes that have been made to express human ferroportin are able to export iron, cobalt, and zinc in appreciable quantities. Following hepcidin treatment, zinc export was reduced. However, four hours following hepcidin treatment in a murine model in the same study elicited hypoferremia, but not hypozincemia[90]. This indicates that if hepcidin is responsible for a significant portion of the hypozincemic response, its mechanism for doing so must lie with other transporters. Determining how large of a role hepcidin plays in the onset of the hypozincemia of inflammation in vivo may go a long way in elucidating the key mechanisms of zinc homeostasis.

A recent study attempted to determine the role hepcidin plays in zinc efflux from enterocytes[5]. In this study, differentiated human intestinal Caco-2 cells were grown on Transwell inserts. To better simulate the human intestine, hepcidin was added to the media in the basolateral chamber and ^{67}Zn was added to the apical chamber corresponding to the brush border expressed by the Caco-2 cells. After 3-24 h of exposure to 1 μM hepcidin in growth medium containing 100 μM $ZnSO_4$, the media from both chambers was collected and compared with cells not exposed to hepcidin for an equivalent time. The apical membrane growth medium was conditioned with tracer ^{67}Zn to read apical to basolateral transport of zinc over time and changes in intracellular zinc. Cells treated with hepcidin for 24 h had a 75% reduction in ZnT1[5]. The downregulation in ZnT1 resulted in a 20% and 26% reduction of zinc transport from the apical to the basolateral chamber at 3 and 24 h, respectively. Concurrent in reduction in zinc transport at 24 h, total cellular ^{67}Zn increased by 27%. Metallothionein mRNA increased by 50% in cells treated with hepcidin for 6 hours compared to control, suggesting a destination for the increased intracellular zinc. Knockdown of ZnT1 with siRNA attenuated hepcidin's effect on apical to basolateral transport of ^{67}Zn while 70% knockdown of ferroportin did not reduce hepcidin's capability to inhibit zinc transport. Importantly, this suggests that hepcidin can inhibit zinc efflux from enterocytes and directly contribute to the hypozincemia of inflammation through post-translational downregulation of ZnT1. This is the first study to show hepcidin inhibiting zinc export to a similar magnitude as its inhibition of iron export. *In vivo* testing is needed to further validate these findings.

ZIP14 is expressed by hepatocytes and enterocytes. It shuttles zinc and NTBI from blood into tissues[91]. In an important study that identified *Slc39a14*, IL-6 was shown as the mediator of Zip14 induction. CD-1 strain and C57BL/6 wildtype and IL-6 KO mice were subjected to

inflammatory responses via injections of either LPS or turpentine. Sixteen hours post-injection, mice were sacrificed and analyzed. In response to LPS, Zip14 mRNA expression was increased 3.7-fold, more than any other zinc transporter[4]. *Mt-1* induction increased approximately 35-fold, highlighting the importance of metallothionein in intracellular zinc sequestration during infection. Subsequent inflammation induction on IL-6 KO mice showed no increase in Zip14 transcript levels, indicating that IL-6 mediates the upregulation of *Slc39a14* during periods of inflammation. *Mt-1* induction was also attenuated by IL-6 KO, and there was an attenuated hypozincemia in those mice exposed to LPS. In totality, these data indicate that IL-6 is just as important for the hypozincemia of infection as it is for the hypoferremia. Hepcidin, another downstream product of IL-6 upregulation, has been so far shown to be ineffective in upregulating ZIP14. Combined with the purported effects of hepcidin on ZnT1, ZIP14 upregulation on enterocytes may be a way to further increase the amount of zinc excreted when duodenal cells are sloughed off. ZnT1 and ZIP14 are also widely expressed on hepatocytes. Zip14 has been shown to be a primary transporter involved in the hypozincemia of infection, as Zip14 KO in mice almost fully attenuates the ~40% decrease in serum zinc following LPS injection[92]. It remains to be seen if hepcidin attenuates ZnT1 expression on hepatocytes in the same way it has been shown to do so on enterocytes.

Conclusion

Hypoferremia and hypozincemia are classic symptoms of infection and evolved as components of nutritional immunity, thereby decreasing the growth and virulence of invading pathogens. The mechanisms of hypoferremia are fairly well studied and rely mainly on hepcidin, a negative regulator of iron absorption and utilization. The mechanisms contributing to the

hypozincemia of infection are not well understood. Previous work has shown that hepcidin inhibits zinc efflux *in vitro* by downregulating the presence of ZnT1 on the basolateral membrane of enterocytes. The primary objective of the proposed study is to determine whether hepcidin inhibits zinc efflux in an *in vivo* model.

Objective and Hypothesis

Objective: To determine whether hepcidin and/or IL-6 attenuate molecular indicators of zinc status and intestinal zinc absorption in mice.

Hypothesis: Hepcidin and IL-6 attenuate molecular indicators of zinc status and intestinal zinc absorption in mice.

CHAPTER 2

METHODS

Experimental Design and General Procedures

This study was approved by the United States Army Research Institute of Environmental Medicine Institutional Animal Care and Use Committee on January 31, 2017. All animal procedures took place at USARIEM and post-collection tissue analysis were conducted at Florida State University. For all studies, male C57BL/6 mice (n=126) were obtained at 6 weeks of age and acclimated for two weeks. C57BL/6 is the most widely used inbred strain and is commonly used as a general-purpose strain. Mice were obtained from Charles River Laboratories (Wilmington, MA), which uses an International Genetic Standardization (IGS) program and pyramid mating to maintain genetic uniformity and decrease variation in experimental results. Important to the objectives of the current study, the initial, seminal work characterizing the effects of hepcidin and inflammation on iron status were conducted in male C57BL/6 mice[75,93]. Moreover, others have used C57BL/6 mice to examine the effects of inflammation (e.g., IL-6) on zinc status[4,92]. Thus, C57BL/6 mice are a suitable model to determine the effects of hepcidin on zinc homeostasis.

Mice were housed in polycarbonate cages in a stable temperature and humidity (25 ± 1ºC/ 40-50%) animal room with a 12 h light/dark cycle and given *ad libitum* access to distilled water throughout the study period. Mice were fed a standard AIN-93G diet containing 30 ppm zinc (Research Diets; New Brunswick, NJ) *ad libitum* for 2 weeks. Dietary iron was reduced in the diet to 10 ppm to lower endogenous hepcidin expression[94]. Following the two-week acclimation period, mice were randomized by bodyweight to one of three treatment groups (n=18/treatment/dose): saline vehicle control, hepcidin-treated, or IL-6-treated. After a 3 h fast[92],

mice were IP injected with saline vehicle (n=18), hepcidin [10 µg/mouse, 50 µg/mouse, or 100 µg/mouse; n=18/dose] or IL-6 [10 µg/kg, 25 µg/kg, or 50 µg/kg; n=18/dose]. The doses of IL-6 were chosen because 25 µg/kg IL-6 has been shown to increase serum hepcidin concentrations from ~18 pg/mL in vehicle-injected mice to ~45 pg/mL in IL-6-injected mice at 48 h post-injection ($P < 0.05$)[95]. Consequently, serum iron concentrations decreased from ~40 µM in vehicle-injected mice to ~30 µM in IL-6-injected mice at 48 h post-injection ($P < 0.05$). These hepcidin concentrations are on the low end of the pM range; however, humans with inflammatory disorders and acute and chronic infection experience hepcidin concentrations in the µM range[96–98]. We suspected the higher dose (50 µg/kg) would be in the µM range. The lower dose (10 µg/kg) was included to determine how sensitive the response is. For hepcidin, 10 µg hepcidin is a dose shown to significantly increase mucosal retention and abolish mucosal transfer of iron at 24 h post-injection[81]. In this same study, serum iron concentrations in vehicle- and hepcidin-injected mice were 40 and 26 µM at 24 h post-injection, respectively. The 50 µg hepcidin dose has been shown to decrease serum iron from ~34 µM in vehicle-injected mice to ~25 µM in hepcidin-injected mice at 3 h post-injection ($P < 0.05$)[99]. And finally, 100 µg hepcidin has been shown to significantly decrease transferrin saturation at 6h and 12h post-injection compared to vehicle-injected controls (serum iron not determined)[100].

Immediately following injection of treatments, mice were given an oral gavage containing the stable isotope ^{67}Zn. Seven hours following isotope administration, feed was provided *ad libitum* to the remaining mice and the amount consumed prior to euthanasia was recorded. Because hepcidin post-translationally regulates ferroportin expression, the decline in serum iron when injecting hepcidin is observed as early as 3 h and up to 24 h. For IL-6, studies have demonstrated that levels of inflammatory mediators, including hepcidin excretion, are

elevated rapidly after IL-6 or LPS infusion (1.5-6 h) with declines in serum iron concentrations at 22-24h[44,75]. Thus, mice were euthanized and tissues collected at 3 h, 6 h [the time of peak hepcidin transcription[101]], and 24 h after treatment.

Euthanasia

Mice were euthanized by CO_2 asphyxiation and exsanguinated by cardiac puncture following thoracotomy according to the American Veterinary Medical Association guidelines[102]. Mice were individually placed in a transparent CO_2 chamber containing 100% atmosphere, which gradually transitioned to 100% CO_2 over several minutes with a CO_2 flow rate between 20 and 30% of the chamber volume. Subsequently, mice were removed from the chamber and underwent bilateral thoracotomy and exsanguination.

^{67}Zn Preparation and Dosing

^{67}Zn (Trace Sciences International; Ontario, Canada) was dissolved in 3 drops of concentrated hydrochloric acid and brought up to 2 mL with distilled water to form a stock solution. The stock solution was added to zinc deficient feed dissolved in distilled water to create a feed slurry containing 10 μg ^{67}Zn/100 μL slurry. After two weeks of feeding, feed was removed for 3 h and mice were given an oral gavage containing a slurry of zinc deficient feed and the stable isotope ^{67}Zn (100 μL containing 10 μg ^{67}Zn). Mice were euthanized as described above and tissues were immediately collected, snap frozen in liquid nitrogen, and stored at -80°C prior to analyses. ^{67}Zn concentrations of digested samples were determined by inductively coupled plasma mass spectrometry (Thermo Fisher Scientific X Series 2).

Plasma Iron Concentration

Iron concentrations of digested plasma were determined by flame atomic absorption spectroscopy. Briefly, ~50 µL of plasma was digested for 24 h in 950 µL of 0.3 N HNO_3. Samples were vortexed then aspirated through the 225ATS (Buck Scientific Atomic Absorption Spectrometer).

Plasma IL-6 and hepcidin

Plasma IL-6 (R&D Systems) and hepcidin (Intrinsic Life Sciences) were determined using enzyme-linked immunoassays (ELISAs) according to manufacturer specifications.

Animal Number Justification and Data Analysis

Power analysis (SPSS Sample Power, version 3.0) indicated that 6 mice/treatment would provide adequate power (alpha = 0.05, power ≥ 0.9) to detect significant changes in plasma zinc with IL-6 treatment. Data used in calculating sample size estimates (i.e., means and standard deviations) were derived from two studies in C57BL/6 mice that demonstrated significant decreases in serum zinc at 6 h post LPS[92] and significant decreases in serum iron at 48h post IL-6 injection[95]. One-way analysis of variance was used to determine effects of treatment and time in body weight and feed intake. Two-way analysis of variance was used to determine effects of treatment, time, and their interaction for all other measures. Post hoc comparisons were completed using Tukey's Test. Data are reported as means ± standard deviations. P-values ≤ 0.05 were considered statistically significant. Data were analyzed using GraphPad Prism version 8.4.1.

CHAPTER 3

RESULTS

Bodyweight and Feed Intake

Following the two-week acclimation period, mice were randomized into seven different treatment groups using the end bodyweight measurement shown in Table 1. There were no significant differences in starting (P = 0.74) or ending (P = 0.26) bodyweight between treatment groups following randomization (**Table 1**).

Table 1. Start, end, and change in bodyweight in young mice[1]

	Saline	IL-6 (µg/kg)			Hepcidin (µg/mouse)			P-value
		10	25	50	10	50	100	
Starting Bodyweight (g)	18.2 ± 2.2	18.2 ± 2.2	17.9 ± 1.7	18.2 ± 1.8	18.4 ± 1.3	17.7 ± 2.0	18.7 ± 1.4	0.74
End Bodyweight (g)	21.8 ± 2.0	21.7 ± 1.2	20.9 ± 1.5	21.0 ± 1.4	21.1 ± 1.2	21.2 ± 1.6	21.4 ± 1.4	0.26
Weight Change (g)	3.7 ± 1.5	3.5 ± 1.9	3.0 ±1.9	2.7 ± 1.5	2.7 ± 1.0	3.5 ± 1.5	2.7 ± 1.8	0.76

[1]Mice were injected with saline, 10, 25, or 50 µg/kg IL-6, or 10, 50, or 100 µg/mouse hepcidin. All values are means ± SD for normally distributed data (n=17-18/group). Data were analyzed by one-way ANOVA and Tukey's post-hoc test (P<0.05).

Seven hours after injection of either saline vehicle, IL-6, or hepcidin, feed was provided ad libitum to the 24-hour group. Feed and iron and zinc intakes were consistent across all treatment groups prior to euthanasia (P = 0.94) (**Table 2**).

Table 2. Feed intake and dietary zinc and iron intake in young mice[1]

	Saline	IL-6 (µg/kg)			Hepcidin (µg/mouse)			P-value
		10	25	50	10	50	100	
Feed Intake (g)	2.6 ± 1.3	2.7 ± 0.4	2.7 ± 0.3	2.3 ± 0.7	2.4 ± 1.1	2.6 ± 0.7	2.8 ± 0.2	0.94
Zinc Intake (mg/day)	0.04 ± 0.021	0.04 ± 0.005	0.04 ± 0.005	0.03 ± 0.010	0.04 ± 0.018	0.04 ± 0.011	0.04 ± 0.003	0.94
Iron Intake (mg/day)	0.08 ± 0.040	0.08 ± 0.011	0.08 ± 0.001	0.07 ± 0.020	0.07 ± 0.033	0.08 ± 0.021	0.08 ± 0.005	0.94

[1]Mice were injected with saline, 10, 25, or 50 µg/kg IL-6, or 10, 50, or 100 µg/mouse hepcidin All values are means ± SD for normally distributed data (n=17-18/group). Data were analyzed by one-way ANOVA and Tukey's post-hoc test ($P<0.05$).

Plasma Hepcidin and IL-6 Concentrations

Plasma concentrations of IL-6 following injections of IL-6 at 3-, 6-, or 24-h post-injection are shown in **Figure 1A**. There was an effect of time ($P = 0.027$), a trend towards a treatment effect ($P = 0.063$), and an interaction effect ($P = 0.009$). Mean IL-6 concentrations at both the 3 h timepoint (36.02 ± 29.29) and 6 h timepoint (34.35 ± 31.63) were significantly elevated compared to the 24 h timepoint (18.92 ± 4.63, $P = 0.027$). Mean plasma IL-6 concentrations were elevated by 41-115% by injection of IL-6 (10 µg/kg: 31.90 ± 28.85, 25 µg/kg: 26.29 ± 23.47, 50 µg/kg: 39.94 ± 31.91) compared to saline (18.5 ± 2.34, $P = 0.063$). The interaction effect was the result of the lower doses of IL-6 showing peak concentrations at the 6 h mark while the highest dose peaked at the 3 h mark.

Plasma concentrations of IL-6 following injections of 10, 50, or 100 µg/mouse exogenous hepcidin at 3-, 6-, or 24-h timepoints are shown in **Figure 1B**. There was an effect of time ($P = 0.044$), a trend towards significance for treatment ($P = 0.071$), and no interaction effect ($P = 0.658$). Plasma IL-6 concentrations were significant elevated at 3 h (36.14 ± 26.18) and 6 h (33.31 ± 36.04) compared to 24 h (17.93 ± 3.30, $P = 0.044$) Mean plasma IL-6 concentrations increased by up to 108% following hepcidin injection compared to control ($P = 0.071$).

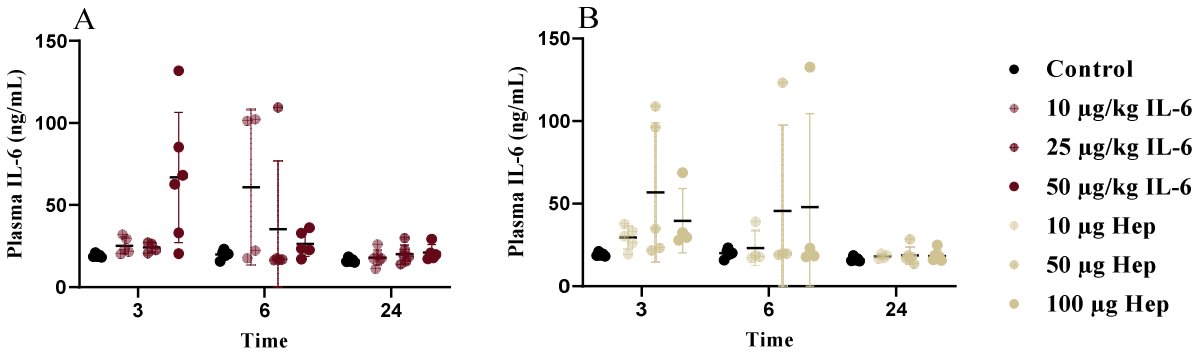

Figure 1. IL-6 concentrations in plasma following injection of varying doses of (A) IL-6 or (B) hepcidin. Mice (n=4-6/group) were injected with saline (control), 10, 25, or 50 µg/kg IL-6, or 10, 50, or 100 µg/mouse hepcidin and euthanized 3, 6, or 24 hours later. IL-6 concentrations were determined by ELISA. All values are means ± SD for normally distributed data. Data were analyzed by two-way ANOVA and Tukey's post-hoc test. Figure A(IL-6): P-time = .027, P-treatment = 0.063, P-interaction = 0.009. Figure B(Hep): P-time = 0.044, P-treatment = 0.071, P-interaction = 0.658. Hep, hepcidin.

Plasma concentrations of hepcidin following injections of 10, 25, or 50 µg/kg IL-6 at 3-, 6-, or 24-h post-injection are shown in **Figure 2A**. There was an effect of time ($P < 0.001$), but no treatment ($P = 0.184$) or interaction effects ($P = 0.432$). Mean concentrations of hepcidin at the 3 h (389.2 ± 138.6) and 6 h (473.4 ± 153.0) timepoints were significantly elevated compared to 24 h (226.4 ± 101.0, $P < 0.001$).

Plasma concentrations of hepcidin following injections of 10, 50, or 100 µg/mouse exogenous hepcidin at 3-, 6-, or 24-h post-injection are shown in **Figure 2B**. There was an effect of time ($P < 0.001$), treatment ($P = 0.021$), and interaction ($P = 0.010$). Mean plasma hepcidin concentrations after 3 h (556.4 ± 343.9) and 6 h (376.1 ± 188.6) were significantly elevated compared to 24 h post-injection (225.8 ± 216.8, $P < 0.001$). Treatment also significantly elevated hepcidin concentrations ($P = 0.021$), with the 100 µg hepcidin injection raising mean concentrations by 77% (524.6 ± 406.2) compared to control (296.4 ± 170.7).

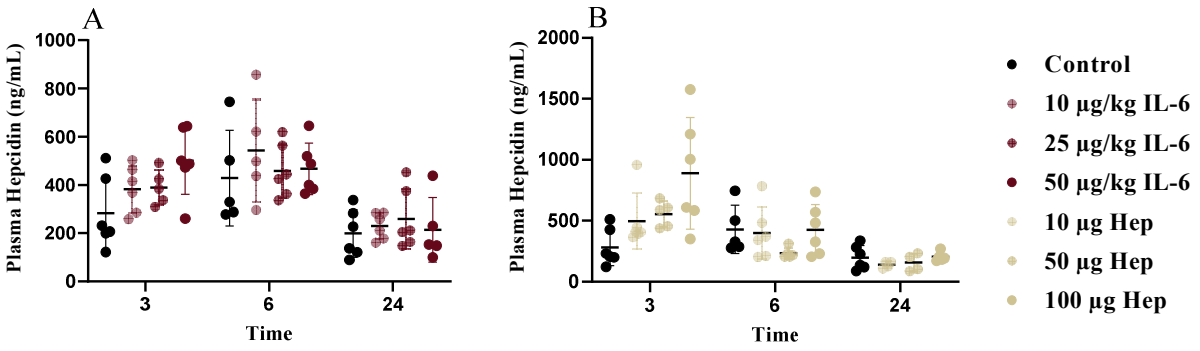

Figure 2. Hepcidin concentrations in plasma following injection of varying doses of (A) IL-6 or (B) hepcidin. Mice (n=5-6/group) were injected with saline (control), 10, 25, or 50 µg/kg IL-6, or 10, 50, or 100 µg/mouse hepcidin and euthanized 3, 6, or 24 hours later. Hepcidin concentrations were determined by ELISA. All values are means ± SD for normally distributed data. Data were analyzed by two-way ANOVA and Tukey's post-hoc test. Figure A(IL-6): P-time < 0.001, P-treatment = 0.185, P-interaction = 0.432. Figure B(Hep): P-time < 0.001, P-treatment = 0.021, P-interaction = 0.010. Hep, hepcidin.

Plasma and Liver Zinc Concentrations

Figure 3A shows plasma zinc concentrations at 3-, 6-, or 24-h following injection of IL-6. There was an effect of treatment (P = 0.035) and time (P = 0.014), but no interaction (P = 0.439). 50 µg/kg IL-6 (1.05 ± 0.16) resulting in a significantly lower plasma zinc level compared to control (1.18 ± 0.16). Plasma zinc concentrations were significantly depressed at 6 h (1.09 ± 0.18) compared to both 3 h (1.17 ± 0.19) and 24 h (1.17 ± 0.12, P = 0.014).

Figure 3B shows plasma zinc concentrations following injection of hepcidin. There is an effect of time (P < 0.001), but not treatment (P = 0.366) or interaction (P = 0.714). Plasma zinc concentrations are significantly reduced at the 6 h timepoint (1.048 ± 0.10) compared to 3 h (1.24 ± 0.20, P < 0.001).

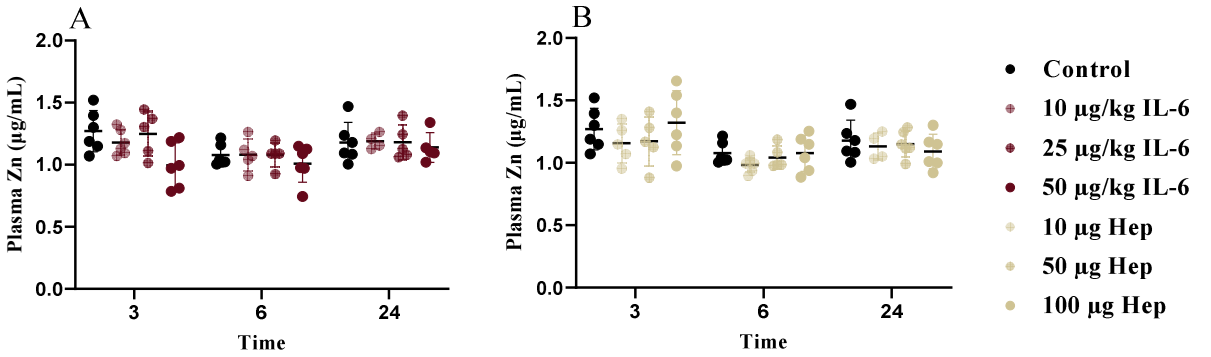

Figure 3. Total zinc concentrations in plasma following injection of varying doses of (A) IL-6 or (B) hepcidin. Mice (n=5-6/group) were injected with saline (control), 10, 25, or 50 µg/kg IL-6, or 10, 50, or 100 µg/mouse hepcidin and euthanized 3, 6, or 24 hours later. Zinc concentrations were determined via ICP-MS. All values are means ± SD for normally distributed data. Data were analyzed by two-way ANOVA and Tukey's post-hoc test. Figure A(IL-6): P-time = 0.014, P-treatment = 0.035, P-interaction = 0.439. Figure B(Hep): P-time < 0.001, P-treatment = 0.366, P-interaction = 0.714. Hep, hepcidin.

Figure 4A shows liver zinc concentrations following treatment with IL-6. There were no treatment (P = 0.335), time (P = 0.339), or interaction (P = 0.434) effects. **Figure 4B** shows liver zinc concentrations following treatment with hepcidin. There were no treatment (P = 0.970), time (P = 0.474), or interaction (P = 0.582) effects.

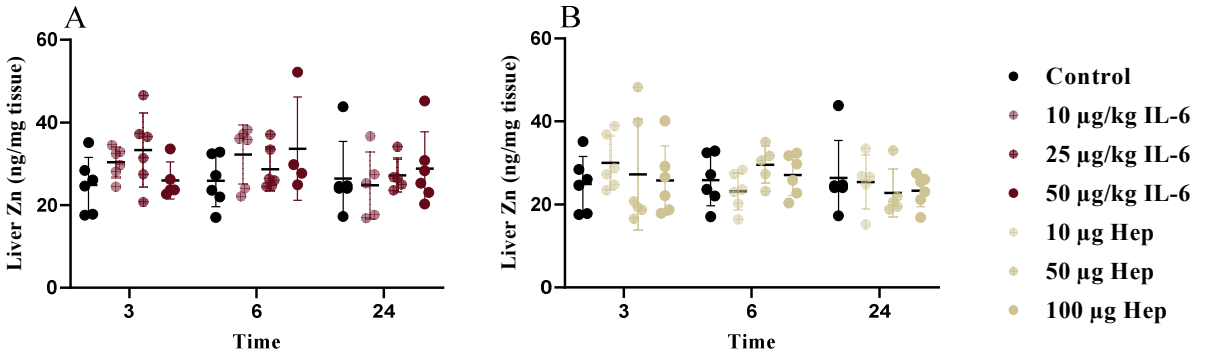

Figure 4. Total zinc concentrations in liver following injection of varying doses of (A) IL-6 or (B) hepcidin. Mice (n=5-6/group) were injected with saline (control), 10, 25, or 50 µg/kg IL-6, or 10, 50, or 100 µg/mouse hepcidin and euthanized 3, 6, or 24 hours later. Zinc

Figure 4 – continued. concentrations were determined via ICP-MS. All values are means ± SD for normally distributed data. Data were analyzed by two-way ANOVA and Tukey's post-hoc test. Figure A(IL-6): P-time = 0.339, P-treatment = 0.335, P-interaction = 0.434. Figure B(Hep): P-time = 0.474, P-treatment = 0.970, P-interaction = 0.582. Hep, hepcidin.

Plasma Iron Concentrations

Figure 5A shows plasma iron concentrations following IL-6 treatment. There is an effect of treatment (P = 0.012), but not time (P = 0.372) or an interaction (P = 0.173). All treatments reduced mean plasma iron concentrations by up to 26% (10 µg/kg: 2.983 ± 0.66, 25 µg/kg: 3.42 ± 0.86, 50 µg/kg: 3.18 ± 0.86) compared to control (4.04 ± 1.35, P = 0.012).

Figure 5B shows plasma iron concentrations following hepcidin treatment. There was an effect of treatment (P = 0.025) and time (P = 0.005), but no interaction (P = 0.568). All concentrations of hepcidin reduced plasma iron, with 100 µg having the greatest reduction (3.08 ± 0.55) in relation to control (4.04 ± 1.35, P = 0.025). The greatest reductions in plasma iron were seen at the 6 h (2.92 ± 0.82) timepoint, which were significantly lower than either 3 h (3.9 ± 1.28) or 24 h (3.6 ± 0.86, P = 0.005).

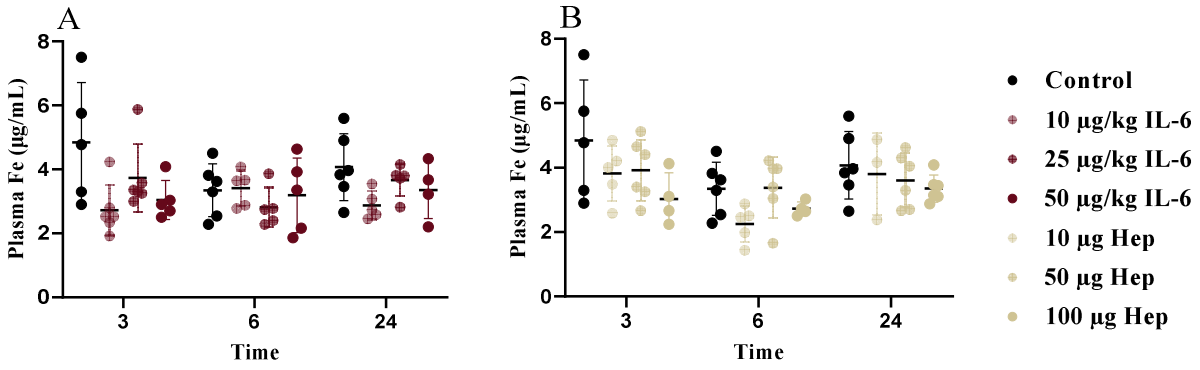

Figure 5. Total iron concentrations in plasma following injection of varying doses of (A) IL-6 or (B) hepcidin. Mice (n=5-6/group) were injected with saline (control), 10, 25, or 50 µg/kg IL-6, or 10, 50, or 100 µg/mouse hepcidin and euthanized 3, 6, or 24 hours later. Iron concentrations were determined via FAAS. All values are means ± SD for normally distributed

Figure 5 – continued. data. Data were analyzed by two-way ANOVA and Tukey's post-hoc test. Figure A(IL-6): P-time = 0.372, P-treatment = 0.012, P-interaction = 0.173. Figure B(Hep): P-time = 0.005, P-treatment = 0.025, P-interaction = 0.568. Hep, hepcidin.

Fractional Zinc Absorption

Figure 6A shows liver $^{67}Zn/^{66}Zn$ ratios following treatment with IL-6. There were no treatment (P = 0.376), time (P = 0.330), or interaction (P = 0.489) effects. **Figure 6B** shows liver $^{67}Zn/^{66}Zn$ ratios following treatment with hepcidin. There were no treatment (P = 0.564), time (P = 0.170), or interaction (P = 0.672) effects.

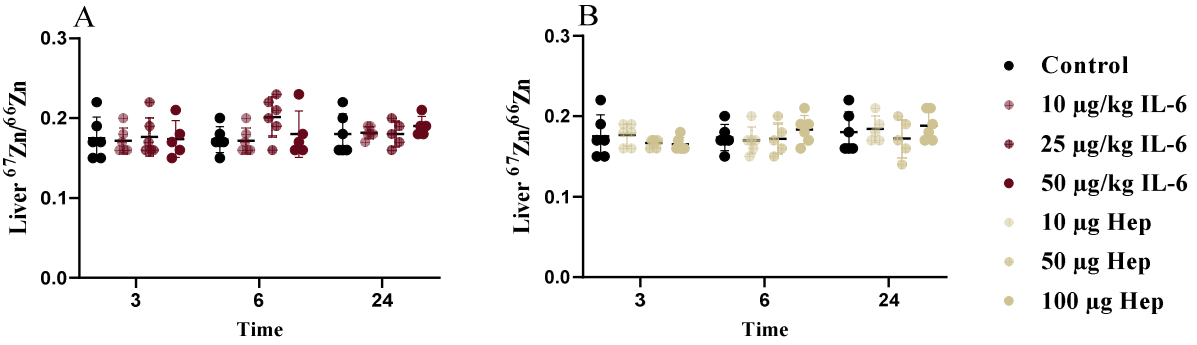

Figure 6. Zinc isotope ratio in liver following injection of varying doses of (A) IL-6 or (B) hepcidin. Mice (n=5-6/group) were injected with saline (control), 10, 25, or 50 µg/kg IL-6, or 10, 50, or 100 µg/mouse hepcidin and euthanized 3, 6, or 24 hours later. Zinc concentrations were determined via ICP-MS. All values are means ± SD for normally distributed data. Data were analyzed by two-way ANOVA and Tukey's post-hoc test. Figure A(IL-6): P-time = 0.330, P-treatment = 0.376, P-interaction = 0.489. Figure B(Hep): P-time = 0.170, P-treatment = 0.564, P-interaction = 0.672. Hep, hepcidin.

Plasma $^{67}Zn/^{66}Zn$ ratio for IL-6 treatments is shown in **Figure 7A**. There was an effect of treatment (P = 0.037) and time (P = 0.002), but no interaction (P = 0.918). Treatment with 25 (0.23 ± 0.06) and 50 (0.24 ± 0.06) µg/kg IL-6 increased the ratio of $^{67}Zn/^{66}Zn$ compared to control (0.20 ± 0.04, P = 0.037). Furthermore, $^{67}Zn/^{66}Zn$ increased with IL-6 treatment at the 3 h (0.24 ± 0.06) and 6 h (0.23 ± 0.05) compared to 24 h (0.19 ± 0.04, P = 0.002).

Plasma $^{67}Zn/^{66}Zn$ ratio for hepcidin treatments is shown in **Figure 7B**. There was an effect of time ($P < 0.001$), but no effect of treatment ($P = 0.104$) or an interaction ($P = 0.430$). A significantly elevated ratio was observed at the 3 h (0.23 ± 0.05) and 6 h (0.23 ± 0.04) compared to 24 h timepoints (0.18 ± 0.02, $P < 0.001$).

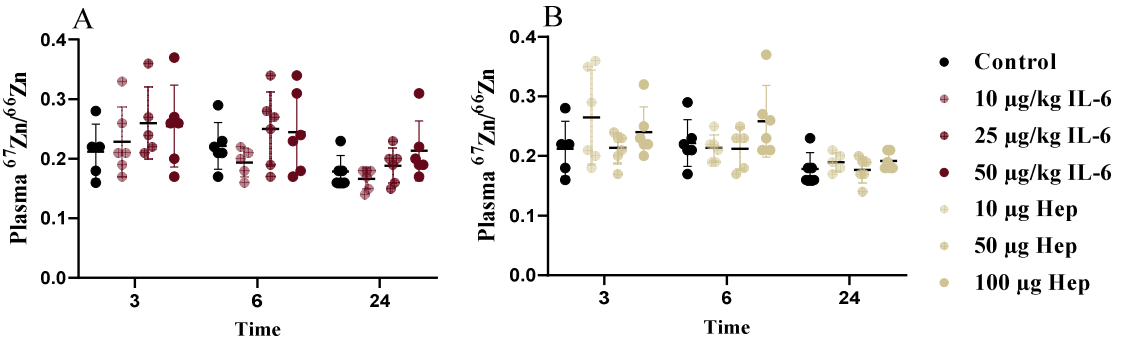

Figure 7. Zinc isotope ratio in plasma following injection of varying doses of (A) IL-6 or (B) hepcidin. Mice (n=5-6/group) were injected with saline (control), 10, 25, or 50 µg/kg IL-6, or 10, 50, or 100 µg/mouse hepcidin and euthanized 3, 6, or 24 hours later. Zinc concentrations were determined via ICP-MS. All values are means ± SD for normally distributed data. Data were analyzed by two-way ANOVA and Tukey's post-hoc test. Figure A(IL-6): P-time = 0.002, P-treatment = 0.037, P-interaction = 0.918. Figure B(Hep): P-time < 0.001, P-treatment = 0.104, P-interaction = 0.430. Hep, hepcidin.

CHAPTER 4

DISCUSSION

The objective of the current study was to determine whether hepcidin and/or IL-6 attenuated indicators of zinc status and intestinal zinc absorption in a murine model. Our findings indicate IL-6, but not hepcidin, is responsible for the hypozincemia observed during periods of inflammation and infection. This is contrary to our hypothesis that hepcidin would inhibit dietary zinc absorption and lead to hypozincemia in a mouse model to a similar degree that has been shown in an in vitro model[5].

A major finding from the current study is that hypozincemia was observed following IL-6 challenge, but not following hepcidin challenge. The effect of IL-6 on diminishing plasma zinc concentrations has been shown before, and is thought to be the result of IL-6-induced increases in ZIP14[4]. Hepatic ZIP14 expression is induced by IL-6 and promotes uptake of zinc from plasma into hepatocytes. However, in the current study, we did not see an increase in liver zinc concentrations with IL-6 injections. Reductions in plasma zinc concentrations may have been caused by ZIP14-mediated zinc uptake into skeletal muscle, which stores approximately 60% of total body zinc[103]. ZIP14 is also expressed on enterocytes. Knockout of ZIP14 has been shown to result in a 35% reduction in zinc absorption[92]. Fractional zinc absorption results from the current study support these data, as mice injected with 25 and 50 µg/kg IL-6 had an approximately 20% higher ratio of plasma $^{67}Zn/^{66}Zn$ at both 3- and 6-h following IP gavage of ^{67}Zn than control mice.

The lack of hypozincemia with hepcidin challenge is an interesting finding in this study. Despite being the master regulator of iron homeostasis, recent in vitro data indicate that hepcidin may play a role in the hypozincemia of inflammation and infection as well[5,75]. These in vitro data

show the possibility of hepcidin signaling for the internalization and degradation of ZnT1, the primary zinc exporter out of the enterocyte, similar to the effect of hepcidin on ferroportin. However, plasma and liver zinc concentrations in the current study were not altered by hepcidin challenge, and the ratio of $^{67}Zn/^{66}Zn$ did not respond to treatment by hepcidin injection following IP gavage of ^{67}Zn. These findings suggest that hepcidin does not play a role in the hypozincemia observed with infection in a biologically complex in vivo environment. Alternatively, the lack of a hypozincemia following hepcidin challenge in the current study may be due to the timepoints chosen in testing and the rapid clearance of excess hepcidin from plasma[104].

The lack of significant response in hepcidin concentrations after IL-6 injection was unexpected, as IL-6 is known to upregulate hepcidin transcription through the JAK/STAT pathway[95,105]. The shorter timeframe (24 h vs 48 h) in the current study may have contributed to the lack of an increase in hepcidin. However, the current study did find a more sustained elevation in hepcidin with IL-6 injections, with plasma levels of hepcidin 6 h following IL-6 treatment averaging 486.53 ng/mL compared to 360.60 ng/mL with hepcidin treatment at the 6 h timepoint. This may be due to the endogenous upregulation IL-6 would provide. However, this increase was not significant due to large amounts of variance and the increase seen may be the result of the diurnal variation discussed below. These data run counter to several previous observations, specifically that hepcidin concentrations reach their peak 6 h following injection and that plasma iron levels remain depressed for up to 48 h following injection[44,101]. In the current study, the average plasma hepcidin levels for all treatments peaked at the 3 h (501.2 ± 276.6) rather than the 6 h (424.2 ± 176.5) timepoint and plasma iron levels saw little difference when comparing the 3 h to the 24 h timepoint.

Overall means for all treatments of both hepcidin and IL-6 concentrations in the plasma are elevated at the 3 h and 6 h timepoint. This could be due to multiple reasons. One, the injection itself produces psychological stress[106] and inflammation, increased cytokine signaling, and a subsequent rise in IL-6 and hepcidin[107]. Interestingly, the overall variability of plasma concentrations of IL-6 and hepcidin were greater at the 3 and 6 h timepoints, but not for saline. These findings could suggest effects specific to IL-6 and hepcidin, where the variability is decreased at 24 h as the body has cleared much of the IL-6 or hepcidin challenge. Two, both IL-6 and hepcidin levels in plasma are subject to diurnal variation[108,109]. Diurnal variation references peaks and valleys in plasma hormone concentrations that relate to the time of day. In the case of both IL-6 and hepcidin, plasma levels appear to be at their lowest in the early morning at approximately 0900 hours. Injections in this study were performed between 0900 and 1030, with the 6 h sacrifice timepoint approaching the peak levels caused by diurnal variation at approximately 1800 hours. This would be consistent with the reduction in plasma iron and zinc observed at the 6 h timepoint regardless of treatment. The diurnal variation of minerals in the body has been observed for decades and concurrent variations in the hormones that regulate them may be a factor in this observation[110,111].

Plasma iron was also determined following injections of IL-6 and hepcidin. As shown previously, both treatments resulted in significant hypoferremia[75,104,112]. Unlike previous studies, however, the hypoferremia expressed in the current study was even more transient in comparison, with plasma iron levels returning to baseline values at the 24 h mark following hepcidin injection. The severity of hypoferremia was also attenuated, with the current study seeing 20-25% reductions in plasma iron and previous studies showing iron concentrations to be less than half of their control counterparts[104]. There were multiple differences in study design

between the current study and Rivera et al, 2005 that may account for this difference. Mice were fed a diet containing less than 4 ppm iron compared to 10 ppm in the current study. Female mice were also studied, and females have been shown to have distinct hepcidin responses compared to males[113]. Mice started the previous study at eight weeks compared to six, which is approximately equivalent to one year of a human's lifespan[114].

Conclusion

Collectively, the current study found that hepcidin is not a mediator of the hypozincemia observed in times of infection and inflammation in a mouse model. The concurrent hypoferremia with hepcidin treatments and hypozincemia shown with IL-6 injections lends credence to the model and suggests downstream effects of IL-6 are the primary mediator of the hypozincemia of infection. Both IL-6 and hepcidin are cornerstones of the processes of nutritional immunity and must be studied further to elucidate the full range of their effects on the body. Future research should determine hepcidin's effects on zinc transporter expression as well as IL-6's ability to alter zinc transport in the body by methods other than the upregulation of ZIP14.

APPENDIX A

ACUC APPROVAL

DEPARTMENT OF THE ARMY
U.S. ARMY RESEARCH INSTITUTE OF ENVIRONMENTAL MEDICINE
10 GENERAL GREENE AVENUE
BUILDING 42
NATICK, MA 01760-5007

REPLY TO
ATTENTION OF

MCMR-EMZ

31 January 2017

MEMORANDUM FOR Stephen Hennigar, PhD, Principal Investigator, Military Nutrition Division

PROJECT TITLE: Effects of inflammation and hepcidin on zinc homeostasis in *Mus musculus*
REFERENCES: USARIEM Protocol 16-21-A

SUBMISSION TYPE: New Project
REVIEW TYPE: Full Committee

ACTION: **Approval to Implement**
EFFECTIVE DATE: 31 January 2017

1. The USARIEM Scientific Review Committee (SRC) reviewed the above-referenced research. The SRC Chair approved the protocol on 8 September 2016. The Chair verified that the protocol was consistent with good scientific practice and that the number of animals to be used was appropriate to obtain sufficient data without being excessive and the statistical design was appropriate for the intent of the study.

2. The USARIEM Institutional Animal Care & Use Committee (IACUC) reviewed the above referenced new project at a convened meeting on 4 October 2016 and required modifications to secure approval. The IACUC reviewed your initial response at a convened meeting on 22 November 2016 and required further modification to secure approval.

3. Your second IACUC response and modified protocol was subsequently reviewed through the designated member review process. The designated reviewer confirmed that all required modifications were made and approved the research on 18 January 2017. All regulatory compliance requirements have been met and the Commander approved the research for implementation 31 January 2017.

4. An Annual Report must be submitted and reviewed by the IACUC prior to **17 January 2018**. A complete de novo review and approval of the protocol is required prior to **17 January 2020**. If the research is to continue beyond three years, you must submit and obtain IACUC approval of a new animal use protocol before this one expires.

5. You are approved for 187 male mice. The approved version of the protocol is dated 9 January 2017. You must obtain prior approval for any planned changes to the approved protocol. Any unanticipated pain and distress, morbidity, or mortality must be reported to the attending veterinarian and the IACUC.

MCMR-EMZ
SUBJECT: Approval to Implement Research under Protocol titled: Effects of inflammation and hepcidin on zinc homeostasis in *Mus musculus*. USARIEM Protocol 16-21-A

6. You may direct any questions to Ms. Enska, Office of Research Quality & Compliance at usarmy.natick.medcom-usariem.mbx.usariem-rqc-protocol@mail.mil. Please include the protocol title and number in all correspondence about this project.

[redacted]
Wayne Matheny, PhD
Designated Member Reviewer
USARIEM Institutional Animal Care and Use Committee

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BIOGRAPHICAL SKETCH

James (Jay) Schairer was born in Key West, Florida. Jay graduated with a Bachelor of Science in Food and Nutrition Science from Florida State University in 2019. After receiving this degree, Jay was accepted into the Master's in Nutrition and Food Science program at Florida State University. In spring of 2020, Jay began pursuing this degree under the mentorship of Dr. Stephen Hennigar. Jay initially planned a thesis working in combination with Dr. Hennigar, Dr. Jeong-Su Kim, and Dr. Erin Simmons of the Navy Experimental Dive Unit in Panama City Beach, Florida, but the COVID-19 pandemic made those plans defunct. After months in limbo with the research shutdown, Jay began working with Dr. Hennigar to continue his work on hepcidin's role in zinc homeostasis during periods of inflammation. In fall of 2020, Jay was awarded the opportunity to serve as a graduate teaching assistant for The Science of Nutrition, an undergraduate-level course through the Department of Nutrition & Integrative Physiology taught by Dr. Haiyan Maier. In spring of 2021, Jay was selected to the Naval Research Enterprise Internship Program, where he spent the summer of 2021 assisting Dr. Simmons at the Navy Experimental Dive Unit. Following graduation, Jay plans to attend Florida Gulf Coast University's Doctorate of Physical Therapy program, where he will leverage his knowledge in nutrition and understanding of research to better assist his patients on their road to recovery.