The Role of Hepcidin in Regulation of Iron Balance in Bats

By

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Iron storage disease is a significant cause of liver disease and mortality in captive Egyptian fruit bats (*Rousettus aegyptiacus*). The nature of the susceptibility in this and other captive exotic species to iron storage disease is not clear. Hepcidin, a key iron regulatory hormone, is involved in the regulation of iron absorption in humans and other mammalian species and a deficiency in hepcidin has been associated with a number of genetic mutations resulting in hemochromatosis in humans. The objectives of this thesis were to identify whether there is a functional mutation in the hepcidin gene in the Egyptian fruit bat that may increase the susceptibility of this species to iron storage disease, and whether there is a functional deficiency in hepcidin gene expression in the Egyptian fruit bat in response to iron challenge. We compared the coding region of the hepcidin gene amongst several species of bats and investigated hepcidin response to intramuscular injection of iron dextran amongst three species of bats with variable susceptibility to iron storage disease; the Egyptian fruit bat, the straw-colored fruit bat (*Eidolon helvum*), and the common vampire bat (*Desmodus rotundus*). While a number of genetic differences were identified amongst species, a functional mutation that could result in decreased hepcidin activity was not identified in the Egyptian fruit bat. Bats exhibited marked variation in hepcidin gene expression, with the highest level of hepcidin response to iron challenge in the common vampire bat. While the Egyptian fruit bat exhibited significant hepcidin response to iron challenge, the magnitude of response was lower than that in the
common vampire bat and lower than expected based on findings in healthy humans. The straw-colored fruit bat did not exhibit any hepcidin response despite a significant increase in iron stores, which suggests this species may have evolved an alternate mechanism for coping with excessive iron or may be more susceptible to iron overload than previously recognized.
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DECLARATION OF WORK PERFORMED

I declare, with the exception of the technical analyses listed below, all the work reported in this thesis was performed by me.

Plasma ferritin analysis was performed by Patricia (Sue) Chavey, at the Veterinary Diagnostic Laboratory, Kansas State University (Manhattan, Kansas). All remaining hematology, plasma iron assay analyses, and hepatic iron content determination were carried out at the Animal Health Laboratory, University of Guelph (Guelph, Ontario). The histotechnology division of the Animal Health Laboratory performed the histological staining of tissue sections. All statistical analyses were performed in consultation with William Sears at the Ontario Veterinary College, University of Guelph (Guelph, Ontario). A portion of the quantitative real-time PCR and DNA sequencing was performed by Jutta Hammermueller at the Ontario Veterinary College, University of Guelph (Guelph, Ontario).
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<th>Full Form</th>
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<tbody>
<tr>
<td>28s rRNA</td>
<td>28S ribosomal RNA</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>AHL</td>
<td>Animal Health Laboratory</td>
</tr>
<tr>
<td>ANCOVA</td>
<td>analysis of covariance</td>
</tr>
<tr>
<td>BID</td>
<td>bis in die (twice a day)</td>
</tr>
<tr>
<td>BLAST</td>
<td>basic local alignment search tool</td>
</tr>
<tr>
<td>CBC</td>
<td>complete blood cell count</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>DMT-1</td>
<td>divalent metal transporter</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxyribonucleotide</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>HAMP</td>
<td>hepcidin</td>
</tr>
<tr>
<td>HFE</td>
<td>high iron (Fe) (human hemochromatosis gene)</td>
</tr>
<tr>
<td>HIV</td>
<td>human immunodeficiency virus</td>
</tr>
<tr>
<td>HJV</td>
<td>hemojuvelin</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>induction coupled plasma mass spectrometry</td>
</tr>
<tr>
<td>IRE</td>
<td>iron regulatory element</td>
</tr>
<tr>
<td>IRP</td>
<td>iron regulatory protein</td>
</tr>
<tr>
<td>MANOVA</td>
<td>multivariate analysis of variance</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pI</td>
<td>isolelectric point</td>
</tr>
<tr>
<td>PO</td>
<td>per os</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>rho</td>
<td>Spearman’s rank correlation coefficient</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>reverse transcriptase quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
</tr>
<tr>
<td>SID</td>
<td>semel in die (once a day)</td>
</tr>
<tr>
<td>TfR2</td>
<td>transferrin receptor 2</td>
</tr>
<tr>
<td>TIBC</td>
<td>total iron-binding capacity</td>
</tr>
<tr>
<td>UIBC</td>
<td>unsaturated iron-binding capacity</td>
</tr>
<tr>
<td>WBC</td>
<td>white blood cell count</td>
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CHAPTER 1: LITERATURE REVIEW

Iron Metabolism

Iron: The Essential Element

Iron is one of the most abundant elements on earth and is intricately interwoven into the fundamental processes that make life possible. In addition to its critical role as a key component of the oxygen carrying molecules hemoglobin and myoglobin, iron acts as a co-factor in numerous cellular processes including DNA synthesis (Albertson, 2006; Andrews, 1999). It exists in one of two elemental forms, the oxidized (ferric) state and the reduced (ferrous) state, which influence how iron is taken up and distributed and its function in the body. Iron can catalyze the formation of oxygen free radicals that cause toxic damage to cell membranes and tissues, as is seen in iron overload conditions in humans and animals (Gatterman, 2009; Lowenstein and Munson, 1999). In order to prevent the damaging effects associated with excess iron accumulation, all living things have evolved mechanisms to tightly regulate iron homeostasis.

Fundamentally, mammals lack an efficient means of eliminating excess iron from the body. Iron is eliminated by the sloughing of epithelial cells at mucosal and skin surfaces and by hemorrhage. Typically, only a small percentage of total body iron is eliminated daily; <0.05 % in humans (Ganz, 2007). The majority of iron in the body (~70 %) is present in the form of hemoglobin in red blood cells, most of which is recycled from senescent erythrocytes by macrophages in the spleen, bone marrow, and liver (Andrews, 2000; Ganz, 2007). Normally,
less than 0.05% of total body iron is acquired each day through dietary intake, which balances iron loss (Brittenham et al., 2000; Ganz 2007).

The majority of dietary iron in humans is present in the oxidized (ferric) state and consists of heme and non-heme iron, the latter being complexed with organic acids or peptides (Ganz, 2007). Heme is a highly bioavailable form of iron and is released as a product of the proteolytic digestion of myoglobin and hemoglobin in the small intestine (Mackenzie and Garrick, 2005). Heme is the major source of dietary iron in carnivorous species and species such as the common vampire bat, which is an obligatory sanguivore (Morton and Janning, 1982). Heme is taken up into the endosomes or lysosomes of enterocytes in an intact form by cellular transporters that are poorly characterized (Mackenzie and Garrick, 2005). Once in the endosome or lysosome, ferrous iron is believed to be cleaved from heme and released into the cytosol by an as yet undescribed mechanism. In the case of non-heme iron, acidity in the stomach facilitates transformation of ferric iron to the reduced state and enhances the solubility and absorption of iron complexes in the small intestine (Andrews, 1999). Dietary vitamin C plays an important role in enhancing iron absorption by reducing ferric iron and forming iron soluble complexes, which are highly bioavailable (Hunt et al., 1994). The majority of intestinal iron absorption takes place at the level of the duodenum and proximal jejunum (Mackenzie and Garrick, 2005). At the apical membrane of the enterocyte, iron in the ferric state is reduced by the enzyme ferric reductase (duodenal cytochrome B) and is taken up by the cellular iron importer divalent metal transporter-1 (DMT-1) (Mackenzie and Garrick, 2005). This key transporter is present in the brush border of mature enterocytes, primarily in the proximal duodenum, as well as in erythroid precursors and many other cell types (Mackenzie and Garrick, 2005). DMT-1 also facilitates the
cellular uptake of other divalent metal ions including cadmium, zinc, manganese, copper, nickel and lead (Andrews, 1999; Mackenzie and Garrick, 2005).

Once iron enters the enterocyte, it is bound to the iron storage protein, ferritin, in the cytosol or is exported into plasma. Ferritin-bound iron is subsequently sloughed with exfoliating epithelium and is thus eliminated through the intestinal tract (Andrews, 1999). Iron export from the enterocyte and transfer to plasma is dependent on ferroportin, a transmembrane protein, which is located in the basolateral cell membrane of the enterocyte. Ferroportin is also highly expressed in macrophages, hepatocytes, and placental trophoblasts (Donovan et al., 2005). Ferroportin deficiencies result in iron accumulation in these cell types and homozygous ferroportin knock-out mice exhibit early embryonic mortality, which supports the vital function of this protein in homeostasis (Donovan et al., 2005).

After being exported, reduced (ferrous) iron in the cell is oxidized by hephaestin, a copper dependent analogue of plasma ceruloplasmin, in order to be taken up by transferrin in blood (Roy and Enns, 2000). Transferrin is the iron transport protein in blood which binds oxidized iron and distributes iron throughout the body. It has two binding sites, which are functionally equivalent and exists in one of three forms; apo-transferrin, when no iron is bound, monoferric transferrin, when one site is complexed with ferric ion, and differic transferrin or holo-transferrin, when saturated (Harris and Aisen, 1975). Transferrin-bound iron is taken up by transferrin receptors on the cell surface, reduced and incorporated into endosomes (Andrews, 2000). The majority of transferrin-bound iron is carried to erythropoietic cells for incorporation into newly forming red blood cells.

Under normal physiologic conditions, up to 25% of total body iron is stored either as ferritin or hemosiderin in macrophages and hepatocytes, and a small fraction is bound to
transferrin in plasma (Albertson, 2006). Hemosiderin is an insoluble intracellular iron-storage complex which predominates in iron-overload states and may represent a degradation product of ferritin (Harrison and Arosio, 1996). It is often found within macrophages in association with hemorrhage, where it is derived from the breakdown of erythrocytes which have been phagocytosed as part of red blood cell turnover, rather than from the uptake of transferrin bound iron (Brittenham et al., 2000; Cullen and MacLachlan, 2001). When the iron binding capacity of circulating transferrin is saturated; i.e., the amount of absorbed iron in plasma exceeds its iron-binding capacity, non-transferrin bound iron is present (Ganz, 2007). This occurs in conditions such in hereditary hemochromatosis in humans. Hepatocytes, which are able to take up both transferrin-bound and non-transferrin bound iron from plasma, are thus the major site of iron storage in iron overload states (Andrews, 2000).

**Regulation of Iron Metabolism**

Iron regulation takes place at a number of levels. In the enterocyte, iron homeostasis is regulated by differential expression of proteins responsible for iron uptake and export. At the post-transcriptional level, the mRNA templates of various proteins involved in iron metabolism contain regions termed iron regulatory elements (IREs), which serve as binding sites for iron regulatory proteins (IRPs) that regulate translation in response to levels of intracellular iron (Pantopoulos, 2004; Roy and Enns, 2000). In response to cellular iron deprivation, IRPs stabilize transferrin receptor mRNA, resulting in increased transferrin receptor production and uptake of transferrin-bound iron from plasma, and inhibit ferritin translation, preventing iron sequestration within the cell (Pantopoulos, 2004). Post-transcriptional control of gene
expression by IREs and IRPs has also been recognized for DMT-1 and ferroportin (Pantopoulos, 2004).

Studies have shown that excessive iron absorption from the diet results in transient impairment of iron uptake, previously coined the ‘mucosal block’ (Andrews, 1999). Intestinal iron absorption also appears to be regulated by tissue iron stores and is decreased in states of iron excess (Andrews, 1999). Erythropoietic activity has been shown to be a powerful positive regulator of intestinal iron uptake; increased absorption results regardless of tissue iron stores (Andrews, 1999). Anemia and hypoxemia have, likewise, been shown to induce iron absorption (Ganz, 2007; Nicolas et al., 2002). The molecular mechanisms underlying the regulatory processes that govern iron absorption were largely undefined until the recent discovery of hepcidin (Park et al., 2001; Pigeon et al., 2001).

**Hepcidin**

Hepcidin, an iron regulatory protein produced by the liver, is the principal regulator of iron homeostasis in the body. Its discovery has greatly expanded our understanding of many of the fundamental processes that govern iron metabolism, leading to new avenues for research into iron physiology and iron-related diseases (Ganz, 2006; Ganz, 2007; Nemeth and Ganz 2004; Park et al., 2001; Pigeon et al., 2001; Rivera et al., 2005). As well as being a potent regulator of iron metabolism, hepcidin exhibits both modest antifungal and antibacterial activity and may have a role in the acute phase response similar to other antimicrobial peptides (Cuesta et al., 2008; Park et al., 2001).

The hepcidin gene contains three exons and two introns and encodes an 84 amino acid prepro-peptide, which is cleaved into a functional 25 amino acid peptide (Park et al., 2001).
Nuclear magnetic resonance imaging has revealed a hairpin structure with four disulfide bonds, with a relatively high number of cysteine residues compared to other cysteine-rich antimicrobial peptides (Hunter et al., 2002; Park et al., 2001). Hepcidin is primarily expressed in the liver, with weaker expression in other tissues including the stomach, small intestine, lungs, heart, and thymus (Badial et al., 2011; Chen et al., 2007; Pigeon et al., 2001). It is excreted in urine, where it has been found mainly as the bioactive 25 amino acid peptide, but also as inactive truncated 22 and 20 amino acid versions that may represent degradation products (Park et al., 2001).

The active hepcidin peptide appears to be highly conserved amongst vertebrate taxa with homologous sequences in over 51 different highly diverse species including dog, sheep, zebrafish, rat, and pig (Hilton and Lambert, 2008; Nemeth and Ganz, 2006; Park et al., 2001). Homology in protein sequence amongst species, particularly in the region of disulfide bonds and the N-terminal region, suggests a conserved functional significance. In some species, including mice and certain species of fish, multiple copies of the hepcidin gene exist that result in the formation of proteins which exhibit differences in their distribution and functional activity (Hilton and Lambert, 2008).

**Mechanism of Action**

Hepcidin acts by causing the internalization and degradation of ferroportin, the sole cellular iron export protein (Donovan et al., 2005; Nemeth et al., 2004). When tissue and plasma iron levels are high, hepcidin expression is induced, resulting in decreased enteric iron absorption and decreased release of iron from macrophages and hepatocytes (Donovan et al., 2005; Nemeth and Ganz, 2006). Elevated hepcidin mRNA levels occur in mice with experimentally induced iron overload (Pigeon et al., 2001). Transient elevations in urinary
hepcidin have also been described in humans dosed with oral iron (Nemeth et al., 2004). When tissue and plasma iron levels are low, hepcidin expression is decreased and ferroportin levels are elevated, increasing enteric iron absorption and release into plasma. Studies in humans and mice have revealed that alterations in hepcidin metabolism can have profound effects on iron uptake and distribution in the body, resulting in a wide spectrum of physiologic disorders (Ganz, 2007). Hepcidin knockout mice develop iron overload with profound iron accumulation in the liver and pancreas, similar to that seen in human hereditary hemochromatosis (Nicolas et al., 2001). In contrast, mice with hepcidin excess develop anemia and hypoferremia (Rivera et al., 2005). The injection of synthetic hepcidin into mice results in profound dose-dependent hypoferremia within one hour, with effects lasting up to 72 hours (Rivera et al., 2005).

**Role in Inflammation**

Iron sequestration and depletion occurs in association with inflammation; however, the functional significance of this is not well elucidated. It has been suggested that iron sequestration in inflammatory states may have a protective role (Jurado, 1997). Pathogenic microbes rely on iron to maintain their metabolic processes and iron depletion may thus prevent or reduce infection (Ratledge and Dover, 2000). In support of this hypothesis, patients with hemochromatosis are more susceptible to infection with agents including *Vibrio cholerae, E.coli*, hepatitis C virus, and HIV (Khan et al., 2007). The anemia of chronic inflammatory disease is normocytic or microcytic and accompanied by hypoferremia despite normal iron stores; these effects are attributed to iron sequestration in enterocytes and macrophages (Rivera et al., 2005, Ganz, 2007). Hepcidin appears to be a key mediator of anemia of chronic disease as it is known to downregulate enteric iron absorption and iron mobilization from cellular stores (Ganz, 2007).
Inadequate erythropoiesis due to restricted iron supply to the erythropoietic precursors in the bone marrow is thought to ensue.

Hepcidin production is induced by inflammatory cytokines, predominantly interleukin-6 (IL-6) (Nemeth et al., 2004). Interleukin-6 knockout mice show a lack of hepcidin induction in response to subcutaneous turpentine injection, whereas wild type mice develop elevations in hepcidin gene expression and two-fold reductions in serum iron (Nemeth et al., 2004). These results support a direct role for hepcidin in the development of iron-deficiency anemia in response to acute phase inflammation and chronic inflammatory conditions (Nemeth et al., 2004; Nicolas et al., 2002; Rivera et al., 2005).

**Role in Anemia and Hypoxemia**

While inflammation induces hepcidin expression, hepcidin levels are suppressed by anemia and hypoxemia (Nemeth and Ganz, 2006; Nicolas et al., 2002). Hepcidin levels decrease in mice kept in hypobaric chambers and in mice subjected to phlebotomy and to phenylhydrazine-induced hemolytic anemia (Nicolas et al., 2002). Both anemia and hypoxemia induce erythropoiesis, which appears to be the single strongest mediator of hepcidin suppression (Finch, 1994; Nicolas et al., 2002). Decreased hepcidin levels increase the amount of iron available for erythropoiesis through increased enteric iron absorption and increased iron mobilization from macrophages and tissue stores. The underlying mechanisms that regulate hepcidin activity are still under investigation, but it has been postulated that hypoxia-inducible factor, a transcription regulator, or erythropoietin itself may be implicated (Nemeth and Ganz, 2006).
**Iron Overload Disease**

**Primary Hemochromatosis**

Hereditary hemochromatosis is the most common genetic disorder in the Caucasian human population, predominantly affecting people of Northern European origin, with a prevalence as high as 1 in 200 (Pietrangelo, 2004; Tavil, 2001). The hereditary basis of this condition has been widely recognized since the 1930s, and an autosomal recessive mode of inheritance was established in the 1970s. In 1996, a mutation in the HFE gene, which encodes for a major histocompatibility type 1 protein, was discovered, located on chromosome 6 (Pietrangelo, 2004). Other less common genetic mutations have subsequently been identified. Currently, there are four types of hereditary hemochromatosis involving distinct mutations in five iron-regulatory genes: HFE, transferrin receptor 2, ferroportin, and hepcidin or hemojuvelin. HFE hemochromatosis remains the most prevalent, accounting for more than 80% of cases (Zoller et al., 2001). The membrane protein encoded by this gene is similar to major histocompatability complex class-I proteins and associates with \( \beta_2 \)-microglobulin (Pietrangelo, 2004). The most common mutation in the HFE gene involves a single base pair substitution, resulting in altered HFE protein conformation and binding to \( \beta_2 \)-microglobulin. Alteration in the HFE-\( \beta_2 \) microglobulin complex results in impaired cell surface expression of HFE and its binding to transferrin receptor 1, which normally facilitates uptake of transferrin-bound iron (Pietrangelo, 2004). The second major form of hemochromatosis, juvenile hemochromatosis, most commonly involves a mutation in hemojuvelin (HJV). This form results in the most severe type of hemochromatosis, with development of cardiomyopathy, hypogonadism, diabetes, and heart failure by thirty years of age (Cazolla et al., 1998; Pietrangelo, 2004). Most cases of
juvenile hemochromatosis have been linked to a mutation in chromosome 1q, although several cases have been associated with a mutation in the hepcidin gene (HAMP) itself (Roetto et al., 2003). A distinct form of adult-onset hemochromatosis involves a mutation in transferrin receptor 2 (TfR2), which may have a similar function to TfR1 in mediating the uptake of transferrin-bound iron (Mattman et al., 2002). A mutation in the ferroportin gene (C326S), results in a distinct form of hereditary hemochromatosis, with an early age of onset (Drakesmith et al., 2005, Sham et al., 2009). Patients with an autosomal dominant mutation in this gene develop iron overload despite elevated serum hepcidin levels (Sham et al., 2009). In this condition, increased iron absorption is the result of ferroportin resistance to hepcidin (Drakesmith et al., 2005).

Histologically, the various forms of hereditary hemochromatosis in humans share similar features. In early stages, iron accumulates in hepatocytes, particularly in the periportal region. As the disease progresses, iron deposits in Kupffer cells and bile duct epithelium, and periportal fibrosis and fibrous bridging between portal tracts develop (Bacon, 2001). Cirrhosis is seen in advanced disease, sometimes accompanied by hepatic neoplasia. Hepatic failure accounts for 75% of mortalities resulting from the disease (Adams et al., 1997; Neideraou et al., 1996; Tavil, 2001).

The Role of Hepcidin

The recent recognition of hepcidin as a regulator of iron uptake has greatly enhanced our understanding of hereditary hemochromatosis in humans. Hepcidin deficiency is present in HFE, hemojuvelin, and TfR2 type hemochromatosis and may be the underlying factor responsible for development of clinical disease (Nemeth and Ganz, 2006). The various
mutations involved in these forms of hereditary hemochromatosis result in diseases of varying severity, which suggests these proteins may have different roles in hepcidin synthesis or regulation (Ramos et al., 2011). Mutations in hemojuvelin hemochromatosis phenotypically resemble homozygous mutations in the hepcidin gene itself, resulting in the most severe form of hemochromatosis, whereas homozygous mutations in the HFE gene result in less severe decreases in hepcidin and a milder form of disease (Roetto et al., 2003). In addition to the specific recognized effects of these genetic mutations, a wide variation in phenotypic expression of iron storage disease exists for each condition suggesting that environmental and epigenetic factors may influence the severity of clinical disease (Beutler, 2007).

**Secondary Hemochromatosis**

Secondary hemochromatosis exists when there is excessive iron storage in the absence of a recognized primary hereditary disorder of iron metabolism. It is most typically seen in association with chronic transfusion therapy; in association with congenital or acquired anemias such as thalassemia, myelodysplastic syndromes and sickle-cell anemia; and with excessive dietary iron intake (Andrews, 1999; Gatterman, 2009). Transfusional iron overload is the most common form of secondary hemochromatosis. Each unit of blood contains a large amount of iron (about 200-250 mg), which is not readily eliminated from the body, and thus iron accumulates with repeated transfusions (Gatterman, 2009). Secondary hemochromatosis differs from hereditary hemochromatosis in terms of the distribution of iron in tissue. In secondary hemochromatosis, iron accumulates in Kupffer cells and macrophages as well as hepatocytes. This is in contrast to hereditary hemochromatosis where reticuloendothelial cells are relatively
less iron loaded. Periportal fibrosis and cirrhosis occur in advanced cases of secondary hemochromatosis as they do in the primary disease (Gordeuk, 1992).

Dietary iron overload, previously coined “Bantu siderosis”, is highly prevalent amongst sub-Saharan African tribal communities who commonly use non-galvanized steel pots to brew alcohol (Gangaidzo et al., 1999). Traditional beer brewed by this method contains excessive iron in a form that is highly bioavailable (Gordeuk, 1992). As iron overload does not develop in all those who partake in beer drinking, a genetic mutation that predisposes to iron overload is thought to contribute to development of disease (Andrews, 1999; Gordeuk, 1992).

**Hemochromatosis in Exotic Species**

Iron overload conditions in exotic species can be classified into two main classes; hemosiderosis, which is characterized by iron accumulation in tissues without associated toxic morphologic changes, or hemochromatosis, which involves morphologic changes (Lowenstine and Munson, 1999). Hemosiderosis is relatively common in a variety of species and has been reported in association with concurrent disease, anemia, and starvation (Borch Iohnsen and Nilseen, 1987; Cork, 2000; Kincaid and Stoskopf, 1987; Lowenstine and Munson, 1999). Seasonal iron accumulation has been reported in common starlings (*Sturnus vulgaris*) and eider ducks (*Somateria mollissima*) at the end of moult and during incubation and brooding; times that are often associated with starvation and stress (Borch-Iohnsen et al., 1991; Garcia et al., 1984). Waterfowl appear to be particularly susceptible to iron accumulation associated with concurrent disease and there have been reports of hemosiderosis in black-necked (*Cygnus melancoryphus*) and whistling swans (*Cygnus columbianus*) with concurrent emaciation, parasitism, and lead toxicity (Cork et al., 1995; Irwin, 1975; Norambuena et al., 2009). Other associations have been
reported in mammals. For examples, in Svalbard reindeer (*Rangifer tarandus*) in Norway, hemochromatosis was associated with seasonal shortages of feed and ingestion of plants that were high in iron content (Borch Iohnsen and Nilseen, 1987). Hemosiderosis is not an uncommon finding in neonatal animals, where it is believed to be associated with a shift from fetal to adult hemoglobin; however, in most cases this does not reflect a true iron overload disorder (Kinsely, 1994). Hepatic hemosiderosis is a common necropsy finding in many captive mammal species including black rhinoceros (*Diceros bicornis*), red panda (*Ailurus fulgens*), coati (*Nasua spp.*), cheetah (*Acinonyx jubatus*), snow leopard (*Panthera uncia*), and captive lowland gorilla (*Gorilla gorilla*) (Kock et al., 1992; Lowenstine and Munson, 1999). In most instances, iron accumulation is considered incidental and is not associated with pathologic changes in these species.

In the black rhinoceros, hemosiderosis has been seen in association with hemolytic anemia, a common cause of mortality in captive individuals (Kock et al., 1992). A causal relationship is suspected, although iron accumulation has also been documented in animals without concurrent hemolysis (Lowenstine and Munson, 1999). Iron accumulation has also been reported in the Sumatran rhinoceros (*Didermocerus sumatrensis*), which is also a browsing species, but not in grazing species such as the white rhinoceros (*Ceratotherium simum*). This suggests dietary factors as well as a genetic predisposition may be implicated (Linzmeier, 2008).

As iron accumulation in humans has been associated with increased incidence of infection and neoplasia, significant iron accumulation is of concern in exotic species (Gordeuk et al. 1996; Khan et al., 2007).

True hemochromatosis has been reported in only a small proportion of species: birds of the families Ramphastidae (toucans), Sturnidae (mynahs and starlings), Paradisaeidae (birds of
paradise), and tanagers (family Thraupidae), Egyptian fruit bats (*Rousettus aegyptiacus*), bongos (*Boocercus euryceros*), Baird’s (*Tapirus bairdii*) and Brazilian tapir (*Tapirus terrestris*), Afghan pika (*Ochotona rufescens*), rock hyrax (*Procavia capensis*), several species of lemurs (family Lemuridae), and marmosets (family Callitrichidae) (Bonar et al., 2006; Crawshaw et al., 1995; Gonzales et al., 1984; Kincaid and Stoskopf, 1987; Lowenstine, 1978; Lowenstine and Munson, 1999; Madarme et al., 1990; Smith et al., 2008; Spelman et al., 1989; Rehg et al., 1990). In bongo, the disease is progressive, developing during the first few weeks of life, and resembles hereditary hemochromatosis in Saler’s cattle (House et al., 1994; Lowenstine and Munson, 1999). In certain species of birds, including mynahs and birds of paradise, iron accumulates primarily in hepatocytes with lesser amounts in Kupffer cells and macrophages in portal areas, resembling hereditary hemochromatosis in humans (Mete, 2005). Ultrastructurally, iron is found free in the cytosol as ferritin moieties and larger hemosiderin aggregates, as well as bound to plasma membranes (Lowenstine, 1978). In advanced stages, there is periportal fibrosis and hepatocellular necrosis culminating in cirrhosis. An increased incidence of hepatic neoplasia has been reported in lemurs, mynahs, Egyptian fruit bats, and marmosets (Crawshaw et al., 1995; Gonzales et al., 1984; Hill et al., 1986; Miller et al., 1997; Spelman et al., 1989). In other species, such as tanagers and lemurs, iron is deposited primarily in Kupffer cells and portal macrophages rather than in hepatocytes; a distribution more characteristic of nutritional secondary hemochromatosis in humans (Gonzales et al., 1984; Kincaid and Stoskopf, 1987; Spelman et al., 1989). In tanagers, an increased incidence of hemochromatosis has been associated with concurrent chronic inflammatory disease and dietary levels of iron (1230 ppm) that far exceed those recommended for poultry (Kincaid and Stoskopf, 1987).
Although diet is clearly a major factor in the development of hemochromatosis in some species, not all species fed an iron-rich diet develop disease. The underlying cause of iron overload in exotic species is likely multifactorial, with both nutritional and metabolic factors and intercurrent disease processes playing a role.

The Role of Diet

There is some evidence in affected avian species that the degree of hepatocellular iron accumulation increases with increasing time in captivity (Taylor, 1984). Hemosiderosis has been reported in captive black rhinoceroses, but not in free ranging or recently caught animals, suggesting that dietary factors or other features of captivity may play an important role in the development of disease (Kock et al., 1992).

From an evolutionary standpoint, a more efficient means of iron absorption in response to low levels of iron in the natural diet may have provided an advantage in the natural environment, resulting in increased susceptibility to iron accumulation in captivity. Many of the species susceptible to iron storage disorders in captivity are frugivores, insectivores, folivores, or browsers, whose natural food items are likely low in iron (Clauss et al., 2007; Cork et al., 1995; Dierenfeld et al., 1994; Wood et al., 2003). Items such as skins of fruit, leaves, foliage, and browse contain high levels of phytates and tannins, which are natural iron chelators. These items, particularly browse, are often limited in captive zoo diets. Other dietary components such as vitamin C enhance iron absorption by increasing the bioavailability of iron in feed (Hunt et al., 1994). Unfortunately, data on the nutrient composition of wild diets is lacking for most species with most available information pertaining solely to the variety of food items selected. In captivity, natural food items are often replaced by cultivated fruit, pelleted feeds, and vitamin
and mineral supplements of varying composition. Dietary nutrient composition for captive zoo species is often based on requirements in domestic species, which can result in nutrient excesses and deficiencies (Dempsey, 2004).

Some work has been done looking at the effects of diet modification on iron metabolism in a variety of exotic species, including lemurs, straw-colored fruit bats, starlings, and toucans (Drews et al., 2004; Lavin et al., 2010; Seibels et al., 2003; Wood et al., 2003). Lemurs are postulated to be adapted to low levels of absorbable iron in their diet because in the wild they eat foods high in tannins. A reduction of dietary iron and vitamin C content and an increase in dietary tannins was found to result in a decreased percentage of plasma transferrin saturation in several lemur species (Wood et al., 2003). Similarly, the provision of tea leaves to starlings fed an iron-enriched diet resulted in decreased liver iron content relative to animals that did not receive tea leaf supplementation (Seibels et al., 2003).

The Role of Genetics

Despite evidence that excessive dietary levels of iron contribute to hemochromatosis in exotic species, the physiologic mechanisms responsible for the development of this condition are not yet well understood. Interestingly, species with similar dietary preferences appear to vary in their susceptibility to hemochromatosis, which suggests genetic factors may be implicated (Mete, 2005).

Considerable experimental work has been undertaken in mynah birds (Gracula religiosa, Acridotheres tristis); species with known susceptibility to hemochromatosis. In response to a high iron diet, iron absorption in the mynah is downregulated to a lesser degree and iron retention is up to eight-fold higher than in the Eurasian collared dove (Streptopelia decaocto)
Similarly, in vivo, mynah enterocytes have higher rates of iron uptake than chicken enterocytes, despite much higher levels of iron in tissues (Mete et al., 2003). Levels of gene expression of DMT-1 in the duodenum and of ferroportin in all tissues are higher in the mynah than the chicken (Mete, 2005). These findings suggest a high efficiency of iron uptake in mynah species, similar to that seen in humans with HFE hemochromatosis (Zoller et al., 2001). Greater intestinal iron uptake has also been described in several species of lemurs and straw-colored fruit bats (Eidolon helvum) relative to normal ranges in humans (Lavin et al., 2010; Wood et al., 2003). In black rhino, a difference in gene sequence, relative to three other rhino species, human, and mouse, has been identified in the HFE gene at the site of interaction with the transferrin receptor (Beutler, 2001). The significance of this genetic difference is currently still under investigation (Linzmeier, 2008). These data support the hypothesis that in some species metabolic pathways are programmed to promote iron absorption, thus predisposing to the development of iron storage disease in situations of dietary iron excess, such as occurs in captivity.

**Diagnosis of Iron Storage Abnormalities**

Early diagnosis of excessive iron storage poses a diagnostic challenge to the clinical veterinarian as clinical signs are often not apparent until advanced stages of disease. Clinical signs in birds and exotic mammals are typically associated with liver failure and include abdominal distension secondary to ascites, hepatomegaly, weight loss, icterus, anorexia and, in birds, dyspnea (Crawshaw et al., 1995; Hill et al., 1986; Lowenstine and Munson, 1999). Early diagnosis and treatment is essential in order to prevent disease progression in susceptible species. Liver biopsy remains the gold standard for diagnosis of iron storage disease in exotic species.
(Lowenstein and Munson, 1999). Limitations exist due to the invasiveness of liver biopsy sampling and the potential for associated surgical complications, especially in small animals. In humans, liver biopsy sampling has become less commonplace with the advent of novel blood-based assays and genetic tests (Bacon, 2001). Histologic evaluation of liver biopsies allows for grading of the severity of iron accumulation and evaluation for associated pathologic changes including fibrosis or neoplasia. In humans, hepatic fibrosis is the most important prognostic marker and normal life expectancy is expected if phlebotomy therapy is commenced before fibrosis develops (Whittington and Kowdley, 2002).

The identification of several genetic mutations responsible for hereditary hemochromatosis in humans has resulted in the development of specific genetic tests, which have become widely available (Bacon, 2001; Brittenham et al., 2000). Screening strategies depend on the availability of genetic testing and population susceptibility to particular mutations, with biological markers and genetic tests often used in concert (Whittington and Kowdley, 2002).

In humans, assessment of serum iron, total iron binding capacity, transferrin saturation, and serum ferritin has proven to be useful in the diagnosis, determination of prognosis, and treatment of iron storage disease (Bacon, 2001, Tavil, 2001). Serum iron levels reflect the total amount of iron in blood, including transferrin-bound and non-transferrin bound iron. Total iron binding capacity is a measurement of the maximal concentration of iron that transferrin can bind. Transferrin saturation can be indirectly estimated by calculating the ratio of serum iron to total iron binding capacity (Tavil, 2001). In mammals, normally almost all serum iron is bound to transferrin and in humans, transferrin is approximately 30% saturated (Lin et al., 2007). Transferrin saturation values of over 50% for women and 60% for men have a high sensitivity
and specificity for the diagnosis of hereditary hemochromatosis (Tavil, 2001). The application of these techniques to exotic species is still under investigation. In normal birds, transferrin saturation values can be as high as 80% (Lowenstine and Munson, 1999). Studies in mynahs and pigeons have shown little correlation amongst serum iron parameters, the degree of liver iron content, and total body iron status (Mete, 2005; Whiteside, 2001). Evaluation of serum iron parameters in nine different species of captive lemurs revealed marked variation in mean values amongst species, suggesting that the utility of serum iron testing needs to be established on a species specific basis (Williams et al., 2006).

Ferritin, the major iron storage protein in the body, is present in only small concentrations in plasma (Harrison and Arosio, 1996). In humans, serum ferritin levels in general reflect tissue iron stores, but will vary with age and gender, and increase in inflammatory processes and conditions such as hyperthyroidism, chronic hepatitis, alcohol-induced liver disease, and neoplasia. The diagnostic usefulness of ferritin assessment is thus limited (Whittington and Kowdley, 2002). As well, variation in the molecular structure of ferritin results in a need for species specific radioimmunoassays. Nonetheless, ferritin assays have been developed for a variety of species including ten species of lemur, the tapir, the black rhinoceros, and the Egyptian fruit bat (Farina et al., 2005; Paglia, 2000; Smith, 1995; Smith et al., 2008; Williams et al., 2006). A study in Egyptian fruit bats found a significant correlation amongst serum iron, transferrin saturation, and plasma ferritin and histologic grades for hepatic iron deposition. The product of transferrin saturation and serum iron had a high sensitivity and specificity for the detection of hemochromatosis in this species (Farina et al., 2005). Likewise, studies in marmosets have shown good correlation amongst both serum ferritin and percent transferrin saturation and liver iron content (Smith et al., 2008).
The use of magnetic resonance imaging (MRI) to assess hepatic iron stores is currently under investigation. MRI has been used in humans for this purpose and is now an accepted modality for assessing liver iron content (Angelucci et al., 1997). The diagnostic potential of this technique in animals is not clear. In pigeons with experimentally induced iron overload, decreased MRI signal intensity corresponding to hepatic iron increase was noted (Matheson et al., 2007). MRI is not likely to prove useful in a clinical setting to monitor response to therapy in exotic patients as cost, the need for restraint and anesthesia, and the small size of many patients limit diagnostic utility.

_Treatment Modalities_

The mainstay of iron overload treatment in humans involves weekly to bi-weekly phlebotomy; the goal of which is to reduce total body iron stores. Guidelines for treatment include a reduction of serum ferritin to < 50 µg/L and of serum transferrin saturation to <35%, as well as evidence of stalled erythropoiesis; i.e., a failure of hemoglobin and hematocrit levels to rebound prior to the subsequent phlebotomy (Bacon, 2001; Brittenham et al., 2000). Once these goals are met, maintenance therapy is initiated, which involves the withdrawal of ~500 ml, or one unit of blood, three or four times per year. Patients undergoing treatment are encouraged to limit vitamin C intake and avoid iron supplementation (Brittenham et al., 2000; Whittington and Kowdley, 2002). The use of iron chelators is typically reserved for patients with congenital or acquired anemias who cannot tolerate regular phlebotomy (Tavil, 2001).

There is little published data on the use of phlebotomy to treat iron overload in exotic species. Limitations depend on the species and situation and include cost, need for anesthesia, frequency of treatment required, and stress inflicted by repeated handling. The successful use of
phlebotomy has been reported in two toucans, a mynah, and domestic horses (Lowenstein and Munson, 1999; Morris et al., 1989).

Deferoxamine is an injectable compound that binds free iron, ferritin, and hemosiderin into stable complexes that are excreted in urine and feces (Whiteside, 2001). It has been used successfully in the treatment of hemochromatosis in a channel-billed toucan (Ramphastos vitellinus) and a Bali mynah (Leucopsar rothschildi), in combination with phlebotomy (Cornellison, 1995; Loomis, 1993). In starlings, treatments with combined weekly phlebotomy and the iron chelator deferoxamine decreased liver iron content to non-toxic levels when iron overload was induced by feeding a high iron diet (Olsen et al., 2006). Its clinical utility in exotic species is limited by the need for frequent administration and the associated stress of handling. Deferiprone is a newer oral iron chelating compound that has been shown to reduce hepatic iron concentrations in white leghorn chickens and pigeons with experimentally induced hemosiderosis (Whiteside et al., 2004). The clinical utility of iron chelating compounds in bats and other mammalian species requires further investigation.

Prevention of iron accumulation in hemochromatosis-susceptible species is primarily based on dietary management. Based on studies in mynahs, diets with iron levels <25 ppm, much lower than that found in most commercial diets, have been recommended for susceptible species (Mete, 2005). Dietary modifications, including the addition of iron chelating compounds, have also been investigated. A decrease in dietary iron and vitamin C and increases in phytates and tannins resulted in decreased transferrin saturation in several species of captive lemurs and toco toucans (Ramphastos toco) (Drews et al., 2004; Wood et al., 2003). The addition of tannic acid to the diet of a group of captive straw-colored fruit bats resulted in a 40% reduction in iron absorption measured using stable isotope analysis of blood (Lavin et al., 2010).
The addition of iron chelating compounds to the diet of hemochromatosis susceptible species warrants further investigation.

**Hemochromatosis in Bats**

*Bats: Nutrition and Ecology*

The order Chiroptera, one of the most diverse in the animal kingdom, is comprised of over 1000 species of bats (Jones et al., 2003; Nowak, 1991). Bats are widely distributed across the temperate and tropical regions of the world and have developed unique adaptations with differing life histories and ecological niches (Jones et al., 2003; Nowak, 1991). The order Chiroptera is subdivided into two suborders, the Megachiroptera and the Microchiroptera (Nowak, 1991). Microchiropterans are found worldwide and rely on echolocation to navigate and locate the position of their prey. They include all the insectivorous bats, which make up 70% of the world’s extant bat species, a small number of frugivores and nectarivores, and several species which feed exclusively on blood, small mammals, or fish (Dempsey, 2004; Jones et al., 2003). Megachiropterans, which include the Egyptian fruit bat and straw-colored fruit bat, are restricted to the tropical and subtropical regions of the world and feed almost exclusively on fruit and leafy vegetation.

The Egyptian fruit bat ranges throughout Arabia, the Persian Gulf, Cyprus, Turkey, and Africa (Nowak, 1991). It is a primarily frugivorous species, although leaves of various plants and pollen are also occasionally consumed, particularly in the winter, when fruit is scarce (Hadjirsterkosis, 2006; Korine, 1999). As a result of exploitation of cultivated fruit resources,
this species is considered an agricultural pest in many countries, which has resulted in a marked reduction in its numbers in many regions.

The straw-colored fruit bat has an extensive range and is found throughout the south-western Arabian Peninsula, sub-Saharan Africa and Madagascar (Nowak, 1991). This species forms enormous colonies comprised of tens of thousands of individuals and undergoes seasonal migrations over hundreds of kilometres in search of ripened fruit. Its diet consists of juices of various fruits, fruit blossoms, and shoots (Nowak, 1991; Richter, 2006). Throughout sub-Saharan Africa, this species has been found to consume the flowers of at least 10 genera of plant, the fruit of 34 genera of plant, and the leaves of 2 genera of plant and to have distinct dietary preferences based on the spatio-temporal availability of food items (Marshall, 1985).

Data on the nutrient composition of wild fruit bat diets is largely lacking, with most available information describing the types of foods consumed based on analysis of fecal and stomach contents (Farina et al., 2005). Figs (Ficus spp.) form the staple of the diet of the majority of megachiropteran species, including the Egyptian fruit bat and straw-colored fruit bat, with fig seeds reported in up to 80% of fecal samples collected from some species (Marshall, 1985). There is some data showing that the nutritional composition of figs, including iron content, is quite variable amongst Ficus species and even between trees of the same species in different geographic regions (Bonaccorso, 2011). In comparison to cultivated fruit, wild figs have been shown to be a superior source of calcium and other essential minerals, including iron (Stier, 2005; Nelson, 2000). Different species of bats are selective of the Ficus species consumed; however, there is some variation in preference within genera and even between males and females within a single species (Shanahan, 2001). It has been proposed that bats select a
combination of fruits, including figs, most suitable to meet their nutritional requirements (Stier, 2005).

Fruit is inherently low in protein and a number of nutritional strategies have been proposed to explain how paleotropical fruit bats consume sufficient protein to meet requirements for growth and development (Courts, 1998; Thomas, 1984). It has been proposed that fruit bats compensate for the low protein content of their diet by increasing their total fruit intake (Thomas, 1984). More recent evidence; however, shows that megachiropteran bats increase protein consumption by supplementing their diet with foliage, pollen, and by hunting insects (Courts, 1998). Insectivory has been reported in captive *Pteropus* spp. in a zoological institution and has been reported as an important means of nutritional supplementation in a number of neotropical frugivorous microchiropteran species (Courts, 1997; Courts, 1998). Insects have been reported to be a rich source of iron, significantly higher than are plants, which suggests that certain species of bats may consume higher levels of iron than previously believed (Christensen et al., 2006).

In captivity, fruit bats are fed mainly cultivated fruit, which may lack essential nutrients present in native plants, necessitating supplementation (Dempsey, 1999). Recommendations for nutrient composition have largely relied on data available for domestic and laboratory animals. Vitamin and mineral levels that have been shown to be effective for other mammalian species have traditionally been used as a guideline for safe levels in bats; however, over and under-supplementation is common (Dempsey, 2004).
Hemochromatosis in Egyptian Fruit Bats

Hemochromatosis is a known cause of morbidity and mortality in captive Egyptian fruit bats (Crawshaw et al., 1995). Since 1989, hemochromatosis has been the leading cause of mortality amongst adult bats at the Toronto Zoo, with over twenty mortalities attributed to this condition. The condition has also been reported in this species of bat at several other zoological institutions, although information regarding the wider prevalence amongst zoological institutions is still lacking (Farina et al., 2005).

Clinical Features

At the Toronto Zoo, clinical signs have generally not been apparent until advanced stages of the disease, at which point euthanasia was generally necessitated by rapid clinical deterioration. The first sign was often an animal that separated itself from the rest of the colony. Clinical signs were characteristic of liver failure and included generalized icterus, weight loss and emaciation, weakness, and dehydration. Several animals were found moribund. In some cases, radiographic evaluation and abdominal palpation revealed hepatomegaly with the liver extending beyond the border of the sternum. Ascites was reported in a number of individuals with advanced disease, as well as elevations in bilirubin and serum iron parameters (Crawshaw et al., 1995).
**Histopathologic Features**

Gross post-mortem findings in affected individuals have included generalized icterus and hepatomegaly. The liver often has rounded edges, a dark rusty coloration, a firm texture, and an irregular granular cut surface. Ascites and hydrothorax have been reported in a small number of cases (Crawshaw et al., 1995). Histologically, there is disruption of hepatic architecture with extensive periacinar and periportal fibrosis and areas of hepatocellular necrosis. Hepatocytes and Kupffer cells in the vicinity of areas of fibrosis contain coarse iron granules. In a majority of cases, excessive iron accumulation is present in the liver and spleen, with stainable iron also present in reticuloendothelial cells in the lung, small intestine, lymph node, and stomach in a small proportion of cases. Hepatic carcinomas, cholangiolar hyperplasia, and hepatocellular dysplasia have also been identified in some affected animals.

The high incidence of hemochromatosis in the Toronto Zoo population has been attributed to high levels of dietary iron (400 mg/kg on a dry matter basis) and vitamin C fed historically (Crawshaw et al., 1995). In a study comparing wild and captive Egyptian fruit bats in South Africa, captive male and female bats had significantly higher plasma iron levels (286-316 ng/dl or 51.2-56.6 µmol/L) than their wild counterparts (175 ng/dl or 31.3 µmol/L), supporting the hypothesis that iron accumulation is a captivity-related problem in this species (Westhuynzen, 1988). Interestingly, hemochromatosis has not been reported in other species of bats in captivity, including other species of fruit bat at the Toronto Zoo fed the same diet (Crawshaw et al., 1995). This suggests that while diet is a major contributing factor to disease, the Egyptian fruit bat may have an inherent inability to downregulate iron absorption.
**Iron Regulation in the Common Vampire Bat**

In contrast to frugivorous species, certain species of bats ingest extremely high levels of iron in their diet, without associated pathologic changes. The common vampire bat (*Desmodus rotundus*), an obligate sanguivore, consumes over 200 mg/kg of iron daily, equivalent to 800 times the mean daily iron consumption for man and 40 times that for the dog (Morton and Wimsatt, 1980). Despite the highest dietary iron intake of any species, less than 4.2 µg of iron per day is absorbed (0.173% of total body iron), about four times less than the laboratory mouse and three times less than the guinea pig on a weight to weight basis (Morton and Janning, 1982). These findings suggest that common vampire bats have evolved an ability to restrict dietary iron absorption. Iron overload has been experimentally induced in this species via intraperitoneal injection of iron dextran, resulting in increased numbers of hemosiderin-laden macrophages in the liver, spleen, and the wall of the gastrointestinal tract (Morton and Wimsatt, 1980). Within the gastrointestinal tract, iron was most abundant within the mucosal epithelium and macrophages in the fundic cecum, a storage vat for ingested blood, suggesting a role for macrophage-linked iron excretion via diapedesis of macrophages across the mucosal epithelium and desquamation of epithelial cells into the gut lumen (Morton and Wimsatt, 1980).

It is clear that bats, comprising one of the largest orders of mammals in the world, exhibit great diversity in feeding adaptations. In addition to specific dietary preferences, different bat species appear to have evolved different mechanisms of iron regulation. These differences may hold the key to understanding iron storage abnormalities in the Egyptian fruit bat and assist in unravelling the pathogenesis of iron-related disease in other species susceptible to hemochromatosis.
CHAPTER 2: CHARACTERIZATION OF THE HEPCIDIN GENE IN EIGHT SPECIES OF BATS

Abstract

Hemochromatosis, or iron storage disease, has been associated with significant liver disease and mortality in captive Egyptian fruit bats (*Rousettus aegyptiacus*). Although evolutionary adaptation to low levels of iron in the native diet has been implied, the physiologic basis for susceptibility to iron storage disease in captivity has not been established. In humans, the regulatory hormone hepcidin influences iron absorption in the intestine, recycling by macrophages, and mobilization from hepatic stores. A deficiency or resistance to hepcidin has been implicated in human hereditary hemochromatosis and may play a role in the Egyptian fruit bat; however, hepcidin has not been characterized in this species. In the present study, we compared the coding sequence of the hepcidin gene in eight species of bats representing three distinct taxonomic families with diverse life histories. Bat hepcidin mRNA encoded a 23 amino acid signal peptide, a 34 or 35 amino acid pro-region, and a 25 amino acid mature peptide, similar to other mammalian species. There were no genetic differences identified which could explain the increased susceptibility of the Egyptian fruit bat to iron storage disease.

Introduction

Bats make up one of the largest orders of mammals in the world, with 18 extant families and over 1,100 species; they exhibit huge diversity in their evolutionary ecology, physiologic adaptations, and nutritional preferences, allowing them to thrive in diverse environments spanning the globe (Clutton-Brock and Wilson, 2001; Kunz and Fenton, 2003). The Egyptian
fruit bat is a megachiropteran fruit bat whose range spans Africa and the Middle East (Burnie, 2001). In captivity, this species is highly susceptible to iron storage disease, which has been associated with high mortality in some zoological institutions (Crawshaw et al., 1995; Farina et al., 2005). The underlying pathophysiology of iron storage disease in this species has thus far not been elucidated.

Hepcidin, a hormone that plays an important role in the regulation of iron homeostasis in humans, has been characterized in a variety of vertebrate species (Ganz, 2011; Hilton and Lambert, 2008). The mature protein is well conserved, with similar amino acid sequence in a variety of vertebrate taxa, including fish and amphibians (Hilton and Lambert, 2008). Hepcidin regulates iron absorption by causing the cellular internalization and degradation of the cellular iron exporter, ferroportin, resulting in down-regulation of iron absorption and inhibition of iron release from macrophages and hepatocytes (Nemeth et al., 2004). Recent research in iron physiology has shown that hepcidin plays an important role in the pathogenesis of iron storage disorders in humans, with down-regulation of hepcidin function associated with a number of hereditary disorders (Roetto and Camaschella, 2005). In a majority of mammalian species investigated thus far, the hepcidin gene is present as a single copy, while in mice and fish, multiple copies are present (Hilton and Lambert, 2008). The hepcidin gene and its orthologue in fish and mice (HAMP1) are highly homologous amongst species and are believed to play similar roles in iron metabolism. Hepcidin also exhibits modest antimicrobial properties and plays a part in the innate immune system (Park et al., 2001). While the hepcidin gene has been characterized in the little brown bat (Myotis lucifugus), a North American insectivore, its genetic structure has not been characterized amongst divergent bat species, including the Egyptian fruit bat (Hilton and Lambert, 2008).
The goal of this study was twofold. First, to identify possible mutations in the hepcidin gene that may explain the increased susceptibility of the Egyptian fruit bat to iron storage disease. Second, the authors attempted to characterize the hepcidin gene in a variety of species of bats with distinct geographic distribution and dietary preferences in order to expand the understanding of the relationship of hepcidin within the order Chiroptera and between bats and other mammalian species.

**Materials and Methods**

*Sample acquisition and species range*

Samples of frozen liver were obtained from seven different species of bats from three distinct families, including two Old World megachiropteran frugivores; the Egyptian fruit bat (*Rousettus aegyptiacus*) and the straw-colored fruit bat (*Eidolon helvum*), two North American microchiropteran insectivores; the little brown bat (*Myotis lucifugus*) and the eastern red bat (*Lasiurus borealis*), as well as three neotropical species; Pallas’ long-tongued bat (*Glossophaga soricina*), a nectarivore, Seba’s short tailed bat (*Carollia perpicillata*), a generalist frugivore-nectarivore, and the common vampire bat (*Desmodus rotundus*), an obligate sanguivore (Table 2-1). All samples were obtained from the tissue banks at the Toronto Zoo (Toronto, Ontario), with the exception of samples from the Pallas’ long-tongued bat which were obtained from the Assiniboine Park Zoo (Winnipeg, Manitoba), and Seba’s short-tailed bat which were obtained from the Montreal Biodome (Montreal, Quebec).
Table 2-1. The taxonomic classification, geographic origin and dietary preferences of eight species of bats.

<table>
<thead>
<tr>
<th>Species</th>
<th>Common Name</th>
<th>Family</th>
<th>Geographic Origin</th>
<th>Dietary Preference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Rousettus aegyptiacus</em></td>
<td>Egyptian fruit bat</td>
<td>Pteropodidae</td>
<td>Africa and Middle East</td>
<td>Frugivore</td>
</tr>
<tr>
<td><em>Eidolon helvum</em></td>
<td>Straw-colored fruit bat</td>
<td>Pteropodidae</td>
<td>Africa</td>
<td>Frugivore</td>
</tr>
<tr>
<td><em>Pteropus vampyrus</em></td>
<td>Large flying fox</td>
<td>Pteropodidae</td>
<td>Southeast Asia</td>
<td>Frugivore</td>
</tr>
<tr>
<td><em>Myotis lucifugus</em></td>
<td>Little brown bat</td>
<td>Vespertilionidae</td>
<td>North America</td>
<td>Insectivore</td>
</tr>
<tr>
<td><em>Lasiurus borealis</em></td>
<td>Eastern red bat</td>
<td>Vespertilionidae</td>
<td>Eastern North America and Northeastern Mexico</td>
<td>Insectivore</td>
</tr>
<tr>
<td><em>Glossophaga soricina</em></td>
<td>Pallas’ long-tongued bat</td>
<td>Phyllostomidae</td>
<td>South and Central America</td>
<td>Nectarivore</td>
</tr>
<tr>
<td><em>Carollia perspicillata</em></td>
<td>Seba’s short-tailed bat</td>
<td>Phyllostomidae</td>
<td>South and Central America</td>
<td>Generalist frugivore</td>
</tr>
<tr>
<td><em>Desmodus rotundus</em></td>
<td>Common vampire bat</td>
<td>Phyllostomidae</td>
<td>South and Central America</td>
<td>Sanguivore</td>
</tr>
</tbody>
</table>

**DNA Isolation**

Genomic DNA was extracted from ~20 mg (wet weight) of frozen liver from three individuals of four species of bat (straw-colored fruit bat, common vampire bat, little brown bat, Egyptian fruit bat) and one individual of three species of bat (Seba’s short-tailed bat, Pallas’ long-tongued bat, and eastern red bat) using the QIAGEN DNeasy Blood and Tissue Kit (Mississauga, Ontario) following the manufacturers’ instructions.

**Primer design and amplification of hepcidin DNA**

Degenerate primers for amplification of the coding region of the hepcidin gene were designed based on available genomic sequences from the large flying fox and little brown bat (Ensembl database, Flicek et al., 2012), which showed 86% homology in the coding region.
Sequence alignments from each species of bat were used to identify conserved regions of the hepcidin gene using Geneious™. Primers were designed using Primer 3 software (Rozen and Skaletsky, 2000). The oligonucleotides used as forward and reverse primers are listed in Table 2-2.

PCR was performed to amplify the full-length coding sequence of the hepcidin gene in the little brown bat, Egyptian fruit bat, straw-colored fruit bat, common vampire bat, and eastern red bat. Only coding gene sequences for exons two and three were obtained from Seba’s short-tailed bat and Pallas’ long-tongued bat.

PCR was performed in a 50 µL reaction containing 2 µL of the template DNA, PCR buffer (20 mM Tris-HCl, 50 mM KCl), 1.5 mM MgCl₂, 0.2 µM each degenerate primer (Table 2-1), 0.2 mM dNTP, 2 units Platinum® Taq DNA polymerase (Invitrogen, Burlington, Ontario). Cycling parameters were as follows: Initial denaturation for 2 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C (exon 1) and 55 °C (exon 2/3) for 30 s and extension at 72 °C for 45 s, with a final extension of 72 °C for 5 min. Gel electrophoresis was performed on a 1 % agarose gel in 0.5 X TBE buffer with 1 X SYBR SAFE nucleic acid stain to verify the product length. PCR products were purified and sequenced in both forward and reverse directions (Lab Services Division, University of Guelph, Guelph, Ontario). For the common vampire bat, products were purified with a QIAquick® gel extraction kit (QIAGEN, Mississauga, Ontario) prior to being submitted for sequencing.
Table 2-2. Degenerate hepcidin primers used for PCR sequencing of exon 1 and exon 2-3.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Primer Name</th>
<th>Forward Primer (5'→3')</th>
<th>Reverse Primer (5'→3')</th>
<th>Amplicon Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HepcEx1Fwd1d_Rev2d</td>
<td>CG(C/T)CAC(C/T)ACCTTCTTGGA AA</td>
<td>TCT(A/C)CCTTGTTCATTGCTTGA</td>
<td>510</td>
</tr>
<tr>
<td>2-3</td>
<td>HepcEx23Fwd1d_Rev1d</td>
<td>CA(G/T)TATTCCCTTCTGCTTACA</td>
<td>GGGC(G/T)GCAGGAATAAATAAG</td>
<td>318</td>
</tr>
</tbody>
</table>

Sequence comparison and alignment

Sequence analysis was performed using Geneious™ software. Intron-exon junctions were deduced based on known hepcidin sequences from the little brown bat and large flying fox (Ensembl). The amino acid sequence deduced from the nucleotide sequence of the genomic DNA was aligned with those of the little brown bat and large flying fox (Ensembl) and mRNA sequences from the NCBI GenBank (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi). Alignment of amino acid sequences was performed through Geneious™ using the Clustal W application. The phylogenetic tree was drawn using the neighbor-joining method to determine the evolutionary relationship of the bat hepcidin protein with that of other species. The putative signal peptide cleavage site for all bat species was predicted using PrediSi software (http://www.predisi.de). The putative propeptide cleavage site was predicted based on homology with published human and mammalian hepcidin amino acid sequences (Hilton and Lambert, 2008; Valore and Ganz, 2008).

As a measure of genetic variability, overall nucleotide diversity amongst bats was calculated for the entire sequence of the coding region of mRNA, as well as mRNA sequences corresponding to the signal peptide, pro-region, and mature peptide regions of the hepcidin protein. Nucleotide diversity represented by the average proportion of nucleotide differences
between two randomly chosen sequences, \( \pi (\pi) \), was calculated using the following formula (Nei and Li, 1979):

\[
\pi = \frac{n}{n - 1} \sum_{i,j} p_i p_j \pi_{ij}
\]

where \( p_i \) and \( p_j \) are the frequencies of the \( i \)th and \( j \)th sequence (the number of times sequence \( i \) occurs divided by the total number of sequences (\( n \)), and \( \pi_{ij} \) is the proportion of nucleotide differences between the \( i \)th and \( j \)th sequences.

**Results**

*Genomic sequence of hepcidin in bats*

Primers used for characterization of the hepcidin sequence amplified 510 bp (exon 1) and 318 bp (exon 2 and 3) fragments which were bidirectionally sequenced. The sequences were aligned with gene sequences from the little brown bat and large flying fox and known mRNA sequences from the chimpanzee, horse, cow, pig, mouse, and sheep using NCBI Blast (http://www.ncbi.nlm.nih.gov). Sequence alignments confirmed a putative 249 bp or 252 bp (common vampire bat) mRNA fragment composed of three exons and two introns shown in Figure 2-1. The deduced bat hepcidin amino acid sequence consists of a 23 aa signal peptide, a 34-35 aa pro-region, and 25 aa mature hepcidin peptide.

Hepcidin has a putative signal peptide cleavage site between alanine (A23) and serine (S24) for the Egyptian fruit bat, straw-colored fruit bat, common vampire bat, large flying fox, and eastern red bat and between valine (V23) and serine (S24) for the little brown bat.
Sequence alignment and phylogenetic analysis

Predicted hepcidin amino acid sequences from all seven species of bats sequenced here and the large flying fox sequence from Ensembl were aligned with six mammalian species using Geneious™ software (Figure 2-2). The hepcidin sequence from the little brown bat generated in this study was 100% identical to the sequence in Ensembl. Bat hepcidin sequences showed average pairwise identity of 88.0% amongst all eight species with a range of 81.0% to 92.8%, with the lowest homology between the eastern red bat and large flying fox and the highest homology between the large flying fox and straw-colored fruit bat, Egyptian fruit bat and straw-colored fruit bat, and little brown bat and eastern red bat (Geneious™).

The putative mature hepcidin peptide sequence had an average 92.6% pairwise identity amongst all eight species of bat. The signal peptide had an average 84.8% pairwise identity and the pro-region an average 77.8% pairwise identity amongst the six species of bat for which the entire sequence of the coding region was available (Egyptian fruit bat, straw-colored fruit bat, large flying fox, little brown bat, eastern red bat, common vampire bat).

Nucleotide diversity, the average proportion of nucleotide differences between all possible sequence combinations, was calculated for each region of mRNA as a measure of genetic variability amongst species. Overall nucleotide diversity was 5.89%. Amongst the six species of bat for which the entire coding sequence was available, nucleotide diversity was 5.11% for the signal peptide region and 7.36% for the pro-region. Diversity in the mature peptide region for all eight bat species, including Seba’s short-tailed bat and Pallas’ long-tongued bat, was 4.72%.
Seventy four single nucleotide differences were identified, of which 37 were silent and 37 were associated with a difference in amino acid. There was one less leucine in position 26 in bats of the family Vespertilionidae (little brown bat and eastern red bat) and one less arginine in position 56, the putative propeptide cleavage recognition site, in members of the Pteropodidae (Egyptian fruit bat, straw-colored fruit bat, and large flying fox), corresponding to the pro-region of the pro-peptide (Park et al., 2001).

Of the 35 amino acid differences identified, eight occurred in the signal peptide region, 21 in the pro-region, and six in the mature peptide region. In Seba’s short-tailed bat, there was an alanine instead of a threonine in position two at the N-terminus of the mature peptide, corresponding to the proposed site of association of hepcidin with ferroportin (Nemeth et al., 2006) (Figure 2-3).

Amongst the bat species examined, four amino acid substitutions were identified that were unique to the Egyptian fruit bat, all of which were in the pro-region (Figure 2-2). The experimentally deduced hepcidin amino acid sequence for the Egyptian fruit bat exhibited variable pairwise identity with that of other mammalian hepcidin genes analysed, ranging from 67.9% to 72.0% (Figure 2-2).

*Isoelectric point variations by region*

The isoelectric points (pI) were calculated for the prepropeptide, propeptide, pro-region, and mature hepcidin protein for all eight species of bats for which data was available using Geneious™ software (Table 2-3). Isoelectric points were also calculated for the hairpin loop region of the mature peptide which contains four disulfide bonds and confers secondary structure
and stability to the mature peptide, and the five N-terminal amino acids which are essential for hepcidin bioactivity (Nemeth et al., 2006).

Isoelectric points were compared to previously published values for those of other eutharian mammals (Hilton and Lambert, 2008). Isoelectric point values for bats were comparable to those of other mammalian species, except for increased acidity in the mature peptide region of the little brown bat and eastern red bat with an isoelectric point of 7.64. The isoelectric points for the five N-terminal amino acids in the conserved region of the active sequence were identical (5.29) in all eight species of bat and highly similar to that of other eutharian mammals (5.51).
Table 2-3. Predicted isoelectric points for bat hepcidin proteins. Bat pI shown in comparison to average isoelectric points and standard deviations in other mammalian species.

<table>
<thead>
<tr>
<th></th>
<th>Whole</th>
<th>Propeptide</th>
<th>Proregion</th>
<th>Mature</th>
<th>1st 5-6aa</th>
<th>Loop</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammals (n=15)*</td>
<td>9.13±0.21</td>
<td>9.12±0.27</td>
<td>11.16±1.15</td>
<td>8.16±0.26</td>
<td>5.51±1.5</td>
<td>8.74±0.45</td>
</tr>
<tr>
<td>Egyptian fruit bat</td>
<td>8.50</td>
<td>8.41</td>
<td>12.02</td>
<td>7.97</td>
<td>5.29</td>
<td>8.21</td>
</tr>
<tr>
<td>Straw-colored fruit</td>
<td>8.51</td>
<td>8.41</td>
<td>12.02</td>
<td>7.97</td>
<td>5.29</td>
<td>8.21</td>
</tr>
<tr>
<td>bat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Large flying fox</td>
<td>8.50</td>
<td>8.41</td>
<td>10.99</td>
<td>7.97</td>
<td>5.29</td>
<td>8.21</td>
</tr>
<tr>
<td>Little brown bat</td>
<td>8.51</td>
<td>8.41</td>
<td>12.32</td>
<td>7.64</td>
<td>5.29</td>
<td>7.96</td>
</tr>
<tr>
<td>Eastern red bat</td>
<td>8.51</td>
<td>8.41</td>
<td>12.32</td>
<td>7.64</td>
<td>5.29</td>
<td>7.96</td>
</tr>
<tr>
<td>Common vampire bat</td>
<td>8.87</td>
<td>8.81</td>
<td>12.33</td>
<td>7.98</td>
<td>5.29</td>
<td>8.21</td>
</tr>
<tr>
<td>Pallas’ long-tongued</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>7.98</td>
<td>5.29</td>
<td>8.21</td>
</tr>
<tr>
<td>bat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seba’s short-tailed</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>7.98</td>
<td>5.29</td>
<td>8.21</td>
</tr>
<tr>
<td>bat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

na=not available.

* Isoelectric points for 15 mammalian species were extracted directly from table 3 in the publication by Hilton and Lambert (Hilton and Lambert 2008). A list of species included in the comparison can be found in table 1 in the same publication¹.

¹ Mammalian species include human, chimpanzee, orangutan, monkey, bushbaby, mouse lemur, mouse, rat, guinea pig, hedgehog, little brown bat, cow, pig, cat, and dog. A complete list of species and accession numbers can be found in table 1 in the publication by Hilton and Lambert (Hilton and Lambert 2008).
Figure 2-1. Coding gene sequence of hepcidin mRNA for eight species of bat. Full length coding sequences of the hepcidin gene were obtained for Egyptian fruit bat, straw-colored fruit bat, large flying fox, eastern red bat, little brown bat, and common vampire bat. Partial gene sequences (exon 2 and 3) were obtained for Seba’s short-tailed bat and Pallas’ long-tongued bat. Sequence shown for the large flying fox was obtained from the Ensembl database (Accession number ENSPVAG00000004162).
**Signal Peptide**

<table>
<thead>
<tr>
<th>Species</th>
<th>Signal Peptide</th>
<th>Pro-Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egyptian fruit bat</td>
<td>MSLNTRIQAV</td>
<td>CLLLLL-AS</td>
</tr>
<tr>
<td>Straw-colored fruit bat</td>
<td>MSLNTRIQAV</td>
<td>CLLLLL-AS</td>
</tr>
<tr>
<td>Large flying fox</td>
<td>MSLNTRIQAV</td>
<td>CLLLLL-AS</td>
</tr>
<tr>
<td>Little brown bat</td>
<td>MALTVRPQAQA</td>
<td>CLLLLL-AS</td>
</tr>
<tr>
<td>Eastern red bat</td>
<td>MALNVRPQAQA</td>
<td>CLLLLL-AS</td>
</tr>
<tr>
<td>Common vampire bat</td>
<td>MALNTRPQAQA</td>
<td>CLLLLL-AS</td>
</tr>
<tr>
<td>Pallas' long-tongued bat</td>
<td>--------</td>
<td>-----------</td>
</tr>
<tr>
<td>Seba's short-tailed bat</td>
<td>--------</td>
<td>-----------</td>
</tr>
</tbody>
</table>

**Mature Peptide**

<table>
<thead>
<tr>
<th>Species</th>
<th>Mature Peptide</th>
<th>% Pairwise Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egyptian fruit bat</td>
<td>DTHFPICI</td>
<td>83.3</td>
</tr>
<tr>
<td>Straw-colored fruit bat</td>
<td>DTHFPICV</td>
<td>91.6</td>
</tr>
<tr>
<td>Large flying fox</td>
<td>DTHFPICI</td>
<td>78.6</td>
</tr>
<tr>
<td>Little brown bat</td>
<td>DTHFPICI</td>
<td>78.6</td>
</tr>
<tr>
<td>Eastern red bat</td>
<td>DTHFPICI</td>
<td>76.2</td>
</tr>
<tr>
<td>Common vampire bat</td>
<td>DTHFPICI</td>
<td>82.2</td>
</tr>
<tr>
<td>Pallas' long-tongued bat</td>
<td>DTHFPICI</td>
<td>78.3</td>
</tr>
<tr>
<td>Seba's short-tailed bat</td>
<td>DTHFPICI</td>
<td>74.6</td>
</tr>
<tr>
<td>Chimpanzee</td>
<td>DTHFPICI</td>
<td>67.9</td>
</tr>
<tr>
<td>Cow</td>
<td>DTHFPICI</td>
<td>72.0</td>
</tr>
<tr>
<td>Horse</td>
<td>DTHFPICI</td>
<td>69.8</td>
</tr>
<tr>
<td>Mouse</td>
<td>DTHFPICI</td>
<td>68.3</td>
</tr>
<tr>
<td>Pig</td>
<td>DTHFPICI</td>
<td>69.6</td>
</tr>
<tr>
<td>Sheep</td>
<td>DTHFPICI</td>
<td>72.0</td>
</tr>
</tbody>
</table>

**Figure 2-2. Comparison of predicted bat hepcidin peptide sequence with various mammalian species.** Percentage of pairwise identity relative to the Egyptian fruit bat follows each sequence. Amongst bat species, amino acid differences unique to the Egyptian fruit bat are marked with an asterisk. Bat sequences generated by translation of cDNA from liver tissue for straw-colored fruit bat, common vampire bat, little brown bat, Egyptian fruit bat, Seba’s short-tailed bat, Pallas’ long-tongued bat, and eastern red bat and search of Ensembl database for large flying fox. Homologues in other species identified with BLAST search of GenBank database (Gene ID ENSPVAG00000004162). GenBank accession numbers: horse (ACZ04924), sheep (GQ901053), pig (AAM77745), cow (AA111659), mouse (NP_115930.1), and chimpanzee (ABU75211).
<table>
<thead>
<tr>
<th>Species</th>
<th>Amino Acid Sequence</th>
<th>% Pairwise Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egyptian fruit bat</td>
<td>DTHFPICIFCGCCYSKGICCKT</td>
<td>96.2</td>
</tr>
<tr>
<td>Straw-colored fruit bat</td>
<td>DTHFPICVFCCGCCYSKGICCKT</td>
<td>100.0</td>
</tr>
<tr>
<td>Large flying fox</td>
<td>DTHFPICIFCGCCYSKGICCKT</td>
<td>96.2</td>
</tr>
<tr>
<td>Little brown bat</td>
<td>DTHFPICIFCGCCYPSKGCICCKT</td>
<td>96.2</td>
</tr>
<tr>
<td>Eastern red bat</td>
<td>DTHFPICIFCGCCYPSRGCICCKT</td>
<td>92.3</td>
</tr>
<tr>
<td>Common vampire bat</td>
<td>DTHFPICIFCGCCHKSKGICCKT</td>
<td>96.2</td>
</tr>
<tr>
<td>Pallas' long-tongued bat</td>
<td>DTHFPICIFCGCCHKSKGICCKT</td>
<td>96.2</td>
</tr>
<tr>
<td>Seba’s short-tailed bat</td>
<td>DAHFPICMFCCGCCJKSKGICCKT</td>
<td>88.5</td>
</tr>
<tr>
<td>Chimpanzee</td>
<td>DTHFPICIFCGCCCHRSGCICCKT</td>
<td>88.0</td>
</tr>
<tr>
<td>Cow</td>
<td>DTHFPICIFCGCCCRGTCGMCRT</td>
<td>80.0</td>
</tr>
<tr>
<td>Horse</td>
<td>DTHFPICTLCCGCCNKQKCICCKT</td>
<td>80.0</td>
</tr>
<tr>
<td>Mouse</td>
<td>DTNFPICIFCCCKCNNSQCGICCKT</td>
<td>80.8</td>
</tr>
<tr>
<td>Pig</td>
<td>DTHFPICIFCCCRKAIICGMCCKT</td>
<td>84.0</td>
</tr>
<tr>
<td>Sheep</td>
<td>DTHFPICIFCCCRKGGTCGICCKT</td>
<td>88.0</td>
</tr>
</tbody>
</table>

**Figure 2-3. Comparison of bat mature hepcidin amino acid sequences with other species.** Percentage of pairwise identity relative to the Egyptian fruit bat follows each sequence. The position of cysteine residues (highlighted in grey) is conserved. The underlined five N-terminal amino acids represent amino acids essential for hepcidin bioactivity (Nemeth et al., 2006).
Figure 2-4. Phylogenetic tree showing the relationship between bat hepcidin pre-propeptide amino acid sequences. The amino acid sequences for Pallas’ long-tongued bat and Seba’s short-tailed bat do not include the first 29 amino acids. The analysis was repeated with the proximal 29 amino acids removed and resulted in the same relationships. The numbers next to the branches represent bootstrap consensus values on 100 replicates (Geneious™).
Discussion

Hepcidin has been shown to be a functionally important regulator of iron metabolism in a variety of species, but to date, the hepcidin gene has only been characterized in the little brown bat (Hilton and Lambert, 2008). This study aimed to characterize the hepcidin gene in several species of bats with variable dietary preferences and geographic distribution, including the Egyptian fruit bat, a species with increased susceptibility to iron storage disease in captivity.

Hepcidin gene structure was preserved amongst bat species in this study, with only minor variations in predicted amino acid composition. The first five amino acids of the mature hepcidin peptide, which are essential for the bioactivity of the active peptide, are highly conserved in mammalian taxa (Hilton and Lambert, 2008; Nemeth et al., 2006). With the exception of the Seba’s short-tailed bat, these five amino acids were identical in sequence to that of most mammalian species. Eight highly conserved cysteine residues were also present in all eight bat species examined. These residues form four disulfide bridges which confer a β-sheet and β-hairpin loop structure to the mature peptide and are believed to be a significant characteristic in the activity of the mature peptide (Hunter et al., 2002, Jordan et al., 2009).

In the species examined, the bat hepcidin gene is composed of three exons and two introns. This is consistent with the intron-exon structure found in other mammalian species and fish (Badial et al., 2011; Chen et al., 2007; Park et al., 2001; Pigeon, 2001; Zhang et al., 2004). The mRNA structure consisted of a 249 bp open reading frame for all species examined except the common vampire bat, which had three additional nucleotides. This mRNA encoded an 82 or 83 (common vampire bat) amino acid pre-propeptide, a 59 or 60 (common vampire bat) aa pro-peptide, and a 25 aa mature peptide, similar to other mammalian species (Hilton and Lambert,
The bat putative signal peptide was composed of 23 amino acids; the same number found in the cow, horse, mouse, and pig, and sheep, in contrast to a 24 amino acid sequence in the human (Figure 2-2).

In humans and other eutherian mammals, hepcidin undergoes post-translational modification prior to generation of the mature peptide (Valore and Ganz, 2008). Cleavage of the inactive protein precursor by furin, a prohormone convertase, occurs at an arginine/lysine cleave recognition site (RXRR or RXKR), resulting in release of the bioactive 25 amino acid hepcidin peptide (Valore and Ganz, 2008). This cleavage site is also conserved amongst mammals and fish and was present in all eight species of bats examined (Hilton and Lambert, 2008). Similar to the cow and sheep, in megachiropteran bats (Egyptian fruit bat, straw-colored fruit bat, and large flying fox), there was one less arginine in position 57 at the putative propeptide cleavage site than in the microchiropteran species, however, the furin recognition site was preserved.

Calculation of nucleotide diversity identified the greatest genetic variability in the pro-region of the prepropeptide, with only minor variability in nucleotide composition in the signal peptide and mature peptide regions, which is in accordance with previous findings in eutherian mammals (Hilton and Lambert, 2008). Amongst the six species of bat for which the entire coding sequence was available, nucleotide diversity was 5.11% in the signal peptide region and 7.36% in the pro-region. Diversity in the mature peptide region for all eight bat species investigated was 4.72%. By comparison, nucleotide diversity between four closely related primate species, the chimpanzee (*Pan troglodytes*), orangutan (*Pongo abelii*), gorilla (*Gorilla gorilla*), and human, was 1.00% in the signal peptide region, 0.90% in the pro-region, and 0.32%
in the mature peptide region (Geneious™)². Average pairwise identities in bats were 84.8% in the signal peptide region, 77.8% in the pro-region and 92.6% in the mature peptide region in comparison to 80.0%, 62.0%, and 84.0% within eutharian mammals (Hilton and Lambert, 2008). Greater nucleotide diversity amongst the bat species investigated is attributed to greater overall genetic diversity within the order Chiroptera.

Similarly, isoelectric properties also showed greatest variation in the pro-region and minimal variation in the mature peptide region (Fig 3). Isoelectric point values for bats were comparable to those of other mammalian species, except for increased acidity in the mature peptide region of the little brown bat and eastern red bat with an isoelectric point of 7.64 (in comparison to 8.21 for other bat species and 8.16±0.26 for other eutharian mammals), which was attributed to the presence of anionic proline (pI 6.48) in position 16. Proposed criteria for binding to ferroportin including a net cationic charge and an amino terminal anionic charge, however, were preserved, suggesting this difference is unlikely to be of functional significance (Hilton and Lambert, 2008). Likewise, a substitution of alanine for threonine in position two of the mature peptide was present in Seba’s short-tailed bat, but conferred no influence on the isoelectric properties of the mature peptide and, hence, no functional significance was inferred. Higher sequence homology was identified amongst species within the same taxonomic family and relationships amongst hepcidin sequence corresponded to known molecular phylogeny of bats, supporting the taxonomic relationships amongst species (Teeling et al., 2005).

² Genbank™ Accession Numbers: chimpanzee (NM_001109693), gorilla (EU076444), orangutan (NM_001134204), and human (NM_021175)
The increased occurrence of iron storage disease in the Egyptian fruit bat cannot be explained by a functional mutation of the hepcidin gene. Previously identified mutations in this gene that have been found to be associated with iron overload disorders in humans include a frameshift mutation (93delG), premature stop codon (R56X), out of frame start codon (-25G/A), two missense mutations involving cysteine residues (C78T, C70R), a four nucleotide deletion (M50del ivs2+1 (-G), and two missense mutations (K83R, G71D) in the mature peptide region (Roetto and Camaschella, 2005, Nemeth et al., 2006). These mutations were not identified in the Egyptian fruit bat or any bat species evaluated. Of the genetic differences identified in the Egyptian fruit bat, none were associated with the cleavage recognition sites involved in post-translational processing or the mature peptide region, and as such are less likely to be functionally significant.

Despite the lack of a putatively functional genetic difference in the coding region, the reason for the high susceptibility of the Egyptian fruit bat to iron storage disease may still be related to hepcidin function. Perhaps the expression of the hepcidin gene in the Egyptian fruit bat is lower than in other non-susceptible species of closely related bats, possibly due to genetic differences in regulatory regions of this gene. Alternatively, mutations in other iron regulatory genes, including regulators of hepcidin function, may play a role in the pathogenesis of this disorder in this species.

In summary, we have sequenced and characterized the mRNA region encoding hepcidin in seven species from three distinct families of bats and have shown high sequence homology amongst species. The coding region of the hepcidin gene in the Egyptian fruit bat, a megachiropteron, is similar to other megachiropteron and microchiropteron bats and eutherian mammals and appears to lack a functional genetic difference which would confer increased
susceptibility of this species to iron storage disease in captivity. This study expands our comparative knowledge of hepcidin, extending our understanding of the relationship between hepcidin in bats and other mammalian species.
CHAPTER 3: PILOT STUDY ON THE ROLE OF HEPCIDIN IN THE REGULATION OF IRON BALANCE IN BATS

Introduction

Hemochromatosis, or iron storage disease, has been associated with liver pathology and mortality in captive Egyptian fruit bats (*Rousettus aegyptiacus*). Although evolutionary adaptation to low levels of iron in their diet has been proposed, the physiologic basis for susceptibility has not been established. In humans, the regulatory hormone hepcidin influences iron absorption in the intestine, recycling by macrophages, and mobilization from hepatic stores. A deficiency or resistance to hepcidin has been implicated in human hereditary hemochromatosis and may play a role in susceptibility to iron storage disease in the Egyptian fruit bat. In order to provide a framework for a larger scale study of iron metabolism in bats, a preliminary investigation was carried into the role of hepcidin in iron metabolism in the Egyptian fruit bat and in two other bat species in which hemochromatosis is not a recognized problem; the straw-colored fruit bat (*Eidolon helvum*) and common vampire bat (*Desmodus rotundus*).

The objectives of this study were as follows:

- To develop methodology to measure hepcidin gene expression using qPCR in bats
- To determine which dose of intramuscular iron dextran (25 mg/kg or 100 mg/kg) is most effective for the purposes of increasing liver iron content and histologic iron scores and inducing hepcidin gene expression in bats
To determine if liver biopsy sampling 14 days and 30 days after administration of intramuscular iron dextran allows for detection of an increase in liver hepcidin gene expression in bats

To develop a histologic scoring system for evaluation of stainable iron in Perl’s Prussian blue stained sections of liver tissue from bats

To optimize the surgical protocol for collection of liver biopsies from bats

**Materials and Methods**

The research project and methodology was approved by the Animal Care Committee at the University of Guelph and the Animal Care and Research Committee at the Toronto Zoo, both of which operate under the auspices of the Canadian Council for Animal Care.

**Subjects**

Two male straw-colored fruit bats (*Eidolon helvum*), two male Egyptian fruit bats (*Rousettus aegyptiacus*) and two male common vampire bats (*Desmodus rotundus*) were used for the study. The fruit bats and common vampire bats were obtained from long-standing collections at the Toronto Zoo (Scarborough, Ontario) and The Centre for Conservation of Specialized Species (Havelock, Ontario), respectively.

Animals were housed at the Toronto Zoo where the research was carried out. Colonies of both species of fruit bats were housed in free-flight enclosures at the Wildlife Health Centre. The first two individuals of each species to be manually picked out of the colony by the principal investigator were selected for the study. The fruit bats used in this pilot study were transferred to stainless steel wire-mesh cages (61 cm x 70 cm x 62 cm for Egyptian fruit bats and 49 cm x 49 cm x 60 cm for straw-colored fruit bats), where they were housed in pairs for the duration of the
study. The vampire bats were housed together in a plastic mesh cage (89 cm x 61 cm x 61 cm) with a hutch on the top (23 cm x 23 cm x 22 cm). Animals were acclimatized to experimental housing for six days prior to the start of the experimental study. The fruit bats were fed a diet composed exclusively of fruit and lightly ground Mazuri® ZuLife™ softbill pellets (Appendix C). Vampire bats were fed fresh bovine blood from beef cattle (University of Guelph abattoir, Guelph, Ontario).

One representative of each of the three species of bat was assigned to each of two iron dextran (Dextafer, Vétoquinol, Lavaltrie, Quebec) dosage groups: 25 mg/kg or 100 mg/kg administered via the intramuscular route. All bats underwent general anesthesia and laparotomy for liver biopsy collection on Day 0 (the time of iron administration), and on Day 14 and 30 (subsequent to iron administration). Blood was collected from each Egyptian fruit bat and straw-colored fruit bat at each time point. Blood was not collected from the common vampire bats due to their small body size.

Blood Collection

Shortly after anesthetic induction (see below), 1.0 and 1.5 ml of blood were collected from the prepatagial veins of the Egyptian fruit bats and straw-colored fruit bats, respectively, using a 1 ml syringe and a 27 ga heparinised needle. The amount of blood which could be safely removed was calculated to be <1% of body weight (1 ml per 100 g). Blood was placed in a lithium heparin microhematocrit tube with serum separator (Becton, Dickinson, and Company, Franklin Lakes, New Jersey) and ~300 µl was retained for complete blood counts. The remainder was centrifuged and plasma transferred to 2 ml microtubes (Sarstedt, Numbrecht,
Germany) and frozen at -20 °C for analysis of plasma iron, unsaturated iron-binding capacity (UIBC), total iron-binding capacity (TIBC), and plasma ferritin.

Liver Biopsy Sample Collection

Animals were manually restrained and anesthesia was induced by face mask with 5% isoflurane (Baxter, Mississauga, Ontario) in oxygen. Shortly after induction, animals were weighed and given an intramuscular injection of buprenorphine (Chiron, Guelph, Ontario) (0.05 mg/kg) in the right shoulder muscle. They were placed on a warming air mattress (Bair Hugger®, Eden Prairie, Minnesota) and the surgical site on the abdomen was clipped and prepped with chlorhexidine soap (Ecolab, Mississauga, Ontario), 10% povidone iodine (Rougier, Mirabel, Quebec), and 70% isopropyl alcohol (Commercial Alcohols, Brampton, Ontario) solutions using a standard three part protocol. Local anesthesia was administered via subcutaneous injection of 2 mg/kg of 1% lidocaine (Vetoquinol, Lavaltrie, Quebec) along the incision line. A 1 cm ventral midline incision was made just caudal to the xiphoid process on Day 0. At Day 14 and at Day 30, a ventral paramedian incision was made lateral to the original incision. At each sampling interval, three liver biopsy samples were obtained from the caudal edge of the exposed liver lobe using a number 5 French endoscopic biopsy forceps (Karl Storz, Mississauga, Ontario). One liver biopsy sample was immediately transferred into each of an empty cryovial, a cryovial containing 500 µl RNAlater® solution (Ambion, Austin, Texas), and a vial containing 15 ml of 10% neutral buffered formalin for analysis of liver iron content, hepcidin mRNA expression, and histopathology, respectively. Hemostasis was achieved with gentle pressure at the biopsy site using a sterile cotton tip swab. In the case of excessive bleeding, gelatin foam was inserted at the biopsy site (Spongostan Dental, Ethicon, Somerville,
New Jersey). The body wall was sutured in a simple continuous pattern with 4-0 PDS (Ethicon, Markham, Ontario) (Egyptian fruit bat, straw-colored fruit bat) or 5-0 PDS (Ethicon) (common vampire bat). The skin was closed in a simple continuous intradermal pattern with 4-0 Monocryl (Ethicon) (Egyptian fruit bat, straw-colored fruit bat) or simple continuous external pattern with 5-0 PDS (Ethicon) (common vampire bat). The animals were given a subcutaneous injection of meloxicam (Boehringer Ingelheim, Burlington, Ontario) (0.2 mg/kg), cefazolin (Apotex, Toronto, Ontario) (20 mg/kg) and 30 ml/kg Plasmalyte HCl (Baxter, Mississauga, Ontario) prior to recovery. Fruit bats were administered oral meloxicam (Boehringer Ingelheim) (0.1 mg/kg SID) and tramadol (Chiron, Guelph, Ontario) (2 mg/kg BID) for four days post-operatively for pain control. Common vampire bats did not receive oral pain medication due to challenges associated with oral administration in this species.

Clinical Pathology

Evaluation of plasma iron, UIBC, and TIBC was performed at the Animal Health Laboratory (AHL) (University of Guelph, Guelph, Ontario) using a Cobas C® analyzer (Roche, Indianapolis, Indiana). Percent transferrin saturation was calculated using the following formula:

\[
\text{% transferrin saturation} = \left( \frac{\text{serum iron}}{\text{TIBC}} \right) \times 100
\]

Complete blood counts (CBC) were performed at the AHL using the ADVIA 2120 hematology system (Siemens, Mississauga, Ontario) using reference settings for the mouse (\textit{Mus musculus}). Plasma ferritin analysis was performed at Kansas State University Veterinary Diagnostic Laboratory (Manhattan, Kansas) using an assay similar to one previously described for canine ferritin using polyclonal purified rabbit anti-horse spleen ferritin antibody (Sigma–Aldrich, St. Louis, Missouri) instead of canine ferritin antibody and anti-horse ferritin peroxidase as a
conjugate (Andrews et al., 1992; Farina et al., 2005). Standards used were ferritin type I from horse spleen (Sue Chavey, pers. comm.).

Liver Iron Content

Analysis of liver iron content was performed at the Animal Health Laboratory using inductively coupled plasma mass spectrometry (ICP-MS) using the Varian 820-MS ICP Mass Spectrometer (Varian Canada, Mississauga, Ontario). The samples were digested with 1 mL nitric acid in 2.5 mL microfuge tubes held in a sand bath at 90°C. The digest was diluted to 5 mL with Nanopure® water. The digest was analyzed by ICP-MS, quantitating the iron 57 isotope. Results were expressed in ppm (equivalent to µg/gram of liver) on a dry weight basis.

Histopathology

All samples were processed at the AHL. Formalinized liver samples were embedded in paraffin and 4 µm sections were obtained and stained with hematoxylin and eosin (H&E) and Perl’s Prussian blue stains. H&E stained sections were examined by a single blinded observer (Iga Stasiak) for the presence or absence of fibrosis, nodularity, single cell necrosis, and inflammation. Samples were blinded by covering sample identification numbers and shuffling slides prior to slide examination.

The amount of iron present in hepatocytes and Kupffer cells was graded on Perl’s stained sections using a standardized histological scoring system adapted from Farina (Farina et al., 2005):
Kupffer cells:

- Grade 0: no hemosiderin within Kupffer cells
- Grade 1: occasional or low numbers of Kupffer cells containing fine and/or coarse hemosiderin granules
- Grade 2: moderate numbers of Kupffer cells containing fine and/or coarse hemosiderin granules
- Grade 3: coarse hemosiderin granules in most Kupffer cells with enlargement of the Kupffer cells
- Grade 4: large clusters of hemosiderin-laden Kupffer cells throughout parenchyma

Hepatocytes:

- Grade 0: no stainable iron within hepatocytes
- Grade 1: a few fine iron granules in periportal hepatocytes or diffusely throughout the liver.
- Grade 2: moderate to numerous fine iron granules in most hepatocytes and rare coarse hemosiderin granules in rare hepatocytes
- Grade 3: numerous fine iron granules in most hepatocytes and coarse hemosiderin granules in periportal hepatocytes and/or in hepatocytes scattered within the parenchyma

Samples were not graded for the presence of iron in portal tracts due to absence of portal tracts in many of the sections.

*Hepcidin mRNA Expression*

Biopsies in RNALater® solution were stored overnight at -4 °C, and then were transferred to -20 °C for storage. Tissues were homogenized using the PRO200 tissue homogenizer (PRO Scientific, Oxford, Connecticut) and then total RNA was isolated using the RNeasy Micro Kit (QIAGEN, Mississauga, Ontario). RNA yield and quality were analyzed by spectrophotometry.
at 260 and 280 nm (NanoDrop, Thermo Scientific, Wilmington, Delaware) prior to cDNA synthesis.

The first-strand cDNA was synthesized using the QuantiTect® Reverse Transcription Kit (QIAGEN, Mississauga, Ontario). Prior to cDNA synthesis, genomic DNA contamination was eliminated using 7x gDNA wipeout buffer. cDNA synthesis was performed using 500 ng of total RNA, RT primer mix, 5x Quantiscript RT buffer and Quantiscript reverse transcriptase (QIAGEN) in a volume of 20 µl for 30 min at 42 °C. The reverse transcriptase enzyme was inactivated at 95 °C for 3 min.

*Hepcidin mRNA expression by RT-qPCR*

RT-qPCR was used to determine hepcidin mRNA expression in liver tissue. Total RNA was isolated from the liver and reverse transcribed as described above. One µl of a 1:20 dilution of cDNA was amplified with the primers for the hepcidin gene for the Egyptian fruit bat, straw-colored fruit bat, and common vampire bat (Table 3-1); GAPDH for the Egyptian fruit bat, straw-colored fruit bat, and common vampire bat; and β-actin for the straw-colored fruit bat and common vampire bat (Table 3-2).

qPCR primers for hepcidin and β-actin were designed based on bat hepcidin and β-actin gene sequences generated for the Egyptian fruit bat, straw-colored fruit bat, and common vampire bat as described in Chapter 2. Primers for GAPDH and 28s rRNA were adapted from primers used for dog and horse, respectively.
### Table 3-1. Hepcidin primers used for RT-qPCR

<table>
<thead>
<tr>
<th>Species</th>
<th>Forward Primer (5´-3´)</th>
<th>Reverse Primer (5´-3´)</th>
<th>PCR Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egyptian fruit bat</td>
<td>GCAGCAGACAAGACAGATCG</td>
<td>CAGCAGATCCCACAACCTTTGA</td>
<td>1.893</td>
</tr>
<tr>
<td>Straw-colored fruit bat</td>
<td>ACCAGTGCTCAGTTCTCTTT</td>
<td>GCAGATCCCACAACCTTGATT</td>
<td>1.874</td>
</tr>
<tr>
<td>Common vampire bat</td>
<td>GCCTTACCAGTGCTCACGTT</td>
<td>GCAGCAACCACAACACAGAAGA</td>
<td>1.918</td>
</tr>
</tbody>
</table>

### Table 3-2. Reference gene primers for RT-qPCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5´-3´)</th>
<th>Reverse Primer (5´-3´)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>GTCGCCATCAATGACCCCTTC</td>
<td>TTCAAGTGAGCCCCAGCC</td>
</tr>
<tr>
<td>β-actin</td>
<td>TCGTGACATCAAGGAGAAG</td>
<td>GGAGTTGAAGGTTGTTCTCGT</td>
</tr>
</tbody>
</table>

### Table 3-3. Efficiencies for standard curves for each of two reference genes

<table>
<thead>
<tr>
<th></th>
<th>GAPDH</th>
<th>β-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egyptian fruit bat</td>
<td>1.834</td>
<td>NA*</td>
</tr>
<tr>
<td>Straw-colored fruit bat</td>
<td>1.830</td>
<td>1.911</td>
</tr>
<tr>
<td>Common vampire bat</td>
<td>1.836</td>
<td>1.841</td>
</tr>
</tbody>
</table>

*Efficiency for β-actin was below 1.8.
Each PCR assay was performed in triplicate with PCR parameters as follows: pre-incubation at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 58 °C for 10 s, and elongation at 72°C for 15 s, followed by a melting curve from 58 °C to 95 °C. Basal expression of the hepcidin gene in bats was normalized with GAPDH for all three species and β-actin mRNA for the straw-colored fruit bat and common vampire bat.

All RT-qPCR assays were performed in a 10 μl mixture containing 2.5 μl of cDNA preparation, 5 μl premix including SYBR Green 1 Master (2X conc) (Roche Applied Science, Eugene, Oregon), 0.5 ul of 10 μM of each forward and reverse primer, and 1.5 μl of PCR grade water. Real-Time quantifications were performed using the Lightcycler® 480 system (Roche Applied Science, Salt Lake City, Utah). The fluorescence threshold value was calculated using Lightcycler® 480 system software.

A standard curve was generated for each primer set using serial five-fold dilutions of cDNA for each species used in the qPCR assays. Efficiencies for each standard curve are listed in Tables 1 and 3. Samples were run in quintuplicate. Pooled cDNA (1:25x dilution) from one representative of each species was used as a calibrator for each 96 well plate. Concentrations of hepcidin relative to the reference gene for each sample were calibrator normalized.

Results

Results for the pilot study are presented in tables 3-4 to 3-12. Due to the small sample size in each treatment group (n = 1), statistical analyses were not performed.

Analysis of liver iron content in biopsy samples by ICP-MS was successfully conducted. In all three species and at both dosage levels, liver iron levels were increased above baseline at at
least one time point (Table 3-4). Elevation in liver iron content was higher for bats which received the 100 mg/kg dose compared to those that received the 25 mg/kg dose. Induction of hemosiderosis was successful in all species of bat by Day 14, with a ≥1.5-fold increase in liver iron content in bats administered the 100 mg/kg dose.

The methodology outlined above allowed for measurement of physiologic iron parameters (plasma iron, UIBC, TIBC, ferritin) in the blood of bats (Table 3-5). In both Egyptian and straw-colored fruit bats, administration of 25 mg/kg of iron dextran resulted in a decrease or minimal increase in plasma ferritin, as compared to administration of 100 mg/kg which substantially increased ferritin levels in both species. Baseline plasma iron and % transferrin saturation were above values reported elsewhere for healthy Egyptian fruit bats (Farina et al., 2005), and by Day 30, % transferrin saturation was 100% in both animals. Percent transferrin saturation and plasma iron did not increase in the straw-colored fruit bat at either dose administered. Levels of baseline % transferrin saturation for the straw-colored fruit bats were 91 % and 55 %, greater than values previously reported for Egyptian fruit bats with hemosiderosis (Farina et al., 2005).

The histologic scoring systems presented earlier were deemed appropriate for evaluation of H & E and Perl’s Prussian blue stained histologic liver sections (Tables 6 and 7). There was marked hepatic fibrosis; characteristic of hemochromatosis, in one Egyptian fruit bat prior to iron administration and in subsequent biopsy samples from both animals. Single cell necrosis was present only in the Day 30 sample from the Egyptian fruit bat receiving 100 mg/kg of iron dextran. Inflammation was present in the Day 14 samples from both vampire bats and in the Day 30 sample from the animal receiving the lower dose of iron dextran.
Egyptian fruit bats had baseline iron grades in the top category for both hepatocyte (n=2) and Kupffer cell iron stores (n=1). Kupffer cell iron had increased to the top category by Day 14 in the second animal, which received 25 mg/kg of iron dextran. Both hepatocyte and Kupffer cell iron increased by one grade between Day 0 and Day 14 in the straw-colored fruit bats receiving both doses of iron, and remained unchanged at Day 30. Kupffer cell iron increased by one grade between Day 0 and Day 14 in the common vampire bat which received 100 mg/kg of iron dextran; there was no change in hepatocyte iron scores.

The methodology described allowed for measurement of hepcidin gene expression in bats. The magnitude of change in hepcidin gene expression was greater when hepcidin mRNA expression was normalized relative to the GAPDH reference gene than the β-actin reference gene (Table 3-8). Egyptian fruit bats did not consistently upregulate hepcidin gene expression in response to administration of iron dextran; in fact in three out of four measurements, hepcidin gene expression was down-regulated. Using both reference genes, there was an increase in hepcidin gene expression relative to baseline at Day 14 in the straw-colored fruit bat (100 mg/kg dose) and common vampire bat (25 mg/kg and 100 mg/kg dose). There was a decrease to near baseline levels by Day 30 in the straw-colored fruit bat in both dosage groups relative to the β-actin reference gene.

Hematologic parameters remained relatively constant and within reference intervals throughout the experimental period (Tables 3-9 to 3-12).
Conclusions

The chosen methodology for surgical liver biopsy collection was found to be suitable for the species under investigation, with minimal intra- and post-operative complications. The ferritin assay previously developed for the Egyptian fruit bat and Indian flying fox (*Pteropus giganteus*) also allowed for measurement of plasma ferritin in the straw-colored fruit bat. Blood was not collected from the common vampire bat due to small body size and limitations of blood collection volume in this species. Liver biopsies from rats collected at post-mortem were used to validate the measurement of liver iron content by inductively coupled plasma mass spectrometry. Liver iron content measured from small liver biopsy sections similar to those collected in the study was compared to liver iron content from larger sections of liver tissue from the same individual and measurements were found be highly similar. The histological scoring system adapted from Farina was modified slightly as histologic sections were too small to evaluate for the presence of nodularity and portal tracts were absent in many of the examined sections. Sections were evaluated for the presence of fibrosis, inflammation, and single cell necrosis and histologic iron staining in Kupffer cells and hepatocytes only. β-actin and GAPDH were chosen as the reference genes for evaluation of hepcidin gene expression based on validation of these reference genes for measurement of gene expression in liver tissue in other species (Zhang et al., 2009). PCR efficiencies for GAPDH were suitable (>1.8) for measurement of gene expression in all three species of bat and for β-actin in the straw-colored fruit bat and the common vampire bat, but not in the Egyptian fruit bat. An additional reference gene, 28s rRNA, was selected for inclusion in the full scale study based on research which showed that GAPDH expression is
upregulated by hypoxia and thus, may be influenced by mechanisms that regulate iron metabolism (Zhong and Simons, 1999).

In summary, a 100 mg/kg dosage of intramuscular iron dextran was sufficient to consistently increase liver iron content in Egyptian, straw-colored, and common vampire bats and to induce hepcidin gene expression in the straw-colored fruit bat and common vampire bat (n=1 per species), whereas, a dosage of 25 mg/kg was not. Increase in hepcidin gene expression was more evident 14 days after iron administration compared to 30 days after iron administration. Hence, results of the pilot study supported selection of a 100 mg/kg dose of intramuscular iron dextran and a 14 day sampling interval for evaluation of hepcidin gene expression in bats. Preliminary results using the small numbers of animals described here suggested that there are differences in iron metabolism amongst species; however, the high levels of baseline iron in the Egyptian fruit bats may have affected the response of that species to iron administration.

There was no evidence that the experimental model induced anemia, infection, or inflammation, any of which would confound the results of the study.
Table 3-4. Liver iron content before and at different time points after iron administration

<table>
<thead>
<tr>
<th>Day</th>
<th>Egyptian fruit bat</th>
<th>Straw-colored fruit bat</th>
<th>Common vampire bat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25 mg/kg*</td>
<td>100 mg/kg*</td>
<td>25 mg/kg*</td>
</tr>
<tr>
<td>0</td>
<td>12 000</td>
<td>11 000</td>
<td>530</td>
</tr>
<tr>
<td>14</td>
<td>12 000</td>
<td>0</td>
<td>2200</td>
</tr>
<tr>
<td>30</td>
<td>20 000</td>
<td>1.7x</td>
<td>1800</td>
</tr>
</tbody>
</table>

Fold change relative to Day 0

<table>
<thead>
<tr>
<th>Day</th>
<th>25 mg/kg*</th>
<th>100 mg/kg*</th>
<th>25 mg/kg*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>12 000</td>
<td>11 000</td>
<td>530</td>
</tr>
<tr>
<td>14</td>
<td>12 000</td>
<td>0</td>
<td>2200</td>
</tr>
<tr>
<td>30</td>
<td>20 000</td>
<td>1.7x</td>
<td>1800</td>
</tr>
</tbody>
</table>

Liver iron content (ppm) reported on a dry weight basis.
* n=1
a. fold change relative to Day 0

Table 3-5. Plasma iron parameters before and at different time points after iron administration

<table>
<thead>
<tr>
<th>Plasma ferritin (ng/ml)</th>
<th>Egyptian fruit bat</th>
<th>Straw-colored fruit bat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>15 416</td>
<td>3083</td>
</tr>
<tr>
<td>Day 14</td>
<td>14 265</td>
<td>17 158</td>
</tr>
<tr>
<td>Day 30</td>
<td>24 546</td>
<td>17 826</td>
</tr>
</tbody>
</table>

Fold change relative to Day 0

<table>
<thead>
<tr>
<th>Plasma iron (µmol/L)</th>
<th>Egyptian fruit bat</th>
<th>Straw-colored fruit bat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>60</td>
<td>3083</td>
</tr>
<tr>
<td>Day 14</td>
<td>67</td>
<td>78</td>
</tr>
<tr>
<td>Day 30</td>
<td>81</td>
<td>91</td>
</tr>
</tbody>
</table>

Fold change relative to Day 0

<table>
<thead>
<tr>
<th>% transferrin saturation</th>
<th>Egyptian fruit bat</th>
<th>Straw-colored fruit bat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>92</td>
<td>62</td>
</tr>
<tr>
<td>Day 14</td>
<td>65</td>
<td>80</td>
</tr>
<tr>
<td>Day 30</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Fold change relative to Day 0

* n=1
a. fold change relative to Day 0

62
Table 3-6. Evaluation of histologic liver sections before and at different time points after iron administration

<table>
<thead>
<tr>
<th></th>
<th>Egyptian fruit bat</th>
<th>Straw-colored Fruit bat</th>
<th>Common Vampire Bat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25 mg/kg*</td>
<td>100 mg/kg*</td>
<td>25 mg/kg*</td>
</tr>
<tr>
<td><strong>Single Cell Necrosis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day 14</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day 30</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>Inflammation</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day 14</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day 30</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><strong>Fibrosis</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Day 14</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Day 30</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

* n=1
+ presence of single cell necrosis, inflammation, or fibrosis in histologic section
- absence of single cell necrosis, inflammation, or fibrosis in histologic section

Table 3-7. Comparison of histological iron scores before and at different time points after iron administration

<table>
<thead>
<tr>
<th></th>
<th>Egyptian fruit bat</th>
<th>Straw-colored fruit bat</th>
<th>Common vampire bat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25 mg/kg*</td>
<td>100 mg/kg*</td>
<td>25 mg/kg*</td>
</tr>
<tr>
<td><strong>Hepatocytes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Day 14</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Day 30</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td><strong>Kupffer Cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>3</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Day 14</td>
<td>4</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Day 30</td>
<td>4</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

Iron levels graded in histological liver sections stained with Perl’s Prussian blue stain. The grading scheme ranged from 0-4 for iron in Kupffer cells and 0-3 in hepatocytes.

* n=1
Table 3-8. Relative hepcidin mRNA expression before and at different time points after iron administration

<table>
<thead>
<tr>
<th></th>
<th>Egyptian fruit bat</th>
<th>Straw-colored fruit bat</th>
<th>Common vampire bat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25 mg/kg</td>
<td>Fold change(^b)</td>
<td>100 mg/kg</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Day 0</td>
<td>1.331</td>
<td>3.123</td>
</tr>
<tr>
<td></td>
<td>Day 14</td>
<td>0.653 0.5x</td>
<td>1.077 0.3x</td>
</tr>
<tr>
<td></td>
<td>Day 30</td>
<td>1.757 1.3x</td>
<td>1.477 0.5x</td>
</tr>
<tr>
<td>β-actin</td>
<td>Day 0</td>
<td>NA(^a)</td>
<td>NA(^a)</td>
</tr>
<tr>
<td></td>
<td>Day 14</td>
<td>NA(^a)</td>
<td>NA(^a)</td>
</tr>
<tr>
<td></td>
<td>Day 30</td>
<td>NA(^a)</td>
<td>NA(^a)</td>
</tr>
</tbody>
</table>

Hepcidin mRNA expression normalized relative to GAPDH and β-actin.

* Day 30 levels were not available due to loss during sample processing.
a. Efficiency of qPCR reaction for Egyptian fruit bat below 1.8. qPCR not performed.
b. fold change relative to Day 0
Table 3-9. Complete blood cell counts for Egyptian fruit bats before and at different time points after iron administration

<table>
<thead>
<tr>
<th>Blood Parameter</th>
<th>25 mg/kg (n=1)</th>
<th>100 mg/kg (n=1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 14</td>
</tr>
<tr>
<td>WBC (X10⁹/L)</td>
<td>3.0</td>
<td>4.2</td>
</tr>
<tr>
<td>RBC (X10¹²/L)</td>
<td>12.7</td>
<td>12.7</td>
</tr>
<tr>
<td>Hb (g/L)</td>
<td>170</td>
<td>171</td>
</tr>
<tr>
<td>HCT (L/L)</td>
<td>0.49</td>
<td>0.52</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>38</td>
<td>41</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>MCHC (g/L)</td>
<td>351</td>
<td>329</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>12.1</td>
<td>12.6</td>
</tr>
<tr>
<td>Platelets (X10⁹/L)</td>
<td>51</td>
<td>450</td>
</tr>
<tr>
<td>MPV (fL)</td>
<td>17.8</td>
<td>5.5</td>
</tr>
</tbody>
</table>

Notes: platelet clumps present cellular lysis platelet clumps present platelet clumps present platelet clumps present
Table 3-10. Complete blood cell counts for straw-colored fruit bats before and at different time points after iron administration

<table>
<thead>
<tr>
<th>Blood Parameter</th>
<th>25 mg/kg (n=1)</th>
<th></th>
<th>100 mg/kg (n=1)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 14</td>
<td>Day 30</td>
<td>Day 0</td>
</tr>
<tr>
<td>WBC (X10^9/L)</td>
<td>10.0</td>
<td>11.3</td>
<td>9.4</td>
<td>6.5</td>
</tr>
<tr>
<td>RBC (X10^{12}/L)</td>
<td>10.3</td>
<td>11.0</td>
<td>10.9</td>
<td>11.0</td>
</tr>
<tr>
<td>Hb (g/L)</td>
<td>180</td>
<td>188</td>
<td>185</td>
<td>170</td>
</tr>
<tr>
<td>HCT (L/L)</td>
<td>0.48</td>
<td>0.52</td>
<td>0.49</td>
<td>0.46</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>47</td>
<td>47</td>
<td>45</td>
<td>42</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>18</td>
<td>17</td>
<td>17</td>
<td>15</td>
</tr>
<tr>
<td>MCHC (g/L)</td>
<td>373</td>
<td>360</td>
<td>376</td>
<td>370</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>12.3</td>
<td>12.1</td>
<td>12.7</td>
<td>11.0</td>
</tr>
<tr>
<td>Platelets (X10^9/L)</td>
<td>89</td>
<td>436</td>
<td>540</td>
<td>116</td>
</tr>
<tr>
<td>MPV (fL)</td>
<td>7.8</td>
<td>9.2</td>
<td>7.6</td>
<td>8.4</td>
</tr>
</tbody>
</table>

Notes: few platelet clumps
Table 3-11. Manual differential white blood cell counts for Egyptian fruit bats before and at different time points after iron administration

<table>
<thead>
<tr>
<th>Blood Parameter</th>
<th>25 mg/kg (n=1)</th>
<th></th>
<th>100 mg/kg (n=1)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 14</td>
<td>Day 30</td>
<td>Day 0</td>
</tr>
<tr>
<td>Segmented neutrophil count</td>
<td>2.01</td>
<td>2.73</td>
<td>0.15</td>
<td>0.78</td>
</tr>
<tr>
<td>(X10^9/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphocyte count</td>
<td>0.84</td>
<td>1.26</td>
<td>0.44</td>
<td>0.72</td>
</tr>
<tr>
<td>(X10^9/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monocyte count</td>
<td>0.12</td>
<td>0.08</td>
<td>0.02</td>
<td>0.05</td>
</tr>
<tr>
<td>(X10^9/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eosinophil count</td>
<td>0</td>
<td>0.08</td>
<td>0.11</td>
<td>0</td>
</tr>
<tr>
<td>(X10^9/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basophil count</td>
<td>0.03</td>
<td>0.04</td>
<td>0.072</td>
<td>0.05</td>
</tr>
<tr>
<td>(X10^9/L)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Notes</td>
<td>low WBC, cell lysis</td>
<td>low WBC, platelet clumps</td>
<td>platelet clumps</td>
<td>platelet clumps</td>
</tr>
</tbody>
</table>
Table 3-12. Manual differential white blood cell counts for straw-colored fruit bats before and at different time points after iron administration

<table>
<thead>
<tr>
<th>Blood Parameter</th>
<th>25 mg/kg (n=1)</th>
<th></th>
<th>100 mg/kg (n=1)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 14</td>
<td>Day 30</td>
<td>Day 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Day 14</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Day 30</td>
</tr>
<tr>
<td>Segmented neutrophil count (X10^9/L)</td>
<td>0.70</td>
<td>0.90</td>
<td>0.56</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.55</td>
</tr>
<tr>
<td>Lymphocyte count (X10^9/L)</td>
<td>8.30</td>
<td>10.06</td>
<td>7.90</td>
<td>5.98</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7.48</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6.86</td>
</tr>
<tr>
<td>Monocyte count (X10^9/L)</td>
<td>0.10</td>
<td>0</td>
<td>0.09</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Eosinophil count (X10^9/L)</td>
<td>0.80</td>
<td>0.34</td>
<td>0.85</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.10</td>
</tr>
<tr>
<td>Basophil count (X10^9/L)</td>
<td>0.10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.29</td>
</tr>
<tr>
<td>Notes</td>
<td>Few platelet clumps</td>
<td></td>
<td>Few platelet clumps</td>
<td></td>
</tr>
</tbody>
</table>


CHAPTER 4: EVALUATION OF THE ROLE OF HEPcidIN IN THE REGULATION OF IRON BALANCE IN THE EGYPTIAN FRuit BAT, STRAW-COLORED FRuit BAT, AND COMMON VAMPIRE BAT

Abstract

Hemochromatosis is a significant cause of liver disease and mortality in captive Egyptian fruit bats (Rousettus aegyptiacus). In humans, the iron regulatory protein, hepcidin, plays a crucial role in iron homeostasis and a deficiency or resistance to hepcidin has been implicated in the development of hereditary hemochromatosis. In this study, we investigated the role of hepcidin in iron metabolism in three species of bats with variable susceptibility to hemochromatosis; the Egyptian fruit bat, straw-colored fruit bat (Eidolon helvum), and common vampire bat (Desmodus rotundus).

Baseline blood parameters, liver iron content, and relative gene expression of hepcidin were compared to values obtained 14 days after intramuscular iron administration. Results were compared between all three species, including two distinct populations of Egyptian fruit bat with and without underlying hemochromatosis.

Iron challenge resulted in significantly increased liver iron content and histologic iron scores in all three species of bat and elevated plasma iron parameters in the Egyptian fruit bat and straw-colored fruit bat. Levels of hepcidin mRNA expression increased in response to iron administration in healthy Egyptian fruit bats and the common vampire bat, but not in the straw-colored fruit bat or in Egyptian fruit bats with hemochromatosis. Hepcidin gene expression significantly correlated with liver iron content in the Egyptian fruit bat and common vampire bat and with % transferrin saturation and plasma ferritin in the Egyptian fruit bat.
This study demonstrates that bats exhibit marked variation in hepcidin expression. Hepcidin response is suppressed in Egyptian fruit bats with hemochromatosis and is low in healthy bats of the same species relative to the common vampire bat and healthy humans, which may explain the increased susceptibility of this species to iron storage disease in captivity. Hepcidin response is impaired in the straw-colored fruit bat suggesting that this species may have evolved an alternate mechanism for dealing with excessive iron or is in fact, more susceptible to iron overload than previously recognized. Elevated hepcidin expression in the common vampire bat explains the increased tolerance of this species to an iron-rich diet.

**Introduction**

Iron storage disease, or hemochromatosis, is a recognized condition affecting a variety of captive exotic species and is a leading cause of mortality in the Egyptian fruit bat (*Rousettus aegyptiacus*) in zoological institutions (Crawshaw et al., 1995). While the feeding of a high iron diet and a lack of natural dietary iron chelators have been previously implicated as possible factors contributing to the disease, the underlying pathophysiology of the condition in the Egyptian fruit bat and in other susceptible species has not been elucidated (Crawshaw et al., 1995; Lavin et al., 2010). Interestingly, aside from a single report of hemochromatosis in a straw-colored fruit bat (*Eidolon helvum*), there have been few reports of iron-storage disease in other frugivorous species of bats within the family Pteropodidae that are commonly housed in zoological collections and fed a similar diet (Lavin et al., 2010). The common vampire bat (*Desmodus rotundus*), an obligate sanguivore, tolerates extremely high amounts of iron in its diet without any associated repercussions. This discrepancy begs the question, when it comes to iron metabolism, what is it that sets these species apart?
Hepcidin is the key iron regulatory hormone responsible for the maintenance of iron homeostasis in the body (Ganz et al., 2008). Hepcidin controls dietary iron uptake and iron recycling from macrophages and hepatocytes by binding to the cellular iron export protein ferroportin and inducing its degradation (Donovan et al., 2005). In response to increased body iron stores, hepcidin production in the liver is upregulated, which limits further dietary iron absorption (Ramos et al., 2011). Hepcidin deficiency and resistance to hepcidin have been associated with the development of hereditary hemochromatosis in humans (Nemeth and Ganz, 2006). In humans, a number of mutations have been identified that limit hepcidin response to increased iron levels, resulting in excessive absorption of dietary iron and iron storage disease (Ramos et al., 2011). Mutations in HFE, the human hemochromatosis gene, in transferrin receptor 2, and in hemojulvelin all lead to impairment in hepcidin expression and response to iron (Ramos et al., 2011).

The purpose of this study was to begin to elucidate the pathophysiology of iron storage disease in the Egyptian fruit bat and the comparative role of hepcidin in iron metabolism in three species of bats with variable susceptibility to iron storage disease; the Egyptian fruit bat, the straw-colored fruit bat, and the common vampire bat. Our objective was to determine whether differences in iron metabolism amongst these species can be explained by differences in hepcidin expression.
**Materials and Methods**

The research project and methodology was approved by the Animal Care Committee at the University of Guelph and the Animal Care and Research Committee at the Toronto Zoo, both of which operate under the auspices of the Canadian Council for Animal Care.

**Animal Acquisition and Housing**

*Fruit Bats*

Nineteen Egyptian fruit bats (six females, thirteen males) including six juveniles and thirteen adults were obtained from the Centre for the Conservation of Specialized Species (Havelock, Ontario). A second group of 14 Egyptian fruit bats (five males, ten females) ranging from seven to fourteen years of age was obtained from the long-standing collection at the Toronto Zoo (Scarborough, Ontario), a population of bats with a history of hemochromatosis.

Nineteen straw-colored fruit bats (six males and thirteen females), including one juvenile and eighteen adults ranging from four to eighteen years of age, were obtained from a long-standing collection housed at the Toronto Zoo.

Animals were housed at the Toronto Zoo where the research was carried out. Each of the three colonies of fruit bats was initially housed in a free-flight enclosure at the Wildlife Health Centre. Animals were allocated into one of three (Egyptian fruit bats from Toronto Zoo) or four (Egyptian fruit bats from Havelock, Ontario and straw-colored fruit bats) cage groups in the order that they were randomly picked out of the colony by the principal investigator.

Experimental cages were composed of galvanized wire mesh (62 cm x 122 cm x 76 cm) and were hung from the ceiling. For the Egyptian fruit bats, cages were separated into two compartments
by a median partition and each group of bats had access to one compartment. Due to their larger body size, each straw-colored fruit bat group had access to a full cage. Bats were acclimatized to this experimental housing for two weeks prior to the start of the study. The animals were kept on a natural day-night cycle from March to May of 2012.

Both species of fruit bat were fed a diet composed of fruit and fine-ground Mazuri® ZuLife™ softbill pellets (Mazuri, Guelph, Ontario) (Appendix C). This low iron diet was instituted at the Toronto Zoo in February of 2010. Animals were fed from plastic bowls and were provided water ad lib.

**Common Vampire Bats**

Eight adult common vampire bats (four males, four females) were obtained from the Centre for the Conservation of Specialized Species (Havelock, Ontario). Vampire bats were housed as a single group in two joined side-by-side cages (89 cm x 61 cm x 61 cm) lined by plastic mesh, and with a hutch on the top (23 cm x 23 cm x 22 cm). Bats were acclimated to their new enclosure for two weeks prior to the start of the study. They were fed thawed frozen blood from a Bactrian camel (*Camelus bactrianus*) once daily.

**Experimental Design**

Each cage group was assigned to a surgical day, and within each group the bats were assigned to treatment or control groups according to the order in which they were removed from their cage on the day of surgery. For the straw-colored fruit bats and Egyptian fruit bats from Havelock, the first bat removed from each of the four cage groups and the second bat removed from the first cage group were assigned to the control group; the remaining 14 bats were placed
in the experimental group. For the Egyptian fruit bats from the Toronto Zoo, the first bat removed from each of the three cage groups and the second bat removed from the first and second cage groups were assigned to the control group; the remaining ten bats were placed in the experimental group. For the common vampire bats, the first bat receiving surgery on the first surgical day and the first two bats receiving surgery on the second surgical day were allocated into the control group; the remaining five bats were placed in the experimental group.

All bats underwent general anesthesia and laparotomy for liver biopsy collection on Day 0 (just prior to iron administration) and on Day 14 (subsequent to iron administration). Blood was collected from each Egyptian fruit bat and straw-colored fruit bat at each time point prior to biopsy collection. Blood was not collected from the common vampire bats due to their small body size. On Day 0, immediately prior to reversal of anesthesia, the bats in the treatment groups received an intramuscular injection of iron dextran (Dextafer, Vetoquinol, Lavaltrie, Quebec) at a dose of 100 mg/kg in the left shoulder muscle, as determined based on the results of a pilot study (Chapter 3). Animals in the control group received an intramuscular injection of an equivalent volume of 0.9% NaCl.

Liver Biopsy Sample Collection

Animals were manually restrained and anesthesia was induced by facemask with 5% isoflurane (Baxter, Mississauga, Ontario) in oxygen. Shortly after induction, animals were weighed and given an intramuscular injection of buprenorphine (Chiron, Guelph, Ontario) (0.05 mg/kg) in the right shoulder muscle. They were placed on a warming air mattress (Bair Hugger®, Eden Prairie, Minnesota) and the surgical site on the abdomen was clipped and prepped with chlorhexidine soap (Ecolab, Mississauga, Ontario), 10% povidone iodine (Rougier,
Mirabel, Quebec), and 70% isopropyl alcohol (Commercial Alcohols, Brampton, Ontario) using a standard three part protocol. Local anesthesia was administered via subcutaneous injection of 2 mg/kg of 1% lidocaine (Vetoquinol, Lavaltrie, Quebec) along the incision line. A 1 cm ventral midline incision was made just caudal to the xiphoid process on Day 0. On Day 14, a ventral paramedian incision was made lateral to the original incision. At each sampling interval, three liver biopsy samples were obtained from the caudal edge of the exposed liver lobe using 5 French endoscopic biopsy forceps (Karl Storz, Mississauga, Ontario). One liver biopsy sample was immediately transferred into each of an empty cryovial, a cryovial containing RNAlater® solution (Ambion, Austin, Texas), and a vial containing 10% neutral buffered formalin for analysis of liver iron content, hepcidin mRNA expression, and histopathology, respectively. Hemostasis was achieved with gentle pressure at the biopsy site using a sterile cotton tip swab. In the case of excessive bleeding, gelatin foam was inserted at the biopsy site (Spongostan Dental, Ethicon, Somerville, New Jersey). The body wall was sutured in a simple continuous pattern with 4-0 PDS (Ethicon, Markham, Ontario) (Egyptian fruit bat, straw-colored fruit bat) or 5-0 PDS (Ethicon) (common vampire bat). The skin was closed in a simple continuous intradermal pattern with 4-0 Monocryl (Ethicon) (Egyptian fruit bat, straw-colored fruit bat) or simple continuous external pattern with 5-0 PDS (Ethicon) (common vampire bat). The animals were given a subcutaneous injection of meloxicam (Boehringer Ingelheim, Burlington, Ontario) (0.2 mg/kg), cefazolin (Apotex, Toronto, Ontario) (20 mg/kg) and 30 ml/kg Plasmalyte HCl (Baxter, Mississauga, Ontario) along the midline of the dorsum prior to recovery. Fruit bats were administered oral meloxicam (0.1 mg/kg SID) and tramadol (Chiron, Guelph, Ontario) (2 mg/kg BID) for four days post-operatively for pain control.
Seven Egyptian fruit bats and one straw-colored fruit bat were administered a 7-10 day course of amoxicillin-clavulanic acid (Clavamox, Pfizer, Kirkland, Quebec) (20 mg/kg, BID, PO), several days after the first biopsy procedure due to partial dehiscence of the skin incision, which was allowed to heal by second intention. One Egyptian fruit bat developed a patagial burn on recovery on Day 0 and also received post-operative amoxicillin-clavulanic acid treatment.

**Blood Collection**

Shortly after anesthetic induction, 1.0 and 1.5 ml of blood were collected from the prepatagial veins of the Egyptian fruit bats and straw-colored fruit bats, respectively, using a 1 ml syringe and a 27 ga heparinized needle. The amount of blood that could be safely removed was calculated to be <1% of body weight (1 ml per 100 g). Blood was placed in a lithium heparin microhematocrit tube with serum separator (Becton, Dickinson, and Company, Franklin Lakes, New Jersey) and ~300 µl was retained for complete blood counts. The remainder was centrifuged and plasma transferred to 2 ml microtubes (Sarstedt, Numbrecht, Germany) and frozen at -20 °C for analysis of plasma iron, unsaturated iron-binding capacity (UIBC), total iron-binding capacity (TIBC), and plasma ferritin.
Assay Procedures

Plasma Iron Parameters

Evaluation of plasma iron and unsaturated iron-binding capacity (UIBC) (straw-colored fruit bat and Egyptian fruit bat) was performed at the Animal Health Laboratory (AHL, University of Guelph, Guelph, Ontario) using a Cobas C® analyzer (Roche, Indianapolis, Indiana). The total iron-binding capacity (TIBC) was calculated as the sum of plasma iron and UIBC, and % transferrin saturation was calculated using the following formula:

\[
\text{% transferrin saturation} = \left( \frac{\text{plasma iron}}{\text{TIBC}} \right) \times 100
\]

Complete blood counts (CBC) were performed at the AHL using the ADVIA 2120 hematology system (Seimens, Mississauga, Ontario) using reference settings for the mouse (Mus musculus). Plasma ferritin analysis was performed at Kansas State University Veterinary Diagnostic Laboratory (Manhattan, Kansas) using an assay similar to one previously described for canine ferritin (Andrews et al., 1992). Polyclonal purified rabbit anti-horse spleen ferritin antibody (Sigma–Aldrich, St. Louis, Missouri) was used instead of canine ferritin antibody, with anti-horse ferritin peroxidase as a conjugate (Farina et al., 2005). Standards used were ferritin type I from horse spleen (Sue Chavey, pers. comm.).
Liver Iron Content

Analysis of liver iron content was performed at the AHL using inductively coupled plasma mass spectrometry (ICP-MS) on the Varian 820-MS ICP Mass Spectrometer (Varian Canada, Mississauga, Ontario). The samples were digested with 1 mL nitric acid in 2.5 mL microfuge tubes held in a sand bath at 90 °C. The digest was diluted to 5 mL with nanopure water. The iron 57 isotope was quantitated by ICP-MS. Results were expressed in ppm (equivalent to µg/gram of liver) on a dry weight basis.

Histopathology

All samples were processed at the AHL. Formalinized liver samples were embedded in paraffin and 4 µm sections were obtained and stained with hematoxylin and eosin (H&E) and Perl’s Prussian blue. H&E stained sections were examined by a single blinded observer (Iga Stasiak) for the presence or absence of fibrosis, nodularity, single cell necrosis, and inflammation. Samples were blinded by covering the sample identification numbers and shuffling the slides prior to slide examination.

The amount of iron present in hepatocytes and Kupffer cells was graded on Perl’s stained sections using a standardized histological scoring system adapted from Farina (Farina et al., 2005):
Kupffer cells:
- Grade 0: no hemosiderin within Kupffer cells
- Grade 1: occasional or low numbers of Kupffer cells containing fine and/or coarse hemosiderin granules
- Grade 2: moderate numbers of Kupffer cells containing fine and/or coarse hemosiderin granules
- Grade 3: coarse hemosiderin granules in most Kupffer cells with enlargement of the Kupffer cells
- Grade 4: large clusters of hemosiderin-laden Kupffer cells throughout the parenchyma

Hepatocytes:
- Grade 0: no stainable iron within hepatocytes
- Grade 1: a few fine iron granules in periportal hepatocytes or in hepatocytes diffusely throughout the liver.
- Grade 2: moderate to numerous fine iron granules in most hepatocytes and coarse hemosiderin granules in rare hepatocytes
- Grade 3: numerous fine iron granules in most hepatocytes and coarse hemosiderin granules in periportal hepatocytes and/or in hepatocytes scattered within the parenchyma

Samples were not graded for the presence of iron in portal tracts due to absence of portal tracts in many of the examined sections.

*Hepcidin mRNA Expression*

The liver biopsies used for hepcidin gene expression were transferred into cryovials containing 500 µl of RNAlater® solution (Ambion, Austin, Texas) immediately after collection, stored overnight at -4 °C, and then transferred to -20 °C for storage. Tissues were homogenized.
using the PRO200 tissue homogenizer (PRO Scientific, Oxford, Connecticut) and then total RNA was isolated using the RNeasy Micro Kit (QIAGEN, Mississauga, Ontario). RNA yield and quality were analyzed by spectrophotometry at 260 and 280 nm (NanoDrop, Thermo Scientific, Wilmington, Delaware) prior to cDNA synthesis.

The first-strand cDNA was synthesized using the QuantiTect® Reverse Transcription Kit (QIAGEN, Mississauga, Ontario). Prior to cDNA synthesis, genomic DNA contamination was eliminated using 7x gDNA wipeout buffer. cDNA synthesis was performed using 500 ng of total RNA, RT primer mix, 5x Quantiscript RT buffer and Quantiscript reverse transcriptase (QIAGEN) in a volume of 20 µl for 30 min at 42 °C. The reverse transcriptase enzyme was inactivated at 95 °C for 3 min.

RT-qPCR was used to determine hepcidin mRNA expression in liver tissue. Total RNA was isolated from the liver and reverse transcribed as described above. One µl of a 1:20 dilution of cDNA was amplified with the primers for the hepcidin gene for the Egyptian fruit bat, straw-colored fruit bat, and common vampire bat (Table 4-1); for GAPDH and 28s rRNA for the Egyptian fruit bat, straw-colored fruit bat, and common vampire bat; and for β-actin for the straw-colored fruit bat and common vampire bat (Table 4-2). RT-qPCR primers for hepcidin and β-actin were designed based on bat hepcidin and β-actin gene sequences generated for the Egyptian fruit bat, straw-colored fruit bat, and common vampire bat as described in Chapter 2. Primers for GAPDH and 28s rRNA were adapted from primers used for dog and horse, respectively (Zhang et al., 2009).
Table 4-1. Hepcidin primers used for RT-qPCR on liver samples from three species of bats

<table>
<thead>
<tr>
<th>Species</th>
<th>Forward Primer (5′-3′)</th>
<th>Reverse Primer (5′-3′)</th>
<th>PCR Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egyptian fruit bat</td>
<td>GCAGCAGACAAGACAGATCG</td>
<td>CAGCAGATCCCCACACTTTGA</td>
<td>1.893</td>
</tr>
<tr>
<td>Straw-colored fruit bat</td>
<td>ACCAGTGCCCTCAGTTCTCTT</td>
<td>GCAGATCCCACACTTCGATT</td>
<td>1.874</td>
</tr>
<tr>
<td>Common vampire bat</td>
<td>GCCTTACCAGTGCCCTAGTT</td>
<td>GCAGCAACCACAACAGAAGA</td>
<td>1.918</td>
</tr>
</tbody>
</table>

Table 4-2. Reference gene primers for RT-qPCR on liver samples from three species of bats

<table>
<thead>
<tr>
<th>Gene</th>
<th>GenBank Accession No.</th>
<th>Forward Primer (5′-3′)</th>
<th>Reverse Primer (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>GTCGCCATCAATGACCCCTTC</td>
<td>TTCAAGTGAGCCCCAGCC</td>
<td></td>
</tr>
<tr>
<td>28SrRNA</td>
<td>EU554425</td>
<td>CCGGTAACGGCAGGAGTAAC</td>
<td>TAGGTAAGGACAGTGGAATCTCG</td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
<td>TGCCTGACATCAAGGAGAAG</td>
<td>GGAGTTGAAGGTGCTCTCG</td>
</tr>
</tbody>
</table>
The PCR parameters for hepcidin, β-actin, and GAPDH were as follows: pre-incubation at 95 °C for 5 min, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 58 °C for 10 s, and elongation at 72 °C for 15 s, followed by a melting curve from 58 °C to 95 °C. For 28s rRNA, the annealing temperature was increased to 63 °C and the melting curve ranged from 65 °C to 95 °C. Basal expression of the hepcidin gene in bats was measured relative to GAPDH and 28s rRNA for all three species and relative to β-actin mRNA for the straw-colored fruit bat and common vampire bat.

All RT-PCR reactions were performed in a 10 μL mixture containing 2.5 μl of cDNA preparation, 5 μl premix including SYBR Green 1 Master (2X conc) (Roche Applied Science, Eugene, Oregon), 0.5 μl of 10 μM of each forward and reverse primer, and 1.5 μl of PCR grade water. Real-Time quantifications were performed using the Lightcycler® 480 system (Roche Applied Science, Salt Lake City, Utah). The fluorescence threshold value was calculated using Lightcycler® 480 system software. Each reaction was performed in triplicate.

A standard curve was generated for each primer set using serial five-fold dilutions of cDNA for each species. Efficiencies for each standard curve are listed in Table 4-3. Samples were run in quintuplicate. Pooled cDNA (1:25x dilution) from one representative of each species was used as a calibrator for each 96 well plate. Concentrations of hepcidin relative to the reference gene for each sample were calibrator normalized.

<table>
<thead>
<tr>
<th></th>
<th>28s rRNA</th>
<th>GAPDH</th>
<th>β-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egyptian fruit bat</td>
<td>1.997</td>
<td>1.834</td>
<td>NA*</td>
</tr>
<tr>
<td>Straw-colored fruit bat</td>
<td>2.143</td>
<td>1.830</td>
<td>1.911</td>
</tr>
<tr>
<td>Common vampire bat</td>
<td>2.028</td>
<td>1.836</td>
<td>1.841</td>
</tr>
</tbody>
</table>

*Efficiency for β-actin below 1.8.
**Reference Gene Validation**

Reference gene validation was performed using BestKeeper (Pfaffl et al., 2004) and Normfinder (Andersen et al., 2004) reference gene validation software. Using both programs, the strongest agreement was between calibrator normalized concentrations for GAPDH and β-actin. Treatment effect was measured by evaluating the ratio of Day 14 (post-iron administration) to Day 0 (pre-iron administration) calibrator normalized concentrations for each reference gene for each of the four experimental groups. The effect of hemochromatosis was evaluated by measuring the ratio of calibrator normalized concentrations for each reference gene for both Egyptian fruit bat experimental groups. Although not statistically validated, GAPDH was less influenced by treatment than 28s rRNA and was not induced by hemochromatosis in the Toronto Zoo Egyptian fruit bat experimental group. The level of expression of 28s rRNA was found to be ~11 crossing-points lower or >2,000-fold greater in concentration than hepcidin, whereas GAPDH expression was within one crossing-point of hepcidin for the Egyptian fruit bat and straw-colored fruit bat. Mean GAPDH expression was lower for the common vampire bat (CP= 27.82, range: 26.59-31.54) than the Egyptian fruit bat and straw-colored fruit bat. Based on all reference gene data, GAPDH was chosen as the reference gene for evaluation of hepcidin gene expression in this study.

**Hepcidin Protein Assay**

Two plasma samples from the straw-colored fruit bat and the Egyptian fruit bat were analysed at the David Geffen School of Medicine (Los Angeles, California) using a hepcidin immunoassay developed for mouse sera (Amgen, Thousand Oaks, California). The assay was performed using proprietary monoclonal antibodies, one coating the plate for hepcidin capture
and the other, which was biotinylated, used to detect the captured murine hepcidin. Avidin peroxidase and colorimetric peroxidase reagents were used for development and quantification (Tomas Ganz, personal communication). Detection of bat hepcidin protein was unsuccessful.

Statistics

All statistical analyses were computed using the SAS statistical software (PC-SAS 8.00, SAS Institute Inc., Cary, North Carolina). Multivariate analysis of variance (MANOVA) was used with experimental group, treatment (iron or saline) and sampling day (Day 0 or Day 14) as the independent variables and liver iron content, histologic iron grade (for Kupffer cells and for hepatocytes), plasma iron parameters (iron, % transferrin saturation, ferritin), hepcidin gene expression, and white blood cell count (WBC) as the dependent variables.

To assess MANOVA assumptions, residual analyses were conducted. These included formally testing the residuals for normality using the Shapiro-Wilk, Kolmogorov-Smirnov, Cramér von Mises, and Anderson-Darling tests. In addition, residuals were plotted against the predicted values and explanatory variables used in the model to reveal outliers (more than 2 SDs greater or less than the mean), bimodal distributions, unequal variance, or the need for data transformations. To adequately meet the MANOVA assumptions, a log transformation was applied to results for quantitative liver iron content, hepcidin gene expression, plasma ferritin, and white blood cell count. Because data were recorded as percentage values, a logit transform with a bias correction term was applied to % transferrin saturation data. All terms up to the level of a 3-way interaction were considered; however, if terms were not significant at the 10% level, they were removed from the model. Data were reported as the mean (±SE). In cases where
logarithmic transformations were required to normalize the data to satisfy the assumptions of the model, results were reported as the median (95% confidence interval).

Comparisons between the means or medians for liver iron content, hepcidin gene expression, plasma ferritin, plasma iron, % transferrin saturation, histologic iron scores (for Kupffer cells and for hepatocytes) and white blood cell count for all experimental groups at Day 0 and Day 14 were determined using a split-plot model with experimental group and treatment (iron or saline) as whole-plot factors and day (0 or 14) as a split-plot factor, with day split within each individual. Therefore, each individual was nested within the experimental group by treatment interaction. Histologic iron score data was not normally distributed, however, as there are no non-parametric tests equivalent to the split-plot model, the scores were treated as ranks.

The analysis of covariance (ANCOVA) model was used to compare the predicted response (Day 14) between experimental groups adjusting for the initial conditions on Day 0 for liver iron content, hepcidin gene expression, plasma ferritin, % transferrin saturation, and plasma iron. The Day 0 covariate measure was entered into the model as both a linear and quadratic effect and was allowed to interact with experimental group and treatment.

The Spearman rank correlations (rho) were computed between measured parameters for each species disregarding treatment and day. For all comparisons, the a priori level of significance was set at 0.05.
**Results**

**Comparison of baseline (pre-iron administration) levels of liver iron content, histologic iron scores, plasma iron parameters, and hepcidin gene expression amongst species**

*Liver Iron Content*

Baseline liver iron content was significantly higher in the Egyptian fruit bat (Toronto Zoo) than in the Egyptian fruit bat (Havelock) (7.02-fold, p<0.0001), the straw-colored fruit bat (17.52-fold, p<0.0001), and the common vampire bat (15.50-fold, p<0.0001) (Table A4-1, A4-2). It was also significantly higher in the Egyptian fruit bat (Havelock) than the straw-colored fruit bat (2.49-fold, p<0.0001) and the common vampire bat (2.21-fold, p=0.0053). There was no significant difference in baseline liver iron content between the straw-colored fruit bat and the common vampire bat (p=0.6552).

*Histologic Iron Scores*

A summary of histologic grading of Perl’s iron stained sections of liver is presented in tables A4-3 and A4-4. There was a significant difference in the amount of stainable iron in Kupffer cells amongst the experimental groups (Table A4-5). All Egyptian fruit bats from the Toronto Zoo were assigned a maximal grade of four across all individuals. The straw-colored fruit bat, Egyptian fruit bat (Havelock, Ontario) and the common vampire bat were relatively less iron loaded. Histologic grades for iron in Kupffer cells were significantly higher for the straw-colored fruit bats than either the Egyptian fruit bats (Havelock) or the common vampire bats (p≤0.0084) (Table A4-5). There was no significant difference in baseline histologic scores for iron in Kupffer cells between the Egyptian fruit bat (Havelock) and the common vampire bat.
Histologic scores for the amount of iron in hepatocytes were significantly higher in the Egyptian fruit bat (Toronto Zoo) than the other three experimental groups (p<0.0001) (Table A4-5).

Egyptian fruit bats were sub-divided into two groups on the basis of their hepatic iron concentrations and histologic findings using the classification criteria established by Farina’s group (Farina et al., 2005). Bats with hepatic iron concentrations (on a wet weight basis) greater than 3 SDs of the mean above those reported for wild Egyptian fruit bats were classified as having iron overload (Westhuyzen, 1988). Those bats with histologic evidence of fibrosis were classified as having hemochromatosis. Fourteen of 15 of the Egyptian fruit bats from the Toronto Zoo were classified as having hemochromatosis. The single bat that did not have evidence of fibrosis on histology and was classified into the hemosiderosis group had a liver iron content >14 SDs greater than normal values reported for wild bats, was icteric, and grossly had a liver with tough texture and a mottled granular surface characteristic of bats with hemochromatosis. The absence of fibrosis in this individual was attributed to poor biopsy selection and sampling error. None of the Egyptian fruit bats from Havelock were classified as having hemochromatosis, although, 10/18 had hemosiderosis. These bats were otherwise clinically healthy. For the purposes of the study, the two groups were treated separately and designated as healthy and hemochromatosis and will henceforth be referred to in this manner.
Figure 4-1. Baseline liver iron content. Median baseline liver iron content (ppm) on a dry weight basis prior to administration of iron dextran. Error bars indicate the 95% confidence interval.

Plasma Iron Parameters

Baseline plasma ferritin was significantly higher in the Egyptian fruit bat (hemochromatosis) than either the straw-colored fruit bat (36-fold, p<0.0001) or the Egyptian fruit bat (healthy) (303-fold, p<0.0001); and significantly higher in the straw-colored fruit bat than the Egyptian fruit bat (healthy) (8-fold, p<0.0001) (Table A4-7). Baseline levels of both plasma iron and % transferrin saturation were significantly higher for the Egyptian fruit bat (hemochromatosis) than either the Egyptian fruit bat (healthy) (p=0.0001) or the straw-colored fruit bat (p<0.0001). Mean (±SE) plasma iron and median (95% confidence interval) values for plasma ferritin and % transferrin saturation are presented in table A4-6.
Figure 4-2. **Baseline plasma ferritin.** Median baseline plasma ferritin (ng/ml) prior to administration of iron dextran. Error bars indicate the 95% confidence interval.

Figure 4-3. **Baseline % transferrin saturation.** Median baseline % transferrin saturation prior to administration of iron dextran. Error bars indicate the 95% confidence interval.
**Figure 4-4. Baseline plasma iron.** Mean baseline plasma iron (µmol/L) prior to administration of iron dextran. Error bars indicate the standard error.
**Hepcidin**

Baseline hepcidin mRNA expression was significantly greater for the Egyptian fruit bat (healthy) (12-fold, p<0.0001), Egyptian fruit bat (hemochromatosis) (17-fold, p<0.0001), and common vampire bat (60-fold, p<0.001) as compared to the straw-colored fruit bat (Table A4-8, A4-9). It was also significantly greater for the common vampire bat than the Egyptian fruit bat (healthy) (5-fold, p=0.0329). There was no statistically significant difference in baseline levels of hepcidin mRNA expression between the two groups of Egyptian fruit bat, and between the Egyptian fruit bat (hemochromatosis) and the common vampire bat.

**Figure 4-5. Baseline hepcidin mRNA expression.** Median baseline hepcidin gene expression (relative to GAPDH) prior to administration of iron dextran. Error bars indicate the 95% confidence interval.
Experimental Trial: Comparison of the response of experimental groups to iron administration

The results of the experimental study are presented in tables 4-4 to 4-11 and figures 4-6 to 4-21. There was no significant change between Day 0 and Day 14 in the control groups for the following parameters: liver iron content, histologic grades for iron in Kupffer cells and hepatocytes, % transferrin saturation, and plasma iron. There was a statistically significant increase in hepcidin gene expression between Day 0 and Day 14 in the Egyptian fruit bat (hemochromatosis) control group (1.4-fold, p=0.0264) (Table 4-9). Plasma ferritin was significantly decreased in the control groups for Egyptian fruit bats with (0.4-fold, p=0.0010) and without hemochromatosis (0.3-fold, p=0.0390) at Day 14 relative to Day 0 (Table 4-8).

Liver Iron Content

There was a significant increase in liver iron content in response to iron administration in the Egyptian fruit bat (healthy) (1.84-fold, p=0.0150), straw-colored fruit bat (3.81-fold, p<0.0001), and common vampire bat (2.18-fold, p<0.0001) (Table 4-4, Figure 4-6). There was no statistically significant change in liver iron content in response to iron administration in the Egyptian fruit bat (hemochromatosis).

Histologic Iron Scores

A summary of histologic grading of Perl’s iron stained sections of liver is presented in table 4-5 and 4-6. There was a significant increase in histologic grade of Perl’s iron stained sections for Kupffer cells in response to iron-administration in the Egyptian fruit bat (healthy) (p<0.0001), straw-colored fruit bat (p<0.0001), and common vampire bat (p<0.0001), but not in
the Egyptian fruit bat (hemochromatosis) \((p=1)\). Iron loading was more evident based on scores for Kupffer cells as compared to hepatocytes. Respectively, iron scores for Kupffer cells and hepatocytes increased in 14/14 and 2/14 Egyptian fruit bats (healthy), 11/14 and 9/14 straw-colored fruit bats, and 5/5 and 2/5 vampire bats. The scores for the Egyptian fruit bat (hemochromatosis) were uniformly at maximal grade on Day 0 thus an increase in overall scoring was not possible.

Liver iron content correlated strongly with histologic grade for iron in Kupffer cells and hepatocytes for Egyptian fruit bats (combined) (Kupffer, \(\rho=0.841\); hepatocyte, \(\rho=0.781\)), straw-colored fruit bats (Kupffer, \(\rho=0.806\); hepatocyte, \(\rho=0.903\)), and common vampire bats (Kupffer, \(\rho=0.759\); hepatocyte, \(\rho=0.618\)) (Table 4-7). Iron content was also strongly and positively correlated with plasma ferritin for Egyptian fruit bats (healthy) (\(\rho=0.698\)) and straw-colored fruit bats (\(\rho=0.807\)), and with the product of transferrin saturation and serum iron for the straw-colored fruit bat (\(\rho=0.641\)). Liver iron content was moderately correlated with product of transferrin saturation and serum iron (\(\rho=0.411\)) for Egyptian fruit bats (healthy). A summary of correlation data for plasma iron parameters, histologic iron scores, and liver iron content is presented in Table 4-7.
Figure 4-6. Liver iron content. Comparison of liver iron content on a dry weight basis pre- and 14 days post-administration of 100 mg/kg of intramuscular iron dextran or saline amongst Egyptian fruit bats with and without hemochromatosis, the straw-colored fruit bat, and the common vampire bat. Parameters that changed significantly (p<0.05) between Day 0 and Day 14 are marked with an asterisk. Error bars indicate the 95% confidence interval.
**Plasma Iron Parameters**

There was a significant increase in plasma ferritin after iron administration in the Egyptian fruit bat (healthy) (15-fold, p<0.0001) and the straw-colored fruit bat (46-fold, p<0.0001), but not the Egyptian fruit bat (hemochromatosis) (p=0.8857) (Table 4-8, Figure 4-7).

Percent transferrin saturation and plasma iron were significantly increased in response to iron administration in the Egyptian fruit bat (healthy) and the straw-colored fruit bat (Table 4-8, Figure 4-8 and 4-9). Both the Egyptian fruit bat (healthy) and straw-colored fruit bat reached near complete saturation with median (range) Day 14 transferrin saturation percentages of 90% (75-96) and 94% (86-98), respectively. Percent transferrin saturation for the Egyptian fruit bat (hemochromatosis) was already maximized at Day 0 (100 % (98-100)) and did not change in response to iron administration.

**Hepcidin**

There was significant increase in hepcidin gene expression in response to iron administration in the Egyptian fruit bat (healthy) (2.4-fold, p=0.0012) and common vampire bat (55.5-fold, p=0.0021) (Table 4-9, Figure 4-10). There was no significant increase in hepcidin gene expression in the Egyptian fruit bat (hemochromatosis) (p=0.2634) and straw-colored fruit bat (p=0.9539). The degree of change in hepcidin gene expression was significantly greater for the common vampire bat than the Egyptian fruit bat (healthy) (p<0.0001) (Table 4-10).
Figure 4-7. Plasma ferritin levels. Median plasma ferritin levels in plasma pre- and 14 days post-administration of 100 mg/kg of intramuscular iron dextran or saline in Egyptian fruit bats with and without hemochromatosis and the straw-colored fruit bat. Parameters that changed significantly (p<0.05) between Day 0 and Day 14 are marked with an asterisk. Error bars indicate the 95% confidence interval.
Figure 4-8. % transferrin saturation. Comparison of median % transferrin saturation pre- and 14 days post-administration of 100 mg/kg of intramuscular iron dextran or saline amongst Egyptian fruit bats with and without hemochromatosis and the straw-colored fruit bat. Parameters that changed significantly (p<0.05) between Day 0 and Day 14 are marked with an asterisk. Error bars indicate the 95% confidence interval.
**Figure 4-9. Plasma iron levels.** Comparison of mean plasma iron level pre and 14 days post-administration of 100 mg/kg of intramuscular iron dextran or saline amongst Egyptian fruit bats with and without hemochromatosis, the straw-colored fruit bat, and the common vampire bat. Parameters that changed significantly (p<0.05) between Day 0 and Day 14 are marked with an asterisk. Error bars indicate the standard error.
**Figure 4-10. Hepcidin gene expression.** Comparison of median normalized hepcidin mRNA concentration (relative to GAPDH) from liver tissue pre- and 14 days post-administration of 100 mg/kg of intramuscular iron dextran or saline in Egyptian fruit bats with and without hemochromatosis, the straw-colored fruit bat, and the common vampire bat. Parameters that changed significantly (p<0.05) between Day 0 and Day 14 are marked with an asterisk. Error bars indicate the 95% confidence interval.
There was strong correlation between the level of hepcidin gene expression and % transferrin saturation (rho=0.530) and plasma ferritin (rho=0.627) for the Egyptian fruit bat (healthy), and between hepcidin gene expression and liver iron content for the common vampire bat (rho=0.534). Hepcidin levels were moderately correlated with % transferrin saturation for the straw-colored fruit bat (rho=0.321), with plasma ferritin for Egyptian fruit bats (both groups combined) (rho=0.312), and with liver iron content for Egyptian fruit bats (healthy) (rho=0.436) and straw-colored fruit bat (rho=0.534). Hepcidin correlation data is presented in table 4-11 and figures 4-11 to 4-20.

_Hematological Parameters_

All hematological parameters were within references ranges for the appropriate species (ISIS, Apple Valley, Minnesota, www.isis.org), and there were no significant changes in response to treatment. Hematologic data is presented in Table A4-10 and Appendix B.

_Age and Gender_

Gender was not a significant confounding variable in any of the experimental groups. Age could not be assessed in the statistical model as there was insufficient data available.
Figure 4-11. Correlation between plasma ferritin and relative hepcidin mRNA expression in healthy Egyptian fruit bats.

Figure 4-12. Correlation between plasma ferritin and relative hepcidin mRNA expression in Egyptian fruit bats with hemochromatosis.
Figure 4-13. Correlation between plasma ferritin and relative hepcidin mRNA expression in the straw-colored fruit bat.

Figure 4-14. Correlation between % transferrin saturation and relative hepcidin mRNA expression in healthy Egyptian fruit bats.
Figure 4-15. Correlation between % transferrin saturation and relative hepcidin mRNA expression in Egyptian fruit bats with hemochromatosis.

Figure 4-16. Correlation between % transferrin saturation and relative hepcidin mRNA expression in the straw-colored fruit bat.
Figure 4-17. Correlation between liver iron content (dw) and relative hepcidin mRNA expression in healthy Egyptian fruit bats.

Figure 4-18. Correlation between liver iron content (dw) and relative hepcidin mRNA expression in Egyptian fruit bats with hemochromatosis.
Figure 4-19. Correlation between liver iron content (dw) and relative hepcidin mRNA expression in the straw-colored fruit bat.

Figure 4-20. Correlation between liver iron content (dw) and relative hepcidin mRNA expression in the common vampire bat.
Figure 4-21. **Response to iron challenge.** Comparison of fold change (Day 14/Day 0) in plasma ferritin, hepcidin mRNA expression (normalized relative to GAPDH), and liver iron content in response to administration of 100 mg/kg of intramuscular iron dextran amongst Egyptian fruit bats with and without hemochromatosis, the straw-colored fruit bat, and the common vampire bat. Error bars indicate the 95% confidence interval.
Experimental Trial: Response of experimental groups to iron administration

Table 4-4. Comparison of liver iron content before and after iron administration

<table>
<thead>
<tr>
<th>Species</th>
<th>Iron-Treated*</th>
<th>Control*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egyptian fruit bat (healthy)</td>
<td>13</td>
<td>2119</td>
</tr>
<tr>
<td>Egyptian fruit bat (hemochromatosis)</td>
<td>10</td>
<td>21052</td>
</tr>
<tr>
<td>Straw-colored fruit bat</td>
<td>14</td>
<td>1037.3</td>
</tr>
<tr>
<td>Common vampire bat</td>
<td>5</td>
<td>1088</td>
</tr>
</tbody>
</table>

Liver iron content (ppm) was measured on a dry matter basis on Day 0 (pre-iron) and Day 14 (post iron)
*median (95% confidence interval)
a. significant difference between groups (p-value < 0.05)
b. Ratio of median of day 14 to median of day 0 (95% confidence interval)
Table 4-5. Summary of histological iron scores before and after iron administration

<table>
<thead>
<tr>
<th></th>
<th>Change in Histologic Grade (Day 0 – Day 14)</th>
<th>-1</th>
<th>0</th>
<th>+1</th>
<th>+2</th>
<th>+3</th>
<th>+4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kupffer Cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egyptian fruit bat (hemochromatosis)</td>
<td>Iron</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Egyptian fruit bat (healthy)</td>
<td>Iron</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>6</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Straw-colored fruit bat</td>
<td>Iron</td>
<td>0</td>
<td>3</td>
<td>8</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Common vampire bat</td>
<td>Iron</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Hepatocytes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Egyptian fruit bat (hemochromatosis)</td>
<td>Iron</td>
<td>2</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Egyptian fruit bat (healthy)</td>
<td>Iron</td>
<td>4</td>
<td>8</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>1</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Straw-colored fruit bat</td>
<td>Iron</td>
<td>0</td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Common vampire bat</td>
<td>Iron</td>
<td>0</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Saline</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Iron levels graded in histological liver sections stained with Perl’s Prussian blue stain. The grading scheme ranged from 0-4 for iron in Kupffer cells and -0-3 in hepatocytes.
Table 4-6. Comparison of histological iron scores before and after iron administration

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Species</th>
<th>Iron-Treated</th>
<th>Control</th>
<th></th>
<th></th>
<th>p-value</th>
<th>N</th>
<th>Day 0</th>
<th>Day 14</th>
<th>Difference</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N</td>
<td>Day 0</td>
<td>Day 14</td>
<td>Difference</td>
<td>p-value</td>
<td>N</td>
<td>Day 0</td>
<td>Day 14</td>
<td>Difference</td>
<td>p-value</td>
</tr>
<tr>
<td>Kupffer Cells</td>
<td>Egyptian fruit bat (healthy)</td>
<td>14</td>
<td>0.8 (±0.2)</td>
<td>3.1 (±0.2)</td>
<td>2.3 (±0.2)</td>
<td>&lt;0.0001</td>
<td>5</td>
<td>1.0 (±0.3)</td>
<td>5</td>
<td>1.0 (±0.3)</td>
<td>0.0 (±0.3)</td>
</tr>
<tr>
<td></td>
<td>Egyptian fruit bat (hemochromatosis)</td>
<td>10</td>
<td>4 (±0)</td>
<td>4 (±0)</td>
<td>1.0 (±0)</td>
<td>1.0000</td>
<td>5</td>
<td>4 (±0)</td>
<td>5</td>
<td>4 (±0)</td>
<td>0.0000</td>
</tr>
<tr>
<td></td>
<td>Straw-colored fruit bat</td>
<td>14</td>
<td>1.9 (±0.2)</td>
<td>2.9 (±0.2)</td>
<td>1.1 (±0.2)</td>
<td>&lt;0.0001</td>
<td>5</td>
<td>1.0 (±0.3)</td>
<td>5</td>
<td>1.0 (±0.3)</td>
<td>0.0 (±0.3)</td>
</tr>
<tr>
<td></td>
<td>Common vampire bat</td>
<td>5</td>
<td>0.8 (±0.3)</td>
<td>3.4 (±0.3)</td>
<td>2.6 (±0.3)</td>
<td>&lt;0.0001</td>
<td>3</td>
<td>1.0 (±0.4)</td>
<td>2</td>
<td>1.0 (±0.5)</td>
<td>0.0 (±0.5)</td>
</tr>
<tr>
<td>Hepatocytes</td>
<td>Egyptian fruit bat (healthy)</td>
<td>14</td>
<td>0.8 (±0.2)</td>
<td>0.6 (±0.2)</td>
<td>-0.1 (±0.2)</td>
<td>0.4156</td>
<td>5</td>
<td>0.8 (±0.4)</td>
<td>5</td>
<td>0.6 (±0.4)</td>
<td>-0.2 (±0.3)</td>
</tr>
<tr>
<td></td>
<td>Egyptian fruit bat (hemochromatosis)</td>
<td>10</td>
<td>2.9 (±0.3)</td>
<td>2.7 (±0.3)</td>
<td>-0.2 (±0.2)</td>
<td>0.3361</td>
<td>5</td>
<td>2.6 (±0.4)</td>
<td>5</td>
<td>2.6 (±0.4)</td>
<td>0.0 (±0.3)</td>
</tr>
<tr>
<td></td>
<td>Straw-colored fruit bat</td>
<td>14</td>
<td>1.4 (±0.2)</td>
<td>2.4 (±0.2)</td>
<td>1.0 (±0.2)</td>
<td>&lt;0.0001</td>
<td>5</td>
<td>0.6 (±0.4)</td>
<td>5</td>
<td>0.8 (±0.4)</td>
<td>0.2 (±0.3)</td>
</tr>
<tr>
<td></td>
<td>Common vampire bat</td>
<td>5</td>
<td>1.2 (±0.4)</td>
<td>1.6 (±0.4)</td>
<td>0.4 (±0.3)</td>
<td>0.1756</td>
<td>3</td>
<td>1.3 (±0.5)</td>
<td>2</td>
<td>1.4 (±0.5)</td>
<td>0.1 (±0.4)</td>
</tr>
</tbody>
</table>

Iron levels graded in histological liver sections stained with Perl’s Prussian blue stain. The grading scheme ranged from 0-4 for iron in Kupffer cells and -0-3 in hepatocytes.

a. mean (±SE)
b. significant difference between groups (p-value ≤0.05)
c. no statistically significant interaction between experimental group, treatment (iron or saline), and day (0 and 14). Values are presented to descriptive purposes.
Table 4-7. Correlation between liver iron content (ppm) and plasma iron parameters, relative hepcidin mRNA expression, and histologic grade for iron

<table>
<thead>
<tr>
<th></th>
<th>% transferrin saturation(^a)</th>
<th>Plasma ferritin(^a)</th>
<th>Hepcidin(^a)</th>
<th>Histologic Grade (Kupffer cells)(^a)</th>
<th>Histologic Grade (Hepatocytes)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egyptian fruit bat (healthy)</td>
<td>0.41197 (p=0.0155)</td>
<td>0.69755 (p&lt;0.0001)</td>
<td>0.43613 (p=0.0070)</td>
<td>0.49515 (p=0.0018)</td>
<td>0.32276 (p=0.0514)</td>
</tr>
<tr>
<td>Egyptian fruit bat (hemochromatosis)</td>
<td>0.03611 (p=0.8498)</td>
<td>0.18042 (p=0.3400)</td>
<td>-0.09857 (p=0.6043)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Egyptian Fruit Bat (all)</td>
<td>0.74797 (p&lt;0.0001)</td>
<td>0.85134 (p&lt;0.0001)</td>
<td>0.13909 (0.2616)</td>
<td>0.84108 (&lt;0.0001)</td>
<td>0.78138 (&lt;0.0001)</td>
</tr>
<tr>
<td>Straw-colored fruit bat</td>
<td>0.64646 (p&lt;0.0001)</td>
<td>0.80709 (p&lt;0.0001)</td>
<td>0.32794 (p=0.0475)</td>
<td>0.80628 (&lt;0.0001)</td>
<td>0.90296 (p&lt;0.0001)</td>
</tr>
<tr>
<td>Common vampire bat</td>
<td>NA</td>
<td>NA</td>
<td>0.53364 (p=0.0333)</td>
<td>0.75868 (p=0.0010)</td>
<td>0.61751 (p=0.0142)</td>
</tr>
</tbody>
</table>

Hepcidin mRNA expression was measured relative to GAPDH
\(^a\) Tf sat, transferrin saturation = (serum iron, TIBC x 100). TIBC, total iron binding capacity
\(\rho =\) Spearman's rank correlation coefficient
Table 4-8. Comparison of plasma iron parameters before and after iron administration

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Species</th>
<th>Iron-Treated</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Species</td>
<td>N</td>
<td>Day 0 (µmol/L)</td>
</tr>
<tr>
<td>Plasma ferritin&lt;sup&gt;e,c&lt;/sup&gt; (ng/ml)</td>
<td>Egyptian fruit bat (healthy)</td>
<td>14</td>
<td>27 (14-52)</td>
</tr>
<tr>
<td></td>
<td>Egyptian fruit bat (hemochromatosis)</td>
<td>10</td>
<td>11332 (6980-18397)</td>
</tr>
<tr>
<td></td>
<td>Straw-colored fruit bat</td>
<td>14</td>
<td>238 (138-413)</td>
</tr>
<tr>
<td>% transferrin saturation&lt;sup&gt;a,b,d,g&lt;/sup&gt;</td>
<td>Egyptian fruit bat (healthy)</td>
<td>13</td>
<td>66 (42-84)</td>
</tr>
<tr>
<td></td>
<td>Egyptian fruit bat (hemochromatosis)</td>
<td>10</td>
<td>99 (98-100)</td>
</tr>
<tr>
<td></td>
<td>Straw-colored fruit bat</td>
<td>14</td>
<td>70 (47-86)</td>
</tr>
<tr>
<td>Plasma iron&lt;sup&gt;a,c,g&lt;/sup&gt; (µmol/L)</td>
<td>Egyptian fruit bat (healthy)</td>
<td>13</td>
<td>35 (±4)</td>
</tr>
<tr>
<td></td>
<td>Egyptian fruit bat (hemochromatosis)</td>
<td>10</td>
<td>65 (±5)</td>
</tr>
<tr>
<td></td>
<td>Straw-colored fruit bat</td>
<td>14</td>
<td>34 (±4)</td>
</tr>
</tbody>
</table>

*median (95% confidence interval)

a. mean (±SE)
b. Tf sat, transferrin saturation = (plasma iron/ TIBC x 100). TIBC, total iron binding capacity
c. ratio (Day 14 plasma ferritin: Day 0 plasma ferritin)
d. odds Ratio( Day 14 % transferrin saturation: Day 0 % transferrin saturation)
e. difference (Day 14 plasma iron – Day 0 plasma iron)
f. significant difference between groups (p-value ≤0.05)
g. there was no statistically significant interaction between experimental group, treatment (iron or saline), and day (0 and 14). Data for individual experimental groups is presented for descriptive purposes only.
Table 4-9. Comparison of relative hepcidin mRNA expression before and after iron administration

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Egyptian fruit bat (healthy)</td>
<td>14</td>
<td>19.8 (13.5-28.9)</td>
<td>47.5 (32.5-69.4)</td>
<td>2.4 (1.4-4.0)</td>
<td>0.0012</td>
<td>5</td>
<td>20.9 (11.1-39.4)</td>
<td>16.9 (9.0-31.8)</td>
<td>0.8 (0.3-1.9)</td>
<td>0.6194</td>
</tr>
<tr>
<td>Egyptian fruit bat (hemochromatosis)</td>
<td>10</td>
<td>28.6 (23.5-34.7)</td>
<td>32.3 (26.5-39.2)</td>
<td>1.4 (1.1-3.8)</td>
<td>0.2634</td>
<td>5</td>
<td>24.6 (18.7-32.5)</td>
<td>34.8 (26.4-45.8)</td>
<td>1.4 (1.0-1.9)</td>
<td>0.0264</td>
</tr>
<tr>
<td>Straw-colored fruit bat</td>
<td>14</td>
<td>1.9 (1.4-2.6)</td>
<td>1.9 (1.4-2.7)</td>
<td>1.0 (0.6-1.6)</td>
<td>0.9539</td>
<td>5</td>
<td>1.0 (0.6-1.8)</td>
<td>0.8 (0.5-1.4)</td>
<td>0.8 (0.4-1.6)</td>
<td>0.5022</td>
</tr>
<tr>
<td>Common vampire bat</td>
<td>5</td>
<td>8.2 (1.4-48.6)</td>
<td>464.2 (78.1-2757.7)</td>
<td>55.6 (4.5-714.3)</td>
<td>0.0021</td>
<td>3</td>
<td>12.8 (1.3-128.1)</td>
<td>64.9 (6.5-648.0)</td>
<td>5.0 (0.2-128.2)</td>
<td>0.3199</td>
</tr>
</tbody>
</table>

Hepcidin mRNA expression was measured relative to GAPDH
^*median (95% confidence interval)
^a. significant difference between groups (p-value < 0.05)
^b. Ratio of median of day 14 to median of day 0 (95% confidence interval)
Table 4-10. Comparison of projected Day 14 hepcidin mRNA expression in response to iron administration amongst bat species adjusting for initial hepcidin levels on Day 0

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Egyptian fruit bat (healthy) vs. straw-colored fruit bat</th>
<th>Common vampire bat vs. straw-colored fruit bat</th>
<th>Common vampire bat vs. Egyptian fruit bat (healthy)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepcidin Ratio*</td>
<td>19.43 (10.095-37.42)</td>
<td>208.73 (102.583-424.73)</td>
<td>10.74 (5.479-21.05)</td>
</tr>
<tr>
<td>p-value*</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Hepcidin mRNA expression was measured relative to GAPDH
*median (range) of projected Day 14 hepcidin level in response to iron administration (accounting for differences in hepcidin level at Day 0 between groups). Ratio remains the same regardless of Day 0 value.
Table 4-11. Correlation between levels of hepcidin mRNA expression and % transferrin, plasma ferritin, and histologic scores for iron

<table>
<thead>
<tr>
<th>Species</th>
<th>% transferrin saturation (p)</th>
<th>Plasma ferritin (p&lt;0.0001)</th>
<th>Histologic Grade (Kupffer cells) (p&lt;0.0001)</th>
<th>Histologic Grade (Hepatocytes) (p=0.3177)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egyptian fruit bat (healthy)</td>
<td>0.53056 (p=0.001)</td>
<td>0.62774 (p&lt;0.0001)</td>
<td>0.74875 (p&lt;0.0001)</td>
<td>0.16653 (p=0.3177)</td>
</tr>
<tr>
<td>Egyptian fruit bat (hemochromatosis)</td>
<td>-0.06956 (p=0.7149)</td>
<td>0.41357 (p=0.0231)</td>
<td>NA</td>
<td>-0.01742 (p=0.9272)</td>
</tr>
<tr>
<td>Egyptian fruit bat (all)</td>
<td>0.21289 (0.0886)</td>
<td>0.31157 (p=0.0097)</td>
<td>0.30115 (0.0126)</td>
<td>0.11019 (0.3710)</td>
</tr>
<tr>
<td>Straw-colored fruit bat</td>
<td>0.32104 (p=0.0494)</td>
<td>0.26819 (p=0.1035)</td>
<td>0.34191 (0.0356)</td>
<td>0.32862 (0.0440)</td>
</tr>
<tr>
<td>Common vampire bat</td>
<td>NA</td>
<td>NA</td>
<td>0.27836 (0.3151)</td>
<td>0.31497 (0.2529)</td>
</tr>
</tbody>
</table>

Hepcidin mRNA expression was measured relative to GAPDH
b. TIBC, total iron binding capacity
c. Tf sat, transferrin saturation = (serum iron, TIBC x 100).
Rho = Spearman's rank correlation coefficient
Discussion

The objectives of this study were to investigate the expression of hepcidin, a key iron regulatory hormone, in response to iron challenge in three species of bat with variable susceptibility to iron storage disease (Egyptian fruit bat, straw-colored fruit bat, and common vampire bat) and to compare hepcidin response amongst Egyptian fruit bats with and without hemochromatosis.

Hepcidin gene expression was induced in response to the intramuscular injection of 100 mg/kg of iron dextran in Egyptian fruit bats without hemochromatosis and in the common vampire bat, but not in the straw-colored fruit bat. The dose used in this study was selected based on published literature and the results of a pilot study conducted on two Egyptian fruit bats, two straw-colored fruit bats, and two common vampire bats, which suggested that this dose was appropriate for induction of hepcidin mRNA expression in the species under investigation (Chapter 3). In mice administered a 1 g/kg dose of iron dextran via subcutaneous injection, hepcidin mRNA expression was induced (2.5-fold) as soon as one week after iron administration and remained elevated for ~2 months (Pigeon et al., 2001). In this same study, liver iron content increased > 20-fold over controls in response to iron administration. In our study, administration of a lower (100 mg/kg) dose of iron dextran, resulted in a more modest increase (~2-3-fold) in liver iron content in all three species of bat and was supported by proportional increase in histologic iron staining in Kupffer cells and an elevation in plasma iron parameters (ferritin, iron, and % transferrin saturation), which have been shown to modulate hepcidin expression (Ganz et al., 2008).
A significant correlation was present between hepcidin mRNA expression and % transferrin saturation, plasma ferritin, liver iron content, and iron staining in Kupffer cells in the Egyptian fruit bat (healthy) (p≤0.001), and liver iron content in the common vampire bat (p=0.03). This relationship was expected based on studies in humans and mice, where hepcidin has been shown to increase in response to both extracellular holo-transferrin concentrations and intracellular iron (Ramos et al., 2011). Iron was shown to preferentially accumulate in Kupffer cells relative to hepatocytes, in accordance with previous studies of hepatic distribution in response to iron dextran injection in neonatal pigs (Caperna et al., 1987).

There was no significant increase in physiologic iron parameters in bats that received saline injection. A modest increase (1.4-fold (1-1.9), p=0.0264) in hepcidin mRNA expression was noted in the control group of Egyptian fruit bats with hemochromatosis. Although this change was statistically significant, the 1-fold change was included within the 95% confidence interval and hence, may simply reflect normal variation within the experimental group.

In healthy Egyptian fruit bats, there was a strong correlation between the level of hepcidin gene expression and plasma ferritin (rho=0.63, p<0.0001) and liver iron content (r=0.44, p=0.007). However, relative to the degree of increase in plasma ferritin (15-fold, p<0.0001) in response to iron challenge, the degree of increase in hepcidin mRNA expression (2.4-fold, p<0.0012) was relatively modest (Figure 4-13). In healthy human subjects, serum hepcidin levels typically increase in proportion to the level of serum ferritin (Ganz et al., 2008). This suggests that hepcidin response in the Egyptian fruit bat may be diminished relative to the degree of iron load, as seen in humans with hereditary hemochromatosis and chronically iron
overloaded mice with mutations in genes associated with hereditary hemochromatosis (Ganz et al., 2008; Ramos et al., 2011).

In the straw-colored fruit bat, iron administration resulted in an even more dramatic increase in plasma ferritin (45-fold, p=0.0117) and liver iron content (4-fold, p<0.0001) than in the healthy Egyptian fruit bat, with near maximal saturation of transferrin (94.06 % (85.64-97.68), p=0.0006) 14 days post-iron administration. Despite these changes, there was no accompanying hepcidin response (Figure 4-13). In healthy humans and mice, hepcidin mRNA levels and urinary hepcidin concentrations increase in proportion to transferrin saturation (Gehrke et al., 2003; Lin et al., 2007). The lack of hepcidin response despite elevation in both plasma ferritin and % transferrin saturation parallels that seen in humans with hereditary hemochromatosis. The lack of hepcidin response seen in the straw-colored fruit bat is surprising and argues against the assumption that this species is not susceptible to iron storage disease in captivity. However, there is only a single report of hemochromatosis in this species. It is possible that straw-colored fruit bats have evolved a mechanism for eliminating excessive iron from the body or like mice, become iron overloaded, but do not develop iron-associated disease.

Alternatively, hemochromatosis may simply be underreported in this species amongst zoological collections. A study of iron metabolism in the straw-colored fruit bat found that this species exhibited upregulated absorption of iron relative to normal reference ranges for humans (Lavin et al., 2010). In that study, the bioavailability of iron in blood was measured after oral administration of isotope labelled ferrous sulfate. While this finding was suggestive of a predisposition to iron overload, evidence that straw-colored fruit bats develop iron storage disease amongst zoological collections is largely lacking.
In humans, hepcidin deficiency results from a mutation in a regulator of hepcidin or more rarely, in the hepcidin gene itself (Ganz et al., 2008). Genetic mutations associated with hepcidin deficiency have been associated with mutations in HFE, transferrin receptor 2 (TfR2), and hemojuvelin (HJV) and result in variable degrees of impairment of hepcidin response (Ramos et al., 2011). The hepcidin gene was examined for genetic differences that may result in a deficiency in hepcidin mRNA expression in both the Egyptian fruit bat and straw-colored fruit bat, however, none was identified (Chapter 2) (Nemeth et al., 2006; Roetto and Camaschella, 2005).

Interestingly, hepcidin has been shown to downregulate expression of divalent metal transporter-1 (DMT-1), a protein which transports ferrous iron and other divalent metal ions such as lead across the apical membrane of the enterocyte (Brasse-Lagnel et al., 2011). A relationship between upregulation of DMT-1 and hemochromatosis in Egyptian fruit bats was proposed by Farina based on a finding of elevated hepatic lead levels in Egyptian fruit bats with hemochromatosis (Farina et al., 2005). Studies in mynahs, species with known susceptibility to iron storage disease have shown high levels of expression of DMT-1 in the small intestine (Mete, 2005). A low level of hepcidin gene expression in fruit bats may involve upregulation of DMT-1, which may be a potential mechanism that results in increased dietary iron uptake in these species.

The greatest degree of increase in hepcidin gene expression was seen in the common vampire bat (55.5-fold, p=0.0021). This finding was statistically significant, despite the low sample size (n=5) in this experimental group. By comparison, in a study in mice given a subcutaneous injection of a 10-fold higher dose of iron dextran, hepcidin expression increased
only 2.5-fold over controls (Pigeon et al., 2001). Elevated hepcidin response in the common vampire bat relative to the mouse, the Egyptian fruit bat and the straw-colored fruit bat may be explained, in part by the unique dietary adaptations of this species. The common vampire bat, an obligate sanguivore, consumes over 200 mg/kg of iron daily, equivalent to 800 times the mean daily iron consumption for man on a weight per weight basis (Morton and Wimsatt, 1980). Amazingly, despite the highest dietary iron intake of any species, the percentage of iron absorbed by the common vampire bat is the lowest reported for small mammals (Morton and Janning, 1982). These findings suggest that the common vampire bat has the ability to dramatically upregulate hepcidin expression, which may have evolved in order to restrict dietary iron absorption.

It has been previously proposed that vampire bats eliminate iron through the diapedesis of macrophages across the mucosal epithelium and iron loss via rapid turnover of the mucosal epithelium (Morton and Wimsatt, 1980; Morton and Janning, 1982). This theory was been supported by the finding of large amounts of stainable iron in the cytoplasm of apical enterocytes in the digestive tract of the common vampire bat (Morton and Wimsatt, 1980). Upregulation of hepcidin, which results in iron retention in the enterocyte and prevents iron release into plasma, thus trapping the iron until the enterocyte is shed, may explain these findings (Nemeth et al., 2006).

Diurnal variation in hepcidin levels and time since last meal were considered as factors affecting the large variation in baseline hepcidin levels in the vampire bats seen in this study. In humans, diurnal variation in hepcidin levels exists and there is a transient rise in serum hepcidin in response to iron ingestion (Ganz et al., 2008; Nemeth et al., 2004). This effect may be more
pronounced in the common vampire bat which consumes a single iron-rich meal once daily. A review of the time of liver biopsy collection in relation to time since last meal did not suggest an association between these factors in the vampire bats in this study.

Despite significantly higher baseline liver iron content in Egyptian fruit bats with hemochromatosis (17047 ppm) as compared to Egyptian fruit bats without hemochromatosis (2427 ppm), baseline levels of hepcidin gene expression did not differ between the two groups (Table A4-11). In healthy humans and in mice, hepcidin levels typically increase in proportion to liver iron content (Ramos et al., 2011). In healthy human subjects, there is a strong correlation between serum hepcidin and plasma ferritin and between hepcidin mRNA and plasma ferritin (rho=0.515, p<0.03), similar to that seen in healthy Egyptian fruit bats in our study (rho=0.628, p<0.0001) (Detivaud et al., 2005; Ganz et al., 2008). The lack of a significant correlation (rho=−0.099, p=0.6043) between hepcidin mRNA expression and liver iron content in bats with hemochromatosis reflects that seen in human patients with severe hepatic fibrosis (Detivaud et al., 2005). Our findings likewise suggest that hemochromatosis could result in altered iron metabolism, creating a vicious cycle of increasing iron accumulation and hepatocellular damage.

The lack of a significant positive correlation between hepcidin gene expression and % transferrin saturation in Egyptian fruit bats with hemochromatosis also reflects findings in humans with hereditary hemochromatosis. In human patients with hereditary hemochromatosis and % transferrin saturation above 80%, there is a strong inverse correlation between the level of hepcidin gene expression and serum transferrin saturation (r=−0.861, p<0.01) (Gehrke et al., 2003). In contrast, in patients with normal transferrin saturation, there is upregulation of
hepcidin gene expression in response to iron stores, similar to the response seen in healthy Egyptian fruit bats (Gehrke et al., 2003). These findings have been linked to increased levels of non-transferrin bound iron, which predominates in iron overload states. In *in vitro* models, increasing levels of non-transferrin bound iron result in dowregulation of hepcidin gene expression (Gerhrke, 2003). The presence of increased levels of non-transferrin bound iron may explain the lack of hepcidin response in Egyptian fruit bats with hemochromatosis, whereby transferrin levels are near 100% saturated. It is also possible that hepcidin is maximally expressed in Egyptian fruit bats with hemochromatosis, and like many other physiologic parameters has a ‘ceiling effect’, whereby levels reach a plateau and no further increase is possible.

In the experimental part of our study, a lack of hepcidin response in Egyptian fruit bats with hemochromatosis in response to iron challenge is most likely attributable to the fact that the dose of iron administered was small in relation to total body iron stores and did not result in an identifiable increase in liver iron content or plasma iron parameters. At the start of the study, Egyptian fruit bats with hemochromatosis had nearly 100% transferrin saturation and plasma levels of iron and ferritin far exceeding those of non-iron overloaded bats and other mammalian species (Farina et al., 2005).

There were a number of limitations to this study. The sample size was small, particularly within the common vampire bat group which was composed of eight individuals. Bats were not matched with respect to age or baseline liver iron content at the start of the study, which may have influenced iron loading and hepcidin response to iron challenge. Normally, iron accumulation in the liver increases with age (Deugnier et al., 1993). We have already stated that
initial liver iron content was substantial in many of the Egyptian fruit bats; according to a classification system established by Farina, 14/33 of the Egyptian fruit bats in our study were classified as having hemochromatosis and 15/33 as having hemosiderosis (Farina et al., 2005). Based on the same reference ranges, straw-colored fruit bats were relatively less iron loaded with 2/19 individuals above the reference range and being classified as having hemosiderosis. The vampire bats all fell within the reference range for normal Egyptian fruit bats (Farina et al., 2005). We attempted to correct for this discrepancy, at least in part, by measuring hepcidin response relative to baseline hepcidin levels for each individual bat and using a covariate model of analysis to account for differences in baseline hepcidin levels and liver iron content.

Hepcidin in humans and mice is induced by inflammation (Nemeth et al., 2004). We are aware that the inflammation associated with the laparotomy and biopsy collection may be a confounding factor in this study. In order to assess for the presence of inflammation, blood was taken for CBC at the time of each biopsy sampling. While inflammation may have been present in the period immediately after the initial surgery, at the time of the second biopsy sampling point (Day 14), there were no biologically significant changes in white blood cell count (WBC) in the control or experimental groups.

Histologic grading was greatly limited by the small size of liver biopsy samples and the marked variation in the distribution of iron in histologic sections. The degree of iron staining in sections varied depending on the presence of portal tracts and fibrous connective tissue septa, which were absent in many of the examined sections. The lack of fibrosis in an Egyptian fruit bat with markedly elevated liver iron content may be attributed to inconsistent biopsy sampling.
We considered that blood collection itself results in iron loss which may affect measured iron parameters and hepcidin expression. A statistically significant decrease in plasma ferritin (p<0.04) was noted in both Egyptian fruit bat control groups between Day 0 and Day 14. A decrease in % transferrin saturation in the Egyptian fruit bat (healthy) and straw-colored fruit bat control groups was also noted, although it was not statistically significant. It is possible that these results may reflect a decrease in total body iron as a result of blood collection. A volume equivalent to ~1% of body weight, which is approximately equivalent to one unit of blood for a human, was collected from the Egyptian fruit bats and straw-colored fruit bats on Day 0. In some individual animals, additional internal blood loss could have resulted from the biopsy procedure itself. Based on the iron content of blood (~1 mg of iron per 1 ml of packed red blood cells) in humans, the amount of iron removed through blood collection accounted for less than 4 ±1 % percent of the original 100 mg/kg dose of iron dextran administered and was considered to have a negligible effect on iron challenge in the treatment group (Haskins et al., 1952).

This study demonstrates that hepcidin is a key player in iron metabolism in bats and is differentially expressed in species with diverse life histories, dietary iron intake and recognized susceptibility to iron storage disease. Egyptian fruit bats have low hepcidin gene expression in response to iron load relative to the common vampire bat and healthy human subjects, which may explain their increased susceptibility to iron storage disease in captivity. The straw-colored fruit bat, a species previously believed to be relatively less susceptible to iron overload, is perhaps even less able to upregulate hepcidin gene expression and may also benefit from management actions that limit oversupplementation of dietary iron. Why the disease has not been recognized in this species remains unknown. A genetic difference in the coding region of
the hepcidin gene itself does not appear to be the underlying cause of hepcidin deficiency in either species (Chapter 2); however, further investigation into genetic differences in other iron-regulatory genes that influence hepcidin activity are needed (Chapter 2). The tolerance of the common vampire bat, an obligate sanguivore, to extremely high levels of iron in its diet appears to be related to its ability to greatly upregulate hepcidin gene expression. The findings of this research provide insight into hemochromatosis and the comparative pathophysiology of iron metabolism amongst bat species and may have applications to further our understanding of iron overload conditions in other susceptible species.
GENERAL DISCUSSION

Iron storage disease remains a significant cause of disease and mortality in captive exotic species, including several species of birds and mammals. Two main theories have been proposed regarding the underlying nature of the susceptibility to this condition. Many of the susceptible species are frugivores or insectivores and share a dietary preference for natural food items that are low in iron. The inadvertent addition of iron to the diet during the manufacturing process or oversupplementation in mineral or other supplements in captivity will result in increased dietary iron load, which has been found to exacerbate the condition in a number of species. A second theory involves a genetic predisposition to iron accumulation, related to upregulation of iron regulatory mechanisms that promote iron uptake. A number of studies have shown that susceptible species exhibit increased iron absorption and have a physiologic predisposition to iron accumulation in tissues, similar to humans with hereditary hemochromatosis. It is possible that both theories apply to many of the captive exotic species that are known to be susceptible to iron storage disease.

In humans, hereditary hemochromatosis has a genetic basis and has been associated with mutations in a number of genes that regulate iron metabolism. Hepcidin, a protein which regulates enteric iron absorption and iron release from macrophages and hepatocytes, has been implicated as a key player in the development of the disease. The majority of genetic mutations associated with hemochromatosis in humans involve downregulation of hepcidin activity or hepcidin resistance.
Amongst bat species, the Egyptian fruit bat, a member of the Pteropodidae family, appears particularly susceptible to iron overload. Aside from a single report of upregulation of iron absorption in the straw-colored fruit bat, another species within the family Pteropodidae, reports of iron storage disease in other species of fruit bats have been scant (Lavin et al., 2010). The purpose of our study was to investigate the role of hepcidin in regulation of iron balance in bats as a starting point in understanding the mechanisms underlying iron storage disease in susceptible captive exotic species.

In chapter 2, I characterized the hepcidin gene in eight species of bats and found that hepcidin was well conserved, with differences in genetic composition reflecting the evolutionary relationship amongst bat species. There was no evidence of a functional mutation in the coding region of the hepcidin gene which could explain an increased susceptibility to iron accumulation in the Egyptian fruit bat. Iron storage disease in humans has been associated with mutations in HFE, transferrin receptor 2 (TfR2), ferroportin, or hemojuvelin (Ramos et al., 2011). The role of these iron regulatory proteins in iron metabolism in bats and other susceptible species warrants further investigation and may provide invaluable insights into the pathophysiology of this condition in both exotic species and humans.

In chapter 4, I investigated the functional role of hepcidin in response to iron challenge in three species of bats given an intramuscular infection of iron dextran. Hepcidin response was investigated in the Egyptian fruit bat, straw-colored fruit bat and the common vampire bat, a species with remarkable tolerance to high dietary levels of iron. I found marked variation in hepcidin response amongst the three species of bats and between Egyptian fruit bats with and without hemochromatosis. Hepcidin response to iron challenge was evident in healthy Egyptian
fruit bats, however, appeared to be diminished relative to the degree of iron loading. More surprisingly, there was a lack of hepcidin response to iron challenge in the straw-colored fruit bat, despite a dramatic increase in liver iron content and plasma iron parameters. These findings suggest that fruit bats may have lost the ability to upregulate hepcidin activity in response to increased iron load. Diminished hepcidin response in the fruit bats may reflect an evolutionary adaptation to a low iron diet composed predominantly of fruit. While a reduced ability to upregulate hepcidin gene expression could facilitate increased iron uptake from limited dietary sources of iron in the wild, it may predispose to the development of iron storage disease in captivity, where dietary iron is much more readily available. The lack of hepcidin response in the straw-colored fruit bat is unusual, given the lack of reports of iron storage disease in this species and suggests the straw-colored fruit bat may have evolved an alternate mechanism to deal with excessive iron. A lack of hepcidin response in Egyptian fruit bats with hemochromatosis was attributed to a combination of reduced iron challenge relative to the degree of iron load and altered iron metabolism in this group. Analysis of hepcidin gene expression in the common vampire bat revealed marked upregulation of hepcidin expression in response to iron load. Findings suggest that vampire bats may have evolved the ability to significantly upregulate hepcidin gene expression in response to a high iron diet.

Plasma ferritin, plasma iron and % transferrin saturation have clinical utility in humans for the assessment of body iron status and the diagnosis of iron storage disease. This is in contrast to findings in birds, where the diagnostic utility of these parameters has been limited (Mete, 2005; Whiteside, 2001). In our study, there was a strong correlation between levels of plasma ferritin, % transferrin saturation, plasma iron and liver iron content in the Egyptian fruit.
bat which suggests these tests could serve as useful diagnostic measures of body iron status in this species.

This study lends support to the theory that species that develop iron storage disease may have evolved a genetic predisposition to iron accumulation and may be unable to down regulate iron absorption in states of iron excess. Hepcidin, a key iron regulatory hormone, may be the ‘missing link’ to help explain the susceptibility of the Egyptian fruit bat and other susceptible species to iron overload in captivity. Further investigation into the activities of regulatory proteins that modulate hepcidin function in fruit bats may provide further insights into the pathophysiology of iron storage disease in this and other susceptible species including humans.
SUMMARY AND CONCLUSIONS

The purpose of this study was to investigate the role of hepcidin, a key iron regulatory hormone, in the pathophysiology of iron storage disease in the Egyptian fruit bat and to further our understanding of the role of hepcidin in iron metabolism in bats.

The hepcidin gene was found to be well-conserved amongst eight bat species and no genetic differences were identified which could explain the increased susceptibility of the Egyptian fruit bat to iron storage disease. Intramuscular injection of 100 mg/kg of iron dextran resulted in increased physiologic iron scores and hepcidin activity in the Egyptian fruit bat and common vampire bat. In these species, hepcidin gene expression was significantly positively correlated with liver iron content. In healthy Egyptian fruit bats, there was also a significant positive correlation between the level of hepcidin gene expression and plasma ferritin and % transferrin saturation. Hepcidin response in the Egyptian fruit bat appeared diminished relative to the degree of iron load and was absent in bats with hemochromatosis. Hepcidin induction was not seen in the straw-colored fruit bat in response to iron challenge, despite a significant increase in liver iron content and physiologic iron parameters. The common vampire bat exhibited the most marked baseline variation and showed a dramatic increase in hepcidin levels in response to iron administration. The findings of this study demonstrate that fruit bats may have evolved a decreased ability to upregulate hepcidin activity which may predispose to iron storage disease in states of dietary iron excess. Management actions aimed at the provision of a low iron diet to susceptible species such as the Egyptian fruit bat, should also be considered in other species of bats within the family Pteropodidae, including the straw-colored fruit bat. Upregulated hepcidin
activity in the common vampire bat may explain the high tolerance of this species to increased levels of dietary iron.

In addition to our findings regarding the role of hepcidin, plasma ferritin and % transferrin saturation were found to correlate strongly with liver iron content in Egyptian and straw-colored fruit bats and may serve as useful diagnostic measures of iron storage disease in susceptible bat species.
REFERENCES


Cuesta, Alberto, Jose Moseguer, and Maria Angeles Esteban. 2008. The antimicrobial peptide hepcidin exerts an important role in the innate immunity against bacteria in the bony fish gilthead seabream. *Molecular Immunology* 45: 2333-2342.


APPENDIX A: SUPPLEMENTAL DATA FOR CHAPTER 4

Baseline Parameters

Table A4-1. Baseline liver iron content (ppm)

<table>
<thead>
<tr>
<th>Species</th>
<th>N</th>
<th>Day 0</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egyptian fruit bat (healthy)</td>
<td>18</td>
<td>2427 (1792-3288)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Egyptian fruit bat (hemochromatosis)</td>
<td>15</td>
<td>17047 (12227-23766)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Straw-colored fruit bat</td>
<td>19</td>
<td>973  (724-1307)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Common vampire bat</td>
<td>8</td>
<td>1099 (697-1732)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Liver iron content (ppm) was measured on a dry matter basis
*median (95% confidence interval)
Table A4-2. Comparison of baseline liver iron content (ppm) amongst bat species

<table>
<thead>
<tr>
<th></th>
<th>Egyptian fruit bat (hemochromatosis) vs. Egyptian fruit bat (healthy)</th>
<th>Egyptian fruit bat (healthy) vs. Straw-colored fruit bat</th>
<th>Egyptian fruit bat (hemochromatosis) vs. Straw-colored fruit bat vs.</th>
<th>Common vampire bat vs. Egyptian fruit bat (healthy) vs. Common vampire bat</th>
<th>Egyptian fruit bat (hemochromatosis) vs. Common vampire Bat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio*</td>
<td>7.02 (4.48-11.01)</td>
<td>2.49 (1.63-3.81)</td>
<td>17.51 (11.23-27.32)</td>
<td>1.13 (0.66-1.94)</td>
<td>2.21 (1.28-3.81)</td>
</tr>
<tr>
<td>p-value&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.6552</td>
<td>0.0053</td>
</tr>
</tbody>
</table>

Liver iron content (ppm) was measured on a dry matter basis

<sup>a</sup> ratio of median (95% confidence interval) of baseline (Day 0) values

<sup>a</sup> significant difference between groups (p-value ≤0.05)
Table A4-3. Summary of histologic grading for Kupffer cells

<table>
<thead>
<tr>
<th>Histologic Score*</th>
<th>Egyptian fruit bat (hemochromatosis)</th>
<th>Egyptian fruit bat (healthy)</th>
<th>Straw-colored fruit bat</th>
<th>Common vampire bat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kupffer cells Day 0</td>
<td>0 0 0 0 10 0 0 0 0 5</td>
<td>0 6 6 1 1 0 1 3 1 0 0</td>
<td>1 3 7 3 0 0 2 3 0 0</td>
<td>2 2 1 0 0 0 3 0 0 0</td>
</tr>
<tr>
<td>Kupffer cells Day 14</td>
<td>0 0 0 0 10 0 0 0 0 5</td>
<td>0 0 0 13 1 2 1 2 0 0</td>
<td>0 0 1 13 0 0 2 3 0 0</td>
<td>0 0 0 3 2 0 2 0 0 0</td>
</tr>
</tbody>
</table>

Histologic grades for iron assessed in Perl’s Prussian blue stained liver sections
*each score represents the number of bats in each group that were graded from 0 to 4 for each of the 4 treatment and 4 control groups.

Table A4-4. Summary of histologic iron grading for hepatocytes

<table>
<thead>
<tr>
<th>Histologic Score*</th>
<th>Egyptian fruit bat (hemochromatosis)</th>
<th>Egyptian fruit bat (healthy)</th>
<th>Straw-colored fruit bat</th>
<th>Common vampire bat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatocytes Day 0</td>
<td>0 0 1 9 0 0 2 3 6 5 3 0 2 2 1 0</td>
<td>4 4 3 3 3 1 1 0 0 4 1 0 0 2 1 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatocytes Day 14</td>
<td>0 0 3 7 0 0 2 3 8 4 1 1 3 1 1 0</td>
<td>0 1 7 6 3 1 0 1 0 2 3 0 0 1 1 0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Histologic grades for iron assessed in Perl’s Prussian blue stained liver sections
*each score represents the number of bats in each group that were graded from 0 to 3 for each of the 4 treatment and 4 control groups.
Table A4-5. Comparison of baseline histological iron scores amongst bat species

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Egyptian fruit bat (healthy) vs. Straw-colored fruit bat</th>
<th>Common vampire bat vs. Straw-colored fruit bat</th>
<th>Egyptian fruit bat (healthy) vs. Common vampire bat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histologic Score (Kupffer cells)</td>
<td>Difference b(^\circ) (±SE) -0.9 (±0.3)</td>
<td>-0.9 (±0.3)</td>
<td>0.0 (±0.3)</td>
</tr>
<tr>
<td></td>
<td>p-value a(^\circ)</td>
<td>0.0006</td>
<td>0.0084</td>
</tr>
<tr>
<td>Histologic Score (Hepatocytes)</td>
<td>Difference b(^\circ) (±SE) -0.4 (±0.3)</td>
<td>0.1 (±0.3)</td>
<td>-0.5 (±0.3)</td>
</tr>
<tr>
<td></td>
<td>p-value a(^\circ)</td>
<td>0.1674</td>
<td>0.7888</td>
</tr>
</tbody>
</table>

Histologic grades for iron assessed in Perl’s Prussian blue stained liver sections
* ratio of median (range) of baseline (Day 0) values for treatment groups
a. significant difference between groups (p-value ≤0.05)
b. Difference between means of histologic grade (±SE)
Table A4-6. Baseline plasma iron parameters for three species of bats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Species</th>
<th>N</th>
<th>Day 0</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma ferritin* (ng/ml)</td>
<td>Egyptian fruit bat (healthy)</td>
<td>19</td>
<td>31 (19-512)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Egyptian fruit bat (hemochromatosis)</td>
<td>15</td>
<td>9393 (5270-16742)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Straw-colored fruit bat</td>
<td>19</td>
<td>259 (155-433)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>% transferrin saturation*</td>
<td>Egyptian fruit bat (healthy)</td>
<td>18</td>
<td>60 (40-77)</td>
<td>0.3110</td>
</tr>
<tr>
<td></td>
<td>Egyptian fruit bat (hemochromatosis)</td>
<td>15</td>
<td>100 (99-100)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Straw-colored fruit bat</td>
<td>19</td>
<td>69 (50-83)</td>
<td>0.0462</td>
</tr>
<tr>
<td>Plasma iron* (µmol/L)</td>
<td>Egyptian fruit bat (healthy)</td>
<td>18</td>
<td>33 (±4)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Egyptian fruit bat (hemochromatosis)</td>
<td>15</td>
<td>63 (±4)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td></td>
<td>Straw-colored fruit bat</td>
<td>19</td>
<td>34 (±4)</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*median (95% confidence interval)
a. Tf sat, transferrin saturation = (plasma iron/ TIBC x 100). TIBC, total iron binding capacity
b. mean (±SE)
**Table A4-7. Comparison of baseline plasma iron parameters amongst bat species**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Egyptian fruit bat (hemochromatosis) vs. Egyptian fruit bat (healthy)</th>
<th>Egyptian fruit bat (healthy) vs. straw-colored fruit bat</th>
<th>Egyptian fruit bat (hemochromatosis) vs. straw-colored fruit bat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma ferritin (ng/ml)</td>
<td>Ratio* 303.03 (140.84-666.67) 0.12 (0.06-0.25) 36.26 (16.74-78.55)</td>
<td>p-value &lt;0.0001 &lt;0.0001 &lt;0.0001</td>
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</tr>
<tr>
<td></td>
<td>p-value&lt;sup&gt;c.&lt;/sup&gt;</td>
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</tr>
<tr>
<td>Plasma iron (µmol/L)</td>
<td>Difference&lt;sup&gt;a.&lt;/sup&gt; -1.27 (±5.02) 29.06 (±5.27)</td>
<td>p-value&lt;sup&gt;c.&lt;/sup&gt; 0.8020 &lt;0.0001</td>
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<tr>
<td></td>
<td>p-value&lt;sup&gt;c.&lt;/sup&gt;</td>
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</tr>
<tr>
<td>% transferrin saturation</td>
<td>Odds Ratio&lt;sup&gt;b.&lt;/sup&gt; 147.06 (43.48-500.00) 0.68 (0.22-2.10) 99.40 (30.28-326.31)</td>
<td>p-value&lt;sup&gt;c.&lt;/sup&gt; 0.4917 &lt;0.0001</td>
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<tr>
<td></td>
<td>p-value&lt;sup&gt;c.&lt;/sup&gt;</td>
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* ratio of median (95% confidence interval) of baseline (Day 0) values  
a. difference between mean (±SE) of baseline (Day 0) values  
b. odds ratio (95% confidence interval) of Day 0 values  
c. significant difference between groups (p-value ≤0.05)
Table A4-8. Relative baseline hepcidin mRNA expression

<table>
<thead>
<tr>
<th>Species</th>
<th>N</th>
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<th>p-value</th>
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</thead>
<tbody>
<tr>
<td>Egyptian fruit bat (healthy)</td>
<td>19</td>
<td>20.08 (14.26-28.27)</td>
<td>&lt;0.0001</td>
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<tr>
<td>Egyptian fruit bat (hemochromatosis)</td>
<td>15</td>
<td>27.21 (23.23-31.87)</td>
<td>&lt;0.0001</td>
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<tr>
<td>Straw-colored fruit bat</td>
<td>19</td>
<td>1.61 (1.15-2.25)</td>
<td>0.0062</td>
</tr>
<tr>
<td>Common vampire bat</td>
<td>8</td>
<td>96.92 (23.88-393.29)</td>
<td>&lt;0.0001</td>
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</table>

Hepcidin mRNA expression was measured relative to GAPDH
*median (95% confidence interval)

Table A4-9. Comparison of relative baseline hepcidin mRNA expression amongst bat species

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Egyptian fruit bat (hemochromatosis) vs. Egyptian fruit bat (healthy)</th>
<th>Egyptian fruit bat (hemochromatosis) vs. Straw-colored fruit bat</th>
<th>Egyptian fruit bat (hemochromatosis) vs. Straw-colored fruit bat</th>
<th>Common vampire bat vs. Straw-colored fruit bat</th>
<th>Egyptian fruit bat (healthy) vs. Common vampire bat</th>
<th>Egyptian fruit bat (hemochromatosis) vs. Common Vampire Bat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepcidin Ratio*</td>
<td>1.35 (0.72-2.56)</td>
<td>12.47 (6.85-22.67)</td>
<td>16.90 (8.94-31.93)</td>
<td>60.17 (14.25-254.05)</td>
<td>0.21 (0.05-0.88)</td>
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<td>p-value*</td>
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<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
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Hepcidin mRNA expression was measured relative to GAPDH
*ratio of median (95% confidence interval) of baseline (Day 0) values
a. significant difference between groups (p-value ≤0.05)
**Experimental Trial**

**Table A4-10. Comparison of Day 0 (pre-iron) and Day 14 (post-iron) WBC**

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<thead>
<tr>
<th>Species</th>
<th>N</th>
<th>Day 0</th>
<th>Day 14</th>
<th>p-value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>N</th>
<th>Day 0</th>
<th>Day 14</th>
<th>p-value&lt;sup&gt;a&lt;/sup&gt;</th>
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</thead>
<tbody>
<tr>
<td>Egyptian fruit bat (healthy)</td>
<td>13</td>
<td>7.8 (5.9-10.3)</td>
<td>6.9 (5.2-9.2)</td>
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<td>Egyptian fruit bat (hemochromatosis)</td>
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<td>Straw-colored fruit bat</td>
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<td>4.0 (3.1-5.1)</td>
<td>6.3 (4.9-8.1)</td>
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<td>8.7 (5.7-13.3)</td>
<td>8.9 (5.8-13.7)</td>
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<sup>*median (95% confidence interval)</sup>
<sup>a. significant difference between groups (p-value ≤ 0.05)</sup>
APPENDIX B: RAW DATA

Abbreviations:

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<th>Abbreviation</th>
<th>Description</th>
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<td>Animal ID</td>
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<tr>
<td>juv</td>
<td>Juvenile (&lt; 1 year of age)</td>
</tr>
<tr>
<td>trt</td>
<td>Treatment (iron-i, saline-s)</td>
</tr>
<tr>
<td>iron</td>
<td>Plasma iron (µmol/L)</td>
</tr>
<tr>
<td>UIBC</td>
<td>Unsaturated iron binding capacity</td>
</tr>
<tr>
<td>TIBC</td>
<td>Total iron binding capacity</td>
</tr>
<tr>
<td>sat</td>
<td>% transferrin saturation</td>
</tr>
<tr>
<td>ferritin</td>
<td>Plasma ferritin (ng/ml)</td>
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<tr>
<td>perlhep</td>
<td>Histologic grade for iron in hepatocytes (0-3)</td>
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<tr>
<td>perlkup</td>
<td>Histologic grade for iron in Kupffer cells (0-4)</td>
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<td>presence -1, absence -0</td>
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<td>necrosis</td>
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<tr>
<td>inflam</td>
<td>Inflammation (presence-1, absence-0)</td>
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<td>Liver iron content (ppm) on dry weight basis</td>
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<td>ironwet</td>
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<td>Hb</td>
<td>Hemoglobin</td>
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<tr>
<td>Hct</td>
<td>Hematocrit</td>
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### Egyptian Fruit Bat (Havelock, Ontario)

#### Day 0

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<th>perlhep</th>
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<th>fibrosis</th>
<th>necrosis</th>
<th>inflam</th>
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<th>ironwt</th>
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163
### Egyptian Fruit Bat (Havelock, Ontario)
#### Day 14

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<th>inflam</th>
<th>irondry</th>
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### Common Vampire Bat
#### Day 0

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### Common Vampire Bat
#### Day 14

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<th>ironwet</th>
<th>wbc</th>
<th>hb</th>
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APPENDIX C: NUTRITIONAL COMPOSITION OF TORONTO ZOO FRUIT BAT DIET

**Egyptian Fruit Bat**

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<td>Strawberries</td>
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<td>Cantaloupe</td>
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<td>Honeydew</td>
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**Straw-colored Fruit Bat**

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APPENDIX D: HISTOLOGIC GRADING OF PERL’S PRUSSIAN BLUE STAINED SECTIONS OF LIVER

Hepatocytes

Grade 0

Grade 1

Grade 2

Grade 3