Prolonged Dietary Iron Restriction Alters Total Tissue Iron but Not Heme Iron: Lack of Impact on Blood Pressure and Salt Sensitivity

By

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Abstract

The World Health Organization (WHO) states that iron deficiency (ID) is the preeminent global micronutrient deficiency. Maternal ID is linked to cardiovascular disease and hypertension in offspring, and yet the impact of ID on the kidney is not known. We characterized the impact of ID on circulating and tissue levels of iron and renal function.

Seven and ten week old male and female Wistar rats were fed either control (270ppm) or low iron (3ppm) diet for ten weeks. Total, non-heme and heme iron levels in liver, kidney cortex and kidney medulla were measured following the dietary period. Hematocrit decreased most in males regardless of age (Male 7-17wk: 47%→ 31%; 10-20wk: 47%→ 33.5%; Female 7-17wk: 47%→ 34%; 10-20wk: 47%→ 39%). Dietary ID markedly decreased liver and kidney cortex non-heme iron in both males and females (Female: liver-178±25 to 21±7 ppm; kidney cortex-51±9 ppm to 10±1 ppm; Male: liver-102±18 ppm to 11±1 ppm; kidney cortex 36±14 to 15±8 ppm). In contrast, non-heme iron in the kidney medulla was not significantly decreased

Secondly, in order to determine the impact of ID on renal function, blood pressure was monitored using radio-telemetry starting at six weeks of age (~175g body weight). Dietary salt challenge (5 days Low→5 days High→Normal) was administered to all animals (n=16) starting at eight weeks of age. At ten weeks rats were assigned to either control (225pm) or low (3ppm) iron diet. Dietary salt challenge was repeated at 13 and 18 weeks of age respectively. Despite significant lowering of hematocrit (Control 45% → Low iron 38.6%) hemodynamic changes were minimal, in that, although blood pressure

was lowered following ten weeks of dietary iron restriction, blood pressure did not change in response to dietary salt (Control MAP:105.0 ± 2.5mmHg; ID MAP:100.6 ± 3.2mmHg).

Collectively the tissue and functional analyses demonstrate that the body adapts to lowering of tissue iron supply with ID. The relative sparing of non-heme iron in the kidney medulla suggests that iron in this region of the kidney may be spared because of its importance in the systems responsible for regulating fluid and sodium balance.

Co-Authorship

The following thesis was performed and authored by Matthew R. Twiddy with the following co-authorships and technical assistance:

Chapter 2: Co-authored by Dr. M.A. Adams and Dr. K. Nakatsu. Technical assistance taking animal hematocrits by Danae Benjamin.

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AA- Arachadonic acid
ALA-S-Amino-levulinic acid synthetase-1
cyt c-Cytochrome c oxidase
CYP 450-Cytochrome P450
DBP-Diastolic blood pressure
EET-Epoxyeicosatrienoic acid
ETC- Electron transport chain
FAAS-Flame atomic absorption spectroscopy
Fe^{2+}-Ferrous iron
Fe^{3+}-Ferric iron
Fe-S-Iron-sulfur
FPN-Ferroportin
HCP-1-Heme coupled protein
HETE-Hydroxyeicosatetraenoic acid
HO-1-Heme-oxygenase-1
HR-Heart rate
HS-High salt
ID-Iron deficiency
IDA-Iron deficiency anemia
LS-Low salt
MAP-Mean arterial pressure
NaCl-Sodium chloride
NO-Nitric oxide
NOS-Nitric oxide synthase
NS-Normal salt
PP-Pulse pressure
Pre-LS-Pre low salt period
RBC-Red blood cell
SBP-Systolic blood pressure
sGC-Soluble guanylyl cyclase
TIBC-Total iron binding capacity
TfR-Transferrin receptor
WHO-World health organization

Chapter 1: General Introduction

1.1 Hypertension and Cardiovascular Disease

Blood pressure changes continuously, helping us adapt to the world in which we live- increasing in response to stress and decreasing during sleep[131]. Changes in blood pressure are dictated by multiple regulators consisting of a complex integrated network of local, neural, humoral and hormonal factors which act in concert over both short and long periods of time[131,132]. It is the interplay of these effectors that determines the operating set point of blood pressure. Healthy blood pressure is indicated by a systolic blood pressure (SBP) of ≤ 120mmHg and diastolic blood pressure (DBP) ≤80 mmHg[63]. Hypertension occurs when SBP ≥ 140mmHg and DBP ≥90mmHg[63,64]. Recent reports indicate that hypertension affects over 972 million people worldwide, 333 million in the developed and 639 million in the developing world[1]. At the present rate, despite the advent of new drugs and treatment strategies, it is estimated that by 2025 more than 1.56 billion people will be hypertensive[2]. Not only is hypertension a major health risk factor, it is also strongly associated with major health problems such as obesity, insulin resistance, dyslipidemia, stroke, kidney disease, myocardial infarction, heart failure and dementia[3]. In Canada it is estimated that as much as 25% of the population has hypertension and that 90% of those Canadians that have hypertension also have additional cardiovascular risk factors[4]. Collectively hypertension and cardiovascular disease represent the leading cause of death worldwide[5].

There are two forms of hypertension- secondary hypertension, for which there is a known factor predisposing blood pressure elevation, and essential or primary hypertension for which there is no known cause[5]. Essential hypertension accounts for nearly 95% of all cases of hypertension[6]. It is likely that genetics as well as

environmental factors play a major role in the development of essential hypertension[6]. Twin studies indicate a greater shared prevalence of hypertension in monozygotic as compared to dizygotic twins[7]. Similarly, Lifton and colleagues identified eight mutations associated with hypertension and seven associated with hypotension[8,15]. Although the identified mutations provide insight into the pathways responsible for the pathophysiology of hypertension, they account for only 1% of the known cases of hypertension worldwide[10]. Furthermore, most cases of hypertension occur without symptoms, current estimates suggesting that in Canada over 40% of those with hypertension are unaware of their condition[11].

Although traditionally treatment focused primarily on blood pressure lowering, in more recent years the emphasis of treatment and prevention strategies has shifted, putting a greater focus on altering the progression and development of measurable pathophysiology. In support of this strategy, recent clinical trials (HOPE, ADVANCE, DREAM) have indicated remarkable lowering of cardiovascular endpoints despite only small (i.e. 2-3 mmHg) reductions in blood pressure[12,49.50]. In order to better understand the association between potential risk factors and the development of hypertension it is necessary to examine the interaction of the systems responsible for controlling blood pressure.

1.2 Blood Pressure Control and the Pathophysiology of Hypertension

Neural, humoral, local and hormonal systems act in concert to keep blood pressure within desirable limits in order to achieve an operating set-point pressure[131]. In pathological situations such as hypertension, there is an elevation of the operating

pressure which is paralleled by adaptive changes in the local, neural and humoral controllers of peripheral resistance, in addition to modifications of cardiovascular structure[14,51-56]. The following description will outline the numerous systems of blood pressure regulation and the time frame over which they exert their influence.

Baroreceptors are the fastest acting regulators of blood pressure, changing heart rate, cardiac output and thus blood pressure through rapid parasympathetic nervous system-mediated inhibition of the sympathetic nervous system[117,118]. Short-term fluctuations in blood pressure are also modulated by chemoreceptors[119,120], CNS ischemic response[121], and stress-related relaxation[122,123]. Collectively these effectors function over seconds to minutes to prevent deleterious effects of acute blood pressure fluctuation. Similarly the renin-angiotensin system[124-127], the capillary fluid shift system[128,129] and the aldosterone system[130] act over more extended periods of time, from minutes to hours to modulate blood pressure. Although these systems make important contributions to the control of blood pressure within their respective time frames, it has been shown that over time they adapt and thus reset to a new set-point or reference arterial pressure[131,132].

In 1966, using a computer simulation to model blood pressure adaptations, Guyton suggested that the kidney was the over-riding regulator of the long-term level of arterial pressure. In 1991, Guyton was the first to prove that the kidney dominates long-term blood pressure control through its role in regulating body-fluid volume[9]. Despite the vast number of systems that interact to modulate and moderate changes in blood pressure, it has been shown that the kidney is the only regulatory system that does not

reset in response to changes in blood pressure and therefore, maintains the ability to control the operating set-point over the long-term[132]. The role of the kidney as the over-riding factor in the long-term control of blood pressure has been confirmed from many different experimental perspectives. Kidney transplant studies show that blood pressure follows the phenotype of the kidney[16-20](i.e. a normotensive recipient of a hypertensive kidney will develop hypertension). Similarly, genetic screening studies indicate that the mutated genes common to the development of hypertension alter sodium reabsorption in the kidney[8,15]. Furthermore, it is has been well established that the kidney has a unique ability to control sodium and water retention in response to changes in blood pressure, a phenomenon called pressure-natriuresis, or pressure-induced sodium excretion [21,22].

In all cases of hypertension, there is either a shifting or blunting of the pressure-natriuresis relationship to higher operating pressures, indicating that an elevated arterial pressure is required to achieve the same degree of sodium excretion[9,131,132]. Although there are numerous conflicting opinions regarding the control of the long term level of arterial pressure, the role of pressure-natriuresis is undisputable[9]. This process focuses primarily on the role of factors within the kidney medulla and juxta-medullary nephron and their ability to control and regulate sodium and fluid balance[9,131,132].

There are many risk factors of hypertension including lifestyle choices such as smoking, lack of exercise, stress, alcohol consumption, and an improper diet. Although the common link between these risk factors and the development of hypertension remains

to be established, emerging from among the many etiologies of hypertension is iron deficiency[13,116].

1.3 Iron Deficiency- an Understated Global Epidemic

Iron deficiency (ID), defined as the depletion of tissue iron and circulating iron levels, is a problem of epidemic proportions worldwide[65.67]. It is estimated that greater than two billion people worldwide are ID[1]. Unlike other nutritional problems, ID is the only micronutrient deficiency that represents a significant problem in both developing and industrialized nations[1,65]. Not surprisingly, given the sheer magnitude of this problem, the World Health Organization (WHO) classified ID as one of the top ten greatest global health risks in 2002, along with obesity, hypertension, and high cholesterol. ID is most common during periods of increased iron requirement and loss, and is therefore most common in pregnant women, women of child-bearing age and adolescents [1,135,136].

Iron is an essential micronutrient, playing a variety of important roles as a co-factor in physiological processes that vary from tissue oxygen delivery to DNA synthesis[23,24,25]. Iron in the human body is ubiquitous, most prevalent in hemoglobin of red blood cells, but also found in significant quantities in other organs and tissues[73]. Under normal conditions, in humans, iron intake approximates iron loss, resulting in a state of iron balance[58,73]. In relation to total body iron, dietary iron intake and iron loss, are proportionally small[76]. As a result of the disparity between daily iron intake and daily total body iron requirements for processes such as erythrocyte production, the body has

adapted an integrative and complex network for recycling iron so that tissue storage iron and circulating iron are critically and finely regulated[30,31]. As an adaptive mechanism, tissues such as the liver have the capacity to store up to ten times normal tissue iron levels without any pathological circumstances[26-29].

Despite our substantial capacity to store iron combined with the fact that much of the body's iron is recycled, dietary iron supply is often inadequate. As previously mentioned, ID is a significant problem in both developing and industrialized countries, affecting 66-80% of pregnant women in developing countries and as many as 1 in 3 pregnant women in industrialized countries[137-139]. During pregnancy, blood plasma volume expansion and iron requirement of the fetus result in a natural decline in both hematocrit and hemoglobin, two common circulatory indicators of an individual's iron status[67,138]. In a large percentage of women, the decline in hemoglobin is greater than that regarded as both physiological and safe[67,140,141]. The consequences of anemia during pregnancy are serious both for the mother and her developing fetus, increasing the risk of both maternal mortality and morbidity[142,143]. ID has also been associated with low birth weight, impaired motor and language development, and cardiovascular problems in the offspring of ID mothers[135, 140,144-146]. In this way, the developmental effect of ID fits well with the "fetal programming hypothesis".

Popularized by Dr. David Barker in 1989, the "fetal programming hypothesis" states that the factors to which the fetus is exposed induce changes which increase the risk of developing disease later on in life[147]. In this study, Barker demonstrated that low birth weight was associated with an increased risk of developing cardiovascular disease

later on in life. Since this landmark study, multiple programming agents and developmental effectors such as ID have been linked to persistent changes that in turn increase the risk of developing disease in adulthood[148-152]. In animal models, maternal ID has been shown to cause hypertension in offspring as early as six weeks of age[153-157]. The impact of ID during the developmental period has been shown to cause changes that persist even after iron supplementation[155,156]. Although the nature of these changes has yet to be fully elucidated, these findings suggest that the consequences of developmental iron restriction are persistent, and may be irreversible[158-162].

Not surprisingly, as indicated earlier, the risk of developing ID is increased during periods of growth, and increased iron requirement. Therefore, the most commonly affected populations are adolescents, mothers of child-bearing age and pregnant women. During pregnancy, the mother has an increased risk of developing ID because her iron stores are shared with the fetus at her own expense[161]. Paradoxically mothers are at a greater risk of developing ID than their offspring[160,211]. That is, the mother donates tissue and circulating iron stores to provide adequate iron supply to the fetus to the point that it is common to see a lower circulating iron levels in the mother than her baby[142,143]. Although there are specific populations with an increased risk of developing ID, the impact of ID has important implications to individuals of any age. In total, 0.8 million (1.5%) deaths worldwide are attributable to ID, 1.3% of all male deaths and 1.8% of all female deaths[1]. Considering the individual impacts of ID and hypertension and the cardiovascular risk factors associated with both conditions, the combined impact of these factors may be extensive.

1.4 Determining Iron Status

Despite such staggering global prevalence, ID, like hypertension, often goes unnoticed. This occurs primarily because of the difficulty in accurately and efficiently diagnosing ID[65,67]. Unfortunately, there is not a distinct set of symptoms that would suggest an individual is ID[67,163,164]. ID occurs over a spectrum of severity which can involve the progressive depletion of tissue storage and circulating iron[163,164]. Latent iron deficiency is defined as depletion of tissue storage iron (as determined by liver tissue biopsy and or bone marrow staining) in the absence of changes in circulating iron[67,163,164]. Iron deficiency anemia (IDA) is defined as the depletion of both circulating and tissue iron stores[67,163,164]. Given the spectrum of severities over which ID occurs, in this thesis, ID should be considered to refer to latent iron deficiency and IDA collectively.

Commonly used indicators of circulating iron status include hemoglobin, hematocrit, total iron binding capacity, mean erythrocyte cell volume, serum transferrin, serum ferritin and serum iron[66,68-71]. These circulating iron levels can be readily determined by taking a blood sample. Normal reference ranges are established according to the age and gender of the individual as outlined (Table 1.1). Although circulating iron indicators often serve as accurate tools for the diagnosis of ID, there are several downfalls which make their use less than ideal. The measurement of circulating iron levels is commonly performed when symptoms, such as fatigue, indicate that ID may have progressed to

Table 1.1 Clinical hemoglobin and hematocrit cut-offs for the diagnosis of anemia. Adapted from Looker *et al.* (1997)[133].

	Hemoglobin Concentration (<g/L)	Hematocrit (<%)
Children (Age in years)		
1-<2	110	32.9
2-<5	111	33
5-<8	115	34.5
8-<12	119	35.4
Men (Age in years)		
12-<15	125	37.3
15-<18	133	39.7
≥ 18	135	39.9
Non-Pregnant Women & Women of Lactating Age		
12-<15	118	35.7
15-<18	120	35.9
≥ 18	120	35.7
Pregnant Women		
Weeks Gestation		
12	110	33
16	106	32
20	105	32
24	105	32
28	107	32
32	110	33
36	114	34
40	119	36
Trimester		
1st	110	33
2nd	105	32
3rd	110	33

the point of anemia[71]. Anemia, defined as lowered hemoglobin, may be caused by many factors other than depletion of iron stores[72]. On the other hand, in some cases of incipient ID, ID may not have progressed beyond latent iron deficiency (ie. hemoglobin remains in a normal range). To further make the measurement of iron even more complex, the usual indicators of circulating iron status are also subject to some fluctuation. Serum ferritin, traditionally considered to be the gold standard for initial blood iron assessment changes as a result of stress and inflammation[68]. As well, serum transferrin, total iron binding capacity (TIBC) and hemoglobin change as a result of many different factors (i.e.time of day and hydration) and thus do not necessarily provide an accurate indication of iron status[68,71].

Although circulating iron parameters are the most practical means of diagnosing ID, in order to get the best assessment of an individual's iron status, tissue biopsy or bone marrow staining must be performed[58,71,73]. While accurate, these methods are both time consuming and invasive. It is generally accepted that distinguishing between IDA and anemia imposed by other causes remains a challenge[165]. In order to better understand the effects of ID it is necessary to elucidate the very complex interactions of (1) the distribution of iron in the body, (2) the dietary absorption of iron and (3) circulating and tissue iron levels. In the following sections these important parameters will be outlined in order to provide support and rationale for the structure and design of this research study.

1.5 Iron in the body

In humans, total body iron averages between 3-4g[73]. Functionally, iron is found in three main types of stores in the body in the (1) blood, (2) reticuloendothelial system (liver, bone marrow, spleen and macrophages) and (3) other tissues such as the kidney, brain and skeletal muscle[30,31] (Figure 1.1). Within these functional compartments, iron is found in either heme or non-heme forms. In the heme form iron is complexed with protoporphyrin IX and acts as an important co-factor in many heme-containing proteins or hemoproteins[40]. Iron in the non-heme form is found soluble in iron storage protein ferritin, cytosolic endosomes and as a co-factor in iron-sulfur (Fe-S) proteins, or insoluble, complexed with hydroxyl side chains as $Fe(OH)_3$, called hemosiderin[31,57,58] .

Iron in the blood is found most predominantly in red blood cells in the form of hemoglobin, a protein that accounts for over 2/3 of total body iron[73]. As mentioned previously iron is also found in smaller proportions outside red blood cells, in blood plasma as either transferrin or ferritin. The function of key heme and non-heme iron containing proteins is summarized (Table 1.2). In the reticuloendothelial system iron is prevalent and found predominantly in its storage forms, ferritin or hemosiderin[73]. Hemosiderin is believed to be the final step in the cascade of iron storage resulting from the breakdown of tissue ferritin proteins[74]. Despite the marked heterogeneity in the amounts of iron in some organs and tissues than others, iron is an essential element in every tissue of the body, performing a wide array of functional duties[73].

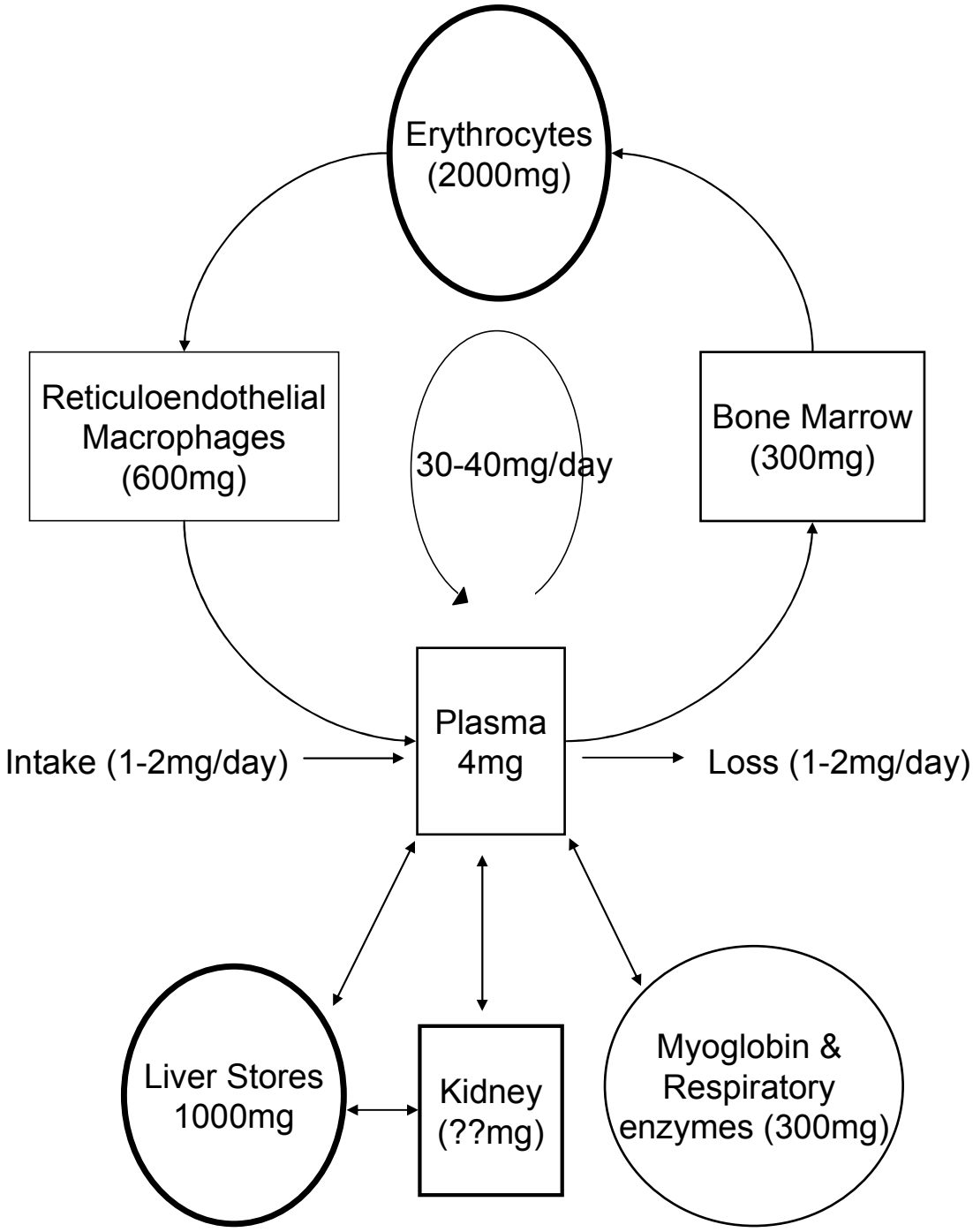

Figure 1.1 Iron distribution in the body. Adapted from Andrews (1999)[31].

Table 1.2 Summary of the function of important heme and non-heme iron proteins found in the human and rat.

Heme Iron	**Function**
Hemoglobin	Oxygen transport
Myoglobin	Oxygen storage
Heme oxygenase	Catalyzes breakdown of heme
Nitric oxide synthase	NO production playing role in regulation of vascular smooth muscle tone. Catalyzes the breakdown of L-arginine to L-citrulline, and in the process releases the gaseous transmitter nitric oxide[40]
Cyclooxygenase	Conversion arachidonic acid to prostaglandin H2. Important in vascular control[40]
Soluble guanylyl cyclase	Catalyses the conversion of GTP to cGMP. Intracellular receptor for nitric oxide. Important in vascular control[269-273]
Cytochromes (P450,a,b,c)	Drug metabolism and redox signalling in the electron tranpsort chain respectively
Catalase	Conversion of H_2O_2 to water and oxygen. Decreases oxidative stress[40]
Hydroxylases	Synthesis of steroid hormones[40]
Non-Heme Iron	**Function**
Ferritin	Spherical iron storage protein holds up to 125 mole Fe^{3+} per mole ferritin. Found primarily in the reticulendothelial system[31]
Transferrin	Iron transport protein. Holds two moles Fe^{3+} per mole transferrin. Shuttles iron from blood plasma to erythroid and non-erythroid tissues[31]
Hemosiderin	Insoluble iron storage form - ferric oxide[74]
NADH dehydrogenase	First enzyme of the mitochondrial electron transport chain. Catalyzes transfer of electrons from NADH to coenzyme Q[31]
Succinate dehydrogenase	Participates in both the citric acid cycle and the mitochondrial electron transport chain. Also contains heme moiety
Xanthine oxidase	Important enzyme for biotransformation in the liver[274]
Ribonucleotide reductase	DNA synthesis. Deoxynucleotide production[31]

1.6 Dietary Iron Absorption and Distribution

Under normal conditions, average dietary iron intake parallels iron loss from the body, each approximately 1-2 mg per day[31]. However, it has also been shown that iron absorption adapts to iron requirement[76]. For example, iron absorption increases to 3-4mg per day in response to the iron demands imposed by pregnancy[32-35]. Likewise, it has been shown that intestinal iron absorption is augmented in response to ID[75]. Whatever the stimuli for increased iron requirement, the adaptation of increased iron absorption alone is not sufficient to meet the daily iron needs for erythropoiesis, production of new proteins, red blood cell recycling and cellular respiration[30,31]. These metabolic demands greatly exceed the amount of iron we get from our diet, requiring as much as 40mg iron per day [30,31]. Consequently, the majority of iron in the body is reused and recycled where it is needed. Specialized mechanisms exist within the body which allow for the sensing, distribution and control of total body iron levels under a range of conditions[76].

As indicated previously, the body has the ability to change the level of dietary absorption, turnover and recycling of circulating iron (ie. red blood cells in splenic and reticuloendothelial macrophages) as well as mobilize tissue iron for use in other areas of the body in response to change in iron supply. This capacity to recycle iron permits the redistribution of iron within local tissues and shuttling from one cellular compartment to another (i.e. endosomal to mitochondrial compartments)[36,40]. Although dietary iron intake appears to be only a small component of total body iron, because the body lacks a natural pathway for the excretion of iron, the regulation of dietary iron absorption is recognized to be a key player in maintaining systemic iron balance[57,58]. The primary site of iron

absorption is the duodenum[77]. Iron is absorbed by intestinal enterocytes and is distributed throughout the body. Similar to within the body, dietary iron is found in two major forms, as either heme or non-heme iron. Heme iron and non-heme iron are absorbed by two different pathways in the intestinal tract, named appropriately, the non-heme and heme iron absorption pathways, respectively. The non-heme iron absorption pathway is much better understood than the more recently discovered heme iron absorption pathway[37].

Non-Heme Iron Absorption:

In the acidic environment of the intestinal tract, non-heme iron is found predominantly in the insoluble ferric (Fe^{3+}) form[30,31]. In order for Fe^{3+} to be absorbed by the intestinal enterocyte it must be reduced to ferrous iron (Fe^{2+}) by a cytochrome-b-like ferric reductase called Dcytb1. Soluble ferrous iron is then transported by Divalent Metal Transporter (DMT-1) across the apical surface of the enterocyte where it is either packaged into an endosome for transport to the rest of the body or compartmentalized by ferritin for storage in the intestinal enterocyte[30,31].

Heme Iron Absorption:

Dietary heme iron is absorbed by a pathway distinct from that of non-heme iron. Although the process has yet to be thoroughly characterized, it has been proposed that heme iron is taken up by the duodenal enterocyte via heme-coupled protein (HCP-1)[36,37]. Following absorption into the duodenal enterocyte, heme is then broken down by heme oxygenase (HO-1) where Fe^{2+} is either packaged into an endosome or compartmentalized by ferritin for storage as in the case of non-heme iron absorption. It has also been

proposed that heme is absorbed directly from dietary sources into the circulation by pathways yet to be thoroughly characterized[37].

Iron Export Pathways:

Although there are multiple routes for dietary iron absorption, to date, there is only one known pathway for intestinal iron export to the rest of the body[36]. Intestinal iron export occurs through the membrane multi-spanning channel ferroportin (FPN), found on the basolateral or serosal side of duodenal enterocytes[97-99]. In fact, FPN is found in all cell types that export iron into plasma including macrophages, hepatocytes, placental trophoblasts, and cells of the CNS[36]. Similar to its brush border counterpart, DMT-1, FPN forms a pore in the basolateral membrane of the duodenal enterocyte, allowing export of Fe^{2+} iron from the cytosol to interstitial plasma. This protein was first elucidated using a FPN knockout model in mice and later in zebrafish, where the absence of FPN was embryonically lethal as a result of the lack of iron transfer across the maternal–fetal interface[38]. Iron export by FPN does not function on its own. FPN iron export is coupled with the multicopper oxidase activity of hephaestin or ceruloplasmin, required to convert soluble Fe^{2+} to insoluble Fe^{3+}, thus facilitating the loading of iron onto transferrin (See Table 1.2 for transferrin function). Ceruloplasmin is involved in iron export from non-intestinal cells and its homolog hephaestin facilitates iron export from intestinal cells[31]. Ceruloplasmin deficiency has been shown in both human and animal models to cause iron accumulation in macrophages, hepatocytes, and cells of the CNS, resulting in iron-restricted erythropoiesis and neurodegeneration[166,167]. Similarly, mutation of the iron absorptive and export mechanisms has also been associated with the

iron overload, caused by hereditary hemochromatosis[78]. On the other hand, hephaestin deficiency has been shown to result in severe IDA[39]. These processes are highly integrated in order to prevent the release of harmful free iron into the surrounding cellular space[99].

1.7 Heme and Non-heme Iron Regulation

As indicated previously, erythrocytes account for over 65% of total body iron, where it is incorporated predominantly in the heme form[30]. In non-erythroid tissues, which comprise the majority of the remaining 35% of total body iron, non-heme iron constitutes a larger component of total iron [40]. It is evident that an interplay between both heme and non-heme iron components within and between erythroid and non-erythroid tissue respectively, is essential for homeostasis and integrated control of systemic iron balance[30]. In order for this relationship to function effectively, there must be coordinated interaction of cellular iron import and export machinery and intracellular heme and non-heme iron components[40,79]. In erythroid cells, heme biosynthesis is controlled by the availability of iron, whereas in non-erythroid tissues the amino-levulinic acid synthetase (ALA-S1) is the major point of control for heme biosynthesis[43]. Accordingly, in erythroid cells, iron is directed immediately to mitochondria or incorporated into hemoglobin[41,42].

In both erythroid and non-erythroid cells the most well described mechanism for the uptake of non-heme iron into the cell is the transferrin receptor (TfR) pathway[80-85]. TfR is expressed on the surface of all cells of the body excluding mature erythrocytes[31,80]. In the TfR pathway, transferrin binds to TfR on the cell's surface, and the transferrin/

TfR complex is internalized via receptor-mediated endocytosis[82,85]. In this process, two molecules of non-heme (Fe^{3+}) iron, per molecule of transferring, are incorporated into a cytosolic endosome. TfR expression is subject to change based on the iron requirements of the cell, and therefore is important in facilitating the distribution of iron throughout the body where it is needed most[81-83,85]. The uptake of heme iron and iron by other means (i.e. hemoglobin directly, ferritin), is still not well understood and much like the absorption of dietary iron, pathways for the direct absorption of iron in the form of heme and molecular hemoglobin forms are only just beginning to be elucidated[37].

Heme iron and non-heme iron have important functions throughout the body, however, one important site, where they are co-localized, is in the mitochondria. Both heme and non-heme iron in the mitochondria are found in the protein complexes of the electron transport chain (ETC). Aside from iron's important roles in the ETC, mitochondrial iron is important for heme synthesis, Fe-S cluster biogenesis and can even be stored as mitochondrial ferritin[44-46]. Communication of mitochondrial and cytosolic iron components, in addition to heme and non-heme components, has been proposed to occur[40,189,190].

It is evident, that the interaction between heme and non-heme iron components is essential within the cell and throughout the body[40]. Similarly, as with any biological system, altering the supply of reagents (i.e. non-heme and heme iron), may alter the dynamics of the biological system[31]. However, despite the lowering of iron supply that

occurs with ID, there are few studies which thoroughly characterize heme and non-heme iron dynamics in response to ID.

1.8 Hemodynamic and Biochemical Alterations of Iron Deficiency and Anemia

Historically, the impact of ID focused primarily on the influence of effects mediated by anemia[24]. It has since been established that there are many important tissue specific biochemical alterations that must be considered that may not be directly associated with the development of anemia[24,59-62,153]. It has also been well established that anemia and ID alter hemodynamic and cardiovascular properties[47,48,153-157]. Despite the association of these factors, few studies have attempted to characterize the connection between tissue biochemical alterations in response to ID and changes in hemodynamics. Furthermore, despite the importance of the kidney in the regulation of blood pressure, and thus its importance in the hemodynamic control, few studies have assessed the impact of ID on the kidney. Therefore, in order to determine the connection between these factors we will characterize the association between (1) tissue specific biochemical changes, (2) circulating iron changes, and (3) hemodynamic changes in response to ID. The following section will detail what is known regarding tissue specific heme and non-heme iron alterations supporting the importance for further characterizing the changes in response to ID within the kidney specifically.

1.9 Tissue Specific Effects of Iron Deficiency

Heme Iron Proteins:

There has been a wide array of tissue specific alterations reported in response to ID[24,35,40]. These include alterations in the expression of both heme and non-heme iron proteins. The impact of ID on cytochrome c (cyt c) expression was the focus of many early studies[200,208]. In general, cyt c is considered an effective indicator of a tissue's capacity for oxidative metabolism. In response to ID cyt c expression has been shown to decrease in the skeletal muscle, intestinal mucosa, liver, and heart[24, 58, 200]. In these studies, decreased expression of cyt c was accompanied by a degree of uncoupling of oxidative phosphorylation. Complicating the issue is the fact that many studies show no change in cyt c expression[188,193]. Similarly, ID has been shown to cause no change in cytochrome P450 (CYP450) expression, whereas marked reductions in the hemoprotein benzopyrene hydroxylase, also responsible for drug metabolism, have been shown[59-62]. Ni et al (1997) showed that there was increased expression of hemoprotein nitric oxide synthase (NOS) in response to dietary ID[204]. Nevertheless, the impact of ID on the expression of hemoproteins appears to be quite variable. This likely relates to differences in the methods used to induce ID (i.e. species, strain, age, severity), and possibly differential depletion of hemoproteins for adaptive or functional reasons.

Non-Heme Iron Proteins:

As previously mentioned (see Table 1.2), non-heme iron proteins have a wide variety of functional roles in the body varying from tissue energy supply to DNA synthesis. Although not fully established, there is a growing body of evidence to suggest

that ID has a greater impact on the expression of non-heme iron-containing proteins in comparison to heme iron containing proteins[24,30]. For example, it is well known that ferritin iron expression is significantly down regulated in response to decreased supply of iron and that hemosiderin is only expressed during times of tissue iron excess[29,36,77]. Similarly, in 1983, McKay *et al.* showed that non-heme iron protein succinate dehydrogenase expression decreased to a greater extent than heme iron containing cytochromes in response to ID[193]. Likewise, decreases in succinate dehydrogenase have been shown to reduce electron flow and ATP production causing early fatigue with exercise[199]. ID has also been shown to cause marked decreases in the expression of non-heme iron containing protein NADH dehydrogenase[198]. As mentioned previously (Table 1.2), NADH dehydrogenase and succinate dehydrogenase are essential components of cellular energy supply, playing important roles in complex I and II of the mitochondrial ETC respectively[193,198,199,202]. Although there were limited changes in CYP450 expression, ID was associated with significant impairment of phase I hepatic detoxification mechanisms, depleting levels of non-heme iron protein microsomal epoxide hydrolase[60,61]. It was proposed that these changes may alter drug responses and in addition increase the risk of carcinogenesis[60-62].

Given the many important roles of heme and non-heme iron proteins within different organs, in addition to their variation in response to iron restriction, the changes that occur between heme and non-heme iron components represent an important consideration of ID. Similarly, there are few studies which characterize the relationship between heme and non-heme iron components in the kidney.

1.10 Heme and Non-heme Iron Balance:

There is an important balance between tissue heme and non-heme iron components. As previously mentioned, there are also specific changes in heme and non-heme iron components in response to ID. It remains to be established whether changes in heme and non-heme iron occur for adaptive reasons and how these changes may be linked specifically, to changes in circulatory control mechanisms[30]. In that regard, in response to dietary ID it has been shown that specific organs such as the heart have a greater capacity to maintain iron in relation to other organs[24, 36,183]. It has also been suggested that the heme iron demands of erythroid production in response to ID may cause exaggerated and damaging effects on non-erythroid tissues in response to the body's attempt to correct anemia[86,87,202]. In the projects described in this thesis we will attempt to determine the extent to which ID induces alterations in the balance of tissue heme and non-heme iron components. Also, given the importance of linking tissue biochemical changes to functional systemic alterations we will determine to what extent tissue changes in response to ID alter hemodynamics and organ function. As mentioned earlier, in order to strengthen our analysis of the tissue specific and functional hemodynamic changes that occur in response to ID a special focus will be given to the impact of ID on the kidney specifically.

1.11 Impact of Iron Deficiency on Hemodynamic and Renal Function

Chronic anemia has been shown to have a significant impact on hemodynamics-decreasing blood viscosity and blood volume and increasing peripheral vasodilation and cardiac output[47,48,88,89]. Collectively, these changes have been referred to as a

hyperkinetic circulation[47]. Similarly prolonged uncorrected anemia has also been associated with specific changes in renal hemodynamics[90]. Alterations in renal function have been reported in the offspring of ID mothers[157]. Lisle and colleagues in 2003, demonstrated that hypertensive offspring had reductions in nephron number and glomerular filtration volume[157]. Likewise, we have shown (unpublished data, Stephane Bourque, 2007), that the offspring of ID mothers develop salt-sensitive blood pressure elevation. That is, there are significant changes in blood pressure in response to alterations in dietary salt content (i.e. high salt= increased blood pressure)[115]. The salt sensitivity of blood pressure has been shown to occur in 51% of the hypertensive population[91]. Likewise, salt sensitivity has been shown extensively in various models of hypertension including the DOCA[100,101], Dahl[95,102], ANP knockout[103,104] and Sabra rats[105,106]. Although salt-sensitivity in these models has been proposed to occur by different specific mechanisms, these models are invariably associated with alterations in renal function. One particular mechanism that has been proposed to cause salt sensitivity in several animal models involves reduction in nitric oxide (NO) production within the kidney.

NO is the major regulatory depressor of blood pressure in the body[108]. It is produced by the hemoprotein, nitric oxide synthase (NOS). The dilatory actions of NO are mediated through a second hemoprotein, soluble guanylyl cyclase (sGC)(See Table 1.2 for descriptions of NOS and sGC). NOS catalyzes the breakdown of L-arginine to L-citrulline, and in the process releases the gaseous transmitter NO[107]. Its close proximity to vascular smooth muscle cells and small, lipophilic nature allow NOS-generated NO to

quickly diffuse into the smooth muscle to exert its actions. Due to its labile nature, $t_{1/2}$ approximately five seconds[108], the distance over which NO can exert its actions is limited. However, its high affinity for the heme-moiety of sGC ensures that it binds to the appropriate target[109]. These hemoproteins are expressed systemically but are also abundant within the renal vasculature[110,204]. Alterations of NOS and sGC within the kidney specifically have been associated with development of hypertension and salt-sensitivity[95,102]. Given the importance of hemoproteins in the NO system, it is possible that this may represent a link between ID and salt sensitivity[107-109]. Although there are few studies characterizing the impact of ID on renal NOS expression, Ni *et al*., (1997) showed a corresponding upregulation of NOS in the kidney in response to ID. However, administration of NOS inhibitors such as L-NAME, in particular into the kidney, has shown reliable acute and chronic experimental hypertension in a variety of animal species[112-114].

Recent studies have also suggested that alterations in another iron dependent process, arachadonic acid (AA) metabolism, may play a role in hypertension and more specifically salt sensitivity[115]. AA metabolism can occur through breakdown mediated by CYP450. The products of this reaction are vasoactive metabolites, 20-hydroxyeicosatetraenoic acid (20-HETE) and epoxyeicosatrienoic acid (EET). It has been shown that 20-HETE and EET can act as modulators of blood pressure[96]. Like the production of NO, AA metabolism occurs systemically, but also within the kidney, specifically[92]. Similarly, alterations in CYP450 associated with AA metabolism, have been shown to cause functional alterations in the regulation of renal sodium channels[92-95].

Given that the breakdown of AA requires the hemoprotein CYP450, the potential exists for changes to occur in response to ID.

There are many mechanisms by which salt sensitivity and thus, alterations in renal function can be produced[115]. However, given the importance of hemoproteins in these processes, it is possible that iron may be a key regulatory component. As mentioned earlier, we have shown that ID during development of the fetus results in salt sensitivity later on in life. It remains to be established whether ID induced following birth, can cause alterations in renal function and in addition salt sensitivity of blood pressure. In order to elucidate the connection of iron deficiency and associated hypertension, the impact of iron deficiency on the development of salt sensitivity needs to be determined. We will also characterize the connection between tissue specific changes in non-heme and heme iron in the kidney and functional alterations that might occur as a result.

1.12 Rationale and Approach

In response to prolonged dietary iron restriction, there is substantial evidence demonstrating that tissue iron stores decrease, followed in turn by a lowering of circulating iron indicators (ie. hemoglobin and hematocrit)[71,73]. In addition there is some evidence, although somewhat incomplete, that suggests progressive depletion of iron-containing entities varies depending on tissue and potentially, iron type (ie heme, non-heme). Although the accepted definition of the progression to ID indicates that there is tissue iron depletion followed by decreases in circulating iron components, the nature of

the interaction between circulating and tissue-specific iron changes has not been thoroughly characterized in key tissues[67,71]. Although there is a probable association between tissue iron deficiency (heme and non-heme components) and cardiovascular and hemodynamic alterations, much remains to be learned about the mechanistic link, particularly involving the kidney. That is despite the fact that the kidney is an essential controller of the long term level of arterial pressure, fluid and sodium balance, little has been done to assess the impact of ID within this organ. Furthermore, the relationship between tissue specific changes in the kidney, and alterations in renal function, has not previously been characterized. In order to assess these relationships the rat has been chosen as an experimental model. Iron metabolism in the rat parallels in many ways iron metabolism in humans, and as a result has been strongly supported as a robust animal model for studying iron metabolism[116]. Therefore, the following studies have been designed to assess the degree to which tissue non-heme iron and heme iron change in response to dietary iron restriction. In particular, we will determine the impact of ID on tissue specific heme and non-heme iron changes within the kidney cortex and medulla. In addition, it is our aim to characterize the impact of ID on renal function. This will be determined indirectly, by assessing changes in hemodynamic parameters in response to alterations in dietary sodium using radio-telemetry blood pressure recordings.

To address these questions the studies were designed to test the following hypotheses:

1) ID will result in differential changes in both circulating and tissue iron levels that are age-, gender-, and pregnancy-dependent.

2) ID will cause tissue specific changes in tissue non-heme iron and heme iron levels that are greater in the liver than the kidney, but that vary within the organs themselves. Further, non-heme iron will decrease to a greater extent than heme iron.

3) Susceptibility to ID will be proportional to tissue iron levels and iron absorption and will vary with respect to age, gender and pregnancy.

4) Tissue specific changes in the level of iron will alter hemodynamic and renal function with the effects reflecting the degree of circulating and tissue iron depletion.

The experiments designed to test these hypotheses have been outlined briefly below. The relative impact of age, gender and pregnancy on the circulating and tissue iron changes that occur in response to prolonged dietary iron restriction was characterized by arranging animals into groups that started on a ten week dietary iron regimen at either seven or ten weeks of age. Animal age was chosen to span the age gap in previous studies on ID performed on either weanling (4 week old) or adult (15+ week old) adult animals[24,116]. Similarly, few studies have tested whether subtle differences in age alter susceptibility to ID. Thus, we induced ID by dietary iron restriction starting at either seven or ten weeks of age, ages intermediary to previous studies. Animals were fed either control or low iron diet for a period of ten weeks. Iron balance was assessed throughout the dietary period and circulating iron levels were monitored weekly by taking blood

samples. At the end of the dietary regimen total, non-heme and heme iron levels were determined in the liver, kidney cortex and kidney medulla in order to assess the relative inter and intra-organ tissue iron changes that occur as a result of dietary iron restriction. This method of inducing ID was chosen because it best mirrors the development of ID in the human population[116].

Subsequently, a second study was performed to determine the impact of tissue and circulating iron depletion on hemodynamics and renal function. In this study only male animals were used, chosen to mirror the oldest group of males studied during tissue iron analysis experiments. Age matched animals were placed on a dietary iron regimen similar to that of the previous tissue iron analysis study. The impact of dietary iron restriction on hemodynamics was assessed by monitoring multiple blood pressure parameters in response to the progressive depletion of tissue and circulating iron using radio-telemetry. Likewise, the impact of dietary iron restriction on the salt sensitivity of blood pressure was assessed by monitoring hemodynamic parameters in response to progressive alterations in dietary salt content. Three different time points for dietary salt manipulation were chosen to reflect progressive depletion of circulating and tissue iron levels. Therefore, by using age and treatment matched animals, the initial tissue iron analysis experiments were used to predict relative tissue iron changes, which in turn were correlated with hemodynamic outcomes in the second study.

Chapter 2: The Impact of Iron Deficiency on Tissue Heme and Non-Heme Iron and Hematocrit

2.1 Introduction

Iron deficiency (ID) is a major global health risk, estimated by the World Health Organization (WHO) to affect up to two billion people[1]. ID is most prevalent during times of increased iron demand and is accordingly most common in pregnant woman, woman of child bearing age and adolescents[169,170]. The potential impact of ID has become more widely appreciated because of the growing body of evidence supporting an association between ID and low birth weight, cardiovascular disease, cardiac hypertrophy or hypertension[154-156,162,174-180]. It is well established using rat models that ID during gestation induces hypertension in offspring as young as six weeks of age[153,154,157]. Iron is an essential co-factor for many proteins in the body, found in either heme or non-heme form[40]. Both mild and severe ID have been shown to affect hemoprotein expression as well as non-heme storage iron levels in various tissues such as the liver, brain, skeletal muscle, heart and kidney [181,182,188]. Severe anemia in weanling rats has also been associated with ultrastructural aberrations in mitochondria and sarcomeres, increased production of reactive nitrogen species, altered expression of hemoproteins in the heart and oxidative decoupling of the electron transport chain (ETC) in hepatic cells [183,189-192]. However, despite the staggering prevalence of ID in both developing and industrialized countries, the diverse age range of the populations at risk and the growing body of evidence indicating undesirable effects on the cardiovascular system, there is a limited understanding of the combined impact of age and sex on tissue iron levels in response to ID. In response to ID it is generally accepted that there is depletion of non-heme tissue iron stores, followed by a subsequent decrease in circulating iron levels[58,67,71]. Recently, it has been proposed that, even in cases of mild ID, limitations in tissue heme supply may

incur pathological consequences[202]. Although, previous studies have determined changes in hemoprotein expression in response to ID, the impact of ID on total tissue heme supply has yet to be assessed. Furthermore, despite the established association of ID with the development of hypertension and critical role of the kidney in the regulation of blood pressure, few studies have characterized the impact of ID on the kidney[9]. Moreover, the relative and collective impact of age, sex and pregnancy on iron levels in the kidney has not previously been characterized. Therefore, in order to address the role of age, sex and pregnancy in the response to ID, we have assessed the effects of a severe, ten week dietary iron restriction on both males and females starting at either seven or ten weeks of age, representing substantial differences in maturity. The impact of ID was assessed according to the following parameters: 1) animal growth, 2) iron balance, 3) circulating iron and 4) liver total, non-heme and heme iron, 5) kidney cortex total, non-heme and heme iron, and 6) kidney medulla total, non-heme and heme iron.

2.2 Methods

1. Animals and Dietary Regimen:

The study received approval by the Animal Care and Use Review Committee at Queen's University. Wistar rats were obtained at seven and ten weeks of age (*Charles River Laboratories Inc, Quebec*), and were housed in individual cages from 22 -24^{o}C on a 12 hour light/ dark cycle. Animals were arranged into four groups according to age (seven or ten weeks) and dietary iron content (control: 270 ppm or low: 3 ppm). Each group consisted of 12 animals (six males and six females). Group1 ($C_{7\text{-}17}$) and group 2 ($C_{10\text{-}20}$), received control diet (270 ppm) *ad libitum* starting at seven and ten weeks of age respectively, while Group 3 ($ID_{7\text{-}17}$) and Group 4 ($ID_{10\text{-}20}$) received low iron diet *ad libitum* starting at seven and ten weeks of age respectively. Control (270 ppm) and low iron (3 ppm) diets, identical aside from iron content, were obtained from *Ren's Feed and Supply limited* (*Oakville,ON*) and *Research Diets Inc.*(*NewBrunswick New Jersey, USA,*) respectively. In each group, following seven weeks of dietary treatment three females were bred (group 1,3=14 weeks of age, group 2, 4=17 weeks of age). Hematocrit, body weight, and iron balance were measured weekly throughout the ten week dietary period. Following ten weeks of control or low iron feeding all animals were euthanized and tissue total, non-heme and heme iron were determined by methods that will be thoroughly described in sections *5-8* that follow.

2. Determination of Hematocrit:

Animals were anaesthetized using halothane anesthesia (dosed by inhalation as needed). The cuticle of each animal was clipped, and blood was collected in a micro-

hematocrit capillary tube (*Fisher Scientific, ON, Canada*). The tube was then sealed with crito-seal, a vinyl plastic putty (*Fisher Scientific*) and centrifuged at 11 500 x g to separate cells from plasma. Hematocrit was determined visually as the percentage of packed cell volume over total volume of blood collected. Weekly, hematocrit measurements in females were stopped upon breeding.

3. Measurement of Iron Balance:

Twenty-four hour food and water intake and feces output were measured weekly throughout the ten week dietary regimen. Food, feces and water iron contents were assayed using the methods described below for measuring total tissue iron content. Food and feces were ashed directly (as described in *5.*). It was not necessary to prepare homogenates. Iron turnover was determined by subtracting total iron output (feces) from total iron input (food and water) and normalized to animal body weight (i.e.iron turnover ug /g body weight).

4. Tissue Collection:

Following ten weeks of dietary treatment, animals (then 17 or 20 weeks of age) were heparinized (5.5mg/kg body weight, i.v) and euthanized via pentobarbital injection (5mg/kg, i.p.) for tissue iron analysis. Likewise, pregnant animals were euthanized immediately following parturition which coincided with ten weeks of control or low iron dietary treatment. Animal carcasses were blanched by bi-directional perfusion through the aortic arch, at a fixed flow rate (5ml/min) with 0.9% saline until it was thoroughly blanched. Liver and kidney were then harvested and immediately frozen in liquid

nitrogen for storage at -80^{o}C. Prior to freezing, kidneys were cut separating medulla and cortex for site specific kidney iron analysis. Total iron, non-heme iron, and heme iron were measured in liver, kidney cortex and kidney medulla of all animals.

5. Measurement of Total Iron:

Liver, kidney cortex and kidney medulla tissue total iron levels were analyzed by flame atomic absorption spectroscopy (FAAS) using an adaptation of the methods described by Gambling and McArdle[159]. In preparation for FAAS, frozen tissue samples were dried at 65^{o}C for 24 hours. Dried samples were then weighed and ashed at 500^{o}C for 12-16 hours in a muffle furnace (Isotemp, *Fisher Scientific, ON ,Canada*). Ashed samples were digested by heating to a slow boil in 1mL concentrated hydrochloric acid (10M, *Fisher Scientific, ON Canada*) and 3mL concentrated nitric acid (6M, *Fisher Scientific, ON, Canada*) over 4-6 hours. One to two drops of hydrogen peroxide (4%w/v) were then added and samples were boiled until only 0.5mL liquid remained. Water was added to concentrated samples (15-20 mL) and absorbance readings were performed at 248.3nm using FAAS (*Spectra AA-10 Varian Spectrophotometer*). Standard curves for iron were prepared from commercially available standards (*Sigma-Aldrich, ON, Canada*). BSA 1577A and Tort-2 (*NIST, MD, USA*) were used as controls for tissue digestion and preparation procedures.

6. Preparation of Tissue Homogenates:

For tissue heme, and non-heme iron measurements, homogenates were prepared from thawed tissue samples (liver, kidney cortex and kidney medulla) in each case 8%

(w/v), tissue/ 20 mM phosphate buffer (20 mM KH_2PO_4, 135 mM KCl, and 0.10mM EDTA titrated to pH 7.4 at 4°C with M KOH). The phosphate buffer was prepared using filtered deionized water. Tissues samples were weighed and sectioned using a razor on a glass petri dish placed on ice. The appropriate volume of 20mM phosphate buffer was added to sectioned tissue in order to achieve 8% (w/v). Tissue mixtures were then homogenized, in a plastic container placed in an ice bath, at 20 000 rpm using a Tissue Tearor mechanical homogenizer (*BioSpec Products Inc., OK, USA*). Samples were then centrifuged for 15 minutes at 10 000 x g to remove any cellular debris. Samples were then frozen at -80^{o}C for storage until heme and non-heme iron measurements were performed.

7. Measurement of Heme Iron Content:

Heme content in the liver, kidney cortex and kidney medulla was determined using the fluorometric method of Morrison (1965) that depends on iron removal from non-fluorescent heme to produce the fluorescent porphyrin ring[187]. For each tissue sample, the stored, frozen aliquot was thawed and 25 μL was added to a test tube. Saturated oxalic acid (1.0 mL) was then added to the test tube, and the tube was heated in a convection oven at 100°C for 30 minutes. The heated sample was then removed from the oven, allowed to cool for ten minutes at room temperature and a 200 μL aliquot of the sample was placed in a well of a 96-microwell plate (Corning Life Sciences Inc., *Acton, MA*). Fluorescence was quantified using a Biotek® plate-reader with excitation settings at 360 nM and emission settings at 595 nM. Heme content was quantified by interpolation on a standard curve generated with 1-10 μg heme/mL where heme was

carried as methemealbumin (*Sigma-Aldrich, ON, Canada*). A set of controls were prepared in an identical manner except that they were not heated to 100°C, but instead were maintained at room temperature. These controls were used to account for any endogenous porphyrins found in the sample. Total heme content per sample was determined by subtracting the heme content found in the heated sample from that in the unheated sample. Heme content was expressed as mg heme/g tissue. Tissue heme iron was determined by performing a mathematical conversion based on the molecular weight of heme (616.5 kDa) and iron (55.8 kDa), respectively[210], given the assumption that each molecule of heme carried one molecule of iron. The accuracy of these calculations was verified by measuring total iron content of the heme standards using FAAS as described previously.

8. Tissue Non-Heme Iron Measurement:

Tissue non-heme iron was analyzed using a spectrophotometric assay using sodium 3-(2-pyridyl)-5,6-bis(4-phenylsulfonate)1,2,4-triazine (ferrozine) as previously described for the microanalysis of tissue non-heme iron[186]. Ferrozine is an extremely sensitive chromogen and thus has been shown to be very accurate for the measurement of non-heme iron in small tissue homogenate volumes[186]. Protein precipitation solution was prepared by combining equal volumes of 1N hydrochloric acid (*Fisher Scientific, ON, Canada*) and 10% tricarboxylic acid (*Fisher Scientific, ON, Canada*). Equal volume of tissue homogenate (300-500uL) and protein precipitation solution were combined in a glass test tube and heated at 95^{o}C for one hour in a convection oven. Heating has been indicated to facilitate the release of iron from complexes within the tissue[186]. Tissue

homogenate/protein precipitation mixtures were weighed before and after heating in order to account for evaporation. Heated samples were cooled for two minutes, vortex mixed, and subsequently centrifuged at 14 000 x g for ten minutes. In order to detect iron and in addition account for background spectrophotometric contamination, ferrozine chromogen solutions and ferrozine reagent blanks were prepared. Ferrozine chromogen reagent was prepared by combining the following reagents in the concentrations indicated: 0.508 mMol/ L ferrozine, 1.5 Mol/L sodium acetate and 1.5% (v/v) thioglycolic acid and filtered deionized water. Likewise, ferrozine reagent blank was prepared by combining 1.5 Mol/L sodium acetate and 1.5% (v/v) thioglycolic acid. All reagents for both ferrozine chromogen and ferrozine reagent blank solutions were obtained from *Fisher Scientific, ON, Canada.* Equal volumes (50uL) of supernatant from centrifuged tissue homogenate/protein precipitation solution were combined with ferrozine chromogen reagent and ferrozine reagent blank respectively in separate wells of a 96 well plate (Costar, *Fisher Scientific, ON Canada*). Reagents were allowed to sit for 30 min at room temperature and absorbance was measured at 562 nm (*Beckman Instruments, Fullerton, CA*). Non-heme iron standard curves (all relationships were found to be linear) were prepared daily from iron atomic absorption spectrometry standard stock solution (1mg iron/mL, 2%HNO_3 , *Fisher Scientific, ON, Canada*) by diluting with filtered deionized water to 0, 2, 4, 6, 8 and 10 ug/mL, respectively. Absorbance of zero iron standards (reagent blanks) ranged between 0.010 and 0.02 AU when read against high-purity water (instrument blank). Reagents and high-purity water used to prepare protein precipitation solution and chromogen solution were chosen to yield absorbance blanks of no more than 0.025 AU.

9. Statistical Analysis:

All hematocrit, iron turn-over, tissue total, heme and non-heme iron measurements were presented as mean ± SD. Animals were grouped according to age, sex, treatment and pregnancy and within group comparison were performed in order to assess the impact of age, sex, iron treatment and pregnancy on tissue iron levels using unpaired Student's t-tests. For example seven week old control males were compared to ten week old control males in order to determine whether tissue iron differed with age. This was repeated for control females, ID males, ID females and between age and treatment matched male and female groups (i.e. Seven week control female vs. seven week control male). The relationship between hematocrit and body weight was assessed by performing a linear regression analysis. The interaction of age, sex and pregnancy with respect to iron treatment was assessed using a two way analysis of variance followed by a Newman- Keuls post-hoc test given statistical significance. The 7-17 and 10-20 week age group data was pooled for non-heme and heme tissue level comparisons between pregnant and non-pregnant animals. All statistical analysis was performed using the program *Graph Pad Prism version 4.03*.

2.3 Results

1. Growth Comparison:

Males grew significantly faster than females in both age groups regardless of dietary iron treatment (Figure 2.1). There were no differences in growth observed between control and ID males of either age group. Similarly, the growth rates of $ID_{7\text{-}17}$ females was not statistically greater than that of the corresponding control females. However, $ID_{10\text{-}20}$ females grew significantly faster than $C_{10\text{-}20}$ females following three weeks of low iron treatment (Figure 2.1). This difference was statistically significant after three to four weeks of restricted iron intake and at ten weeks, the iron restricted animals were 15% heavier than their respective controls.

2. Effect of Iron Deficiency on Hematocrit:

Hematocrit decreased significantly in both males and females following ten weeks of dietary iron restriction, whereas a modest increase was observed in animals fed control diet (Figure 2.2). In the younger ID males ($ID_{7\text{-}17}$), the effects of dietary iron restriction were obvious in the first week as there was significant lowering of hematocrit from controls. In the older, ID males ($ID_{10\text{-}20}$) hematocrit lowering was not evident until week three, at which point there was a steady decline in hematocrit throughout the remainder of the dietary regimen. In the younger female group ($ID_{7\text{-}17}$), hematocrit decreased significantly from controls at three weeks, while in the older females, $ID_{10\text{-}20}$, persistent lowering took five weeks. Similarly, the magnitude of the hematocrit lowering was approximately twice as large in younger females compared with their older counterparts.

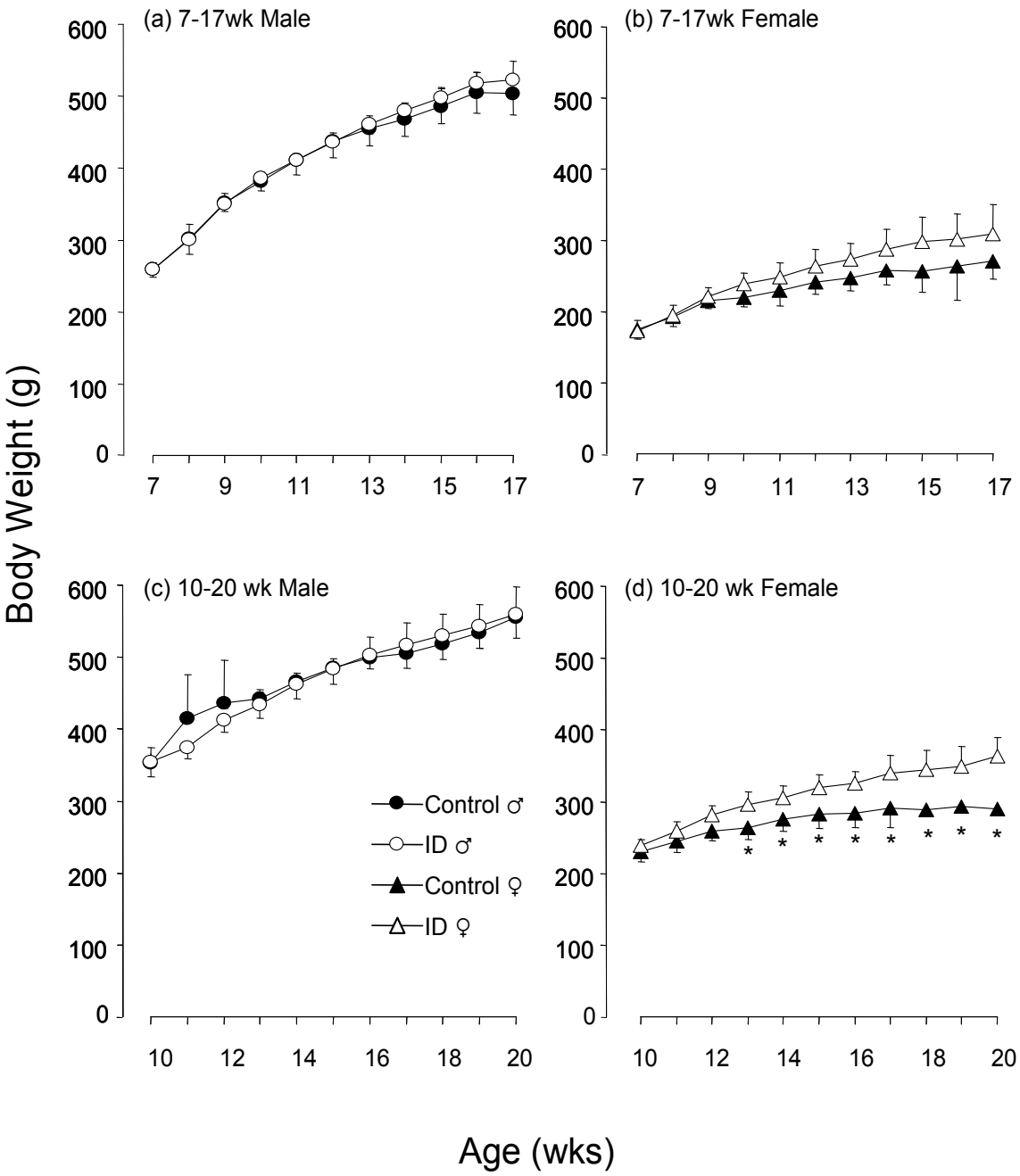

Figure 2.1 Growth profile of male and female rats fed control (270ppm) or low iron (3ppm) diet from age 7-17 weeks and 10-20 weeks. Solid symbols denote control fed and open symbols denote rats fed low iron diet.(a) 7-17 week males, (b) 7-17wk females, (c) 10-20wk males, (d)10-20 wk females. Data are presented as mean ± SD per weekly time point. *$p<0.05$ from control.

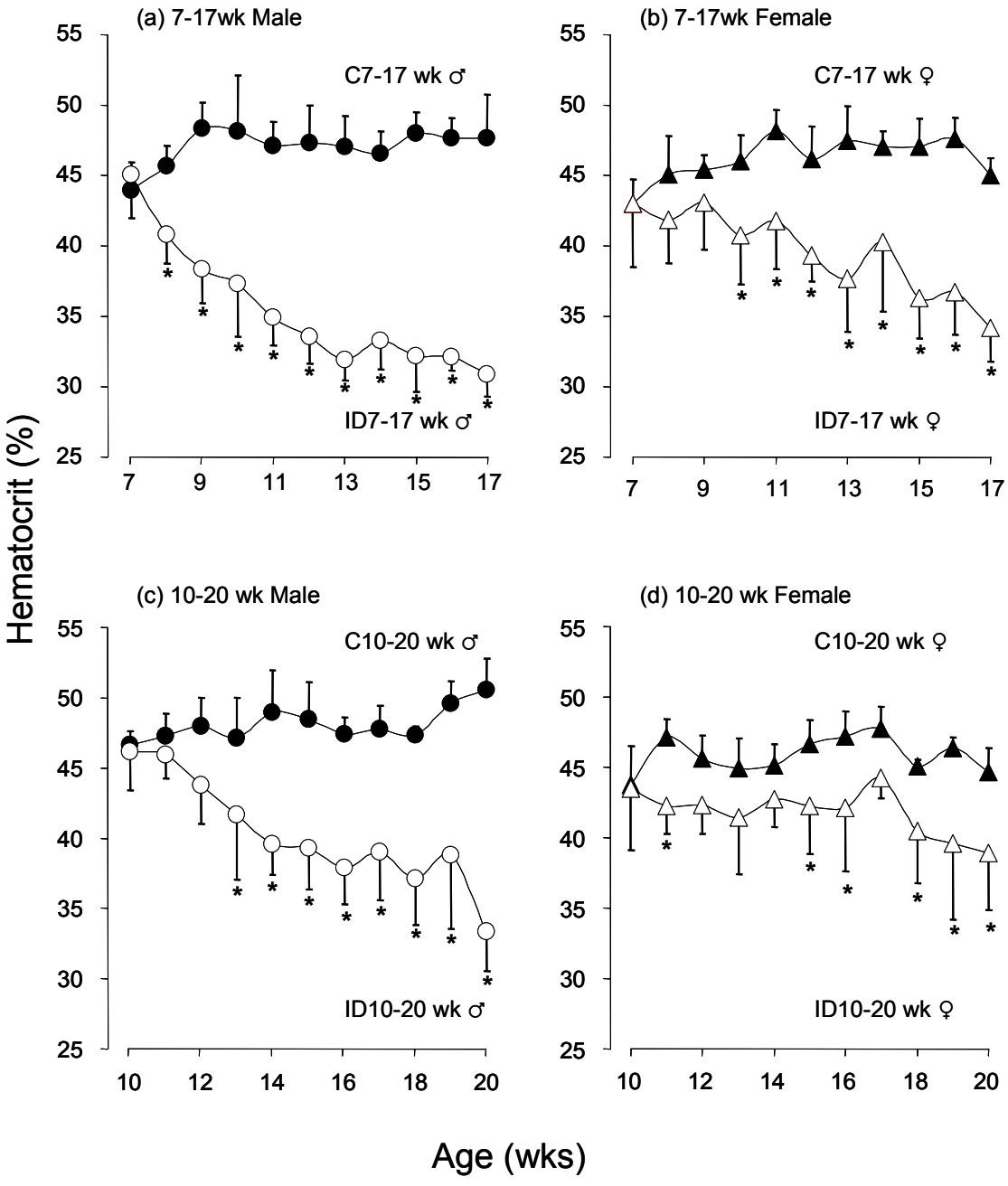

Figure 2.2 Hematocrit profile for male and female rats fed control (270ppm) or low iron (3ppm) diet from age 7-17 weeks and 10-20 weeks. Solid symbols denote control fed and open symbols denote low iron fed rats. a) 7-17 week male, b) 7-17wk female, c) 10-20wk male and d) 10-20 wk female data are presented as mean ± SD at each time point. *p<0.01 versus control.

Normalizing hematocrit change during the ten week dietary period by dividing by change in body weight during this time, eliminated the differences between 7-17 and 10-20 week age groups within each gender, but not between genders. Comparison of hematocrit responses over a continuum of changes in body weight indicated that hematocrit lowering in response to ID in males was directly related to change in body weight in males whereas in females it was not (male R^2=0.693, $p<0.01$; female R^2=0.0763,NS) (Figure 2.3).

3. Iron Balance:

Absolute iron intake was greater in males than females regardless of age, as male food intake averaged 7.02 ± 1.0 mg per day while females averaged 5.1 ± 0.6 mg per day. However, normalizing for body weight indicated that iron intake was greatest in control females. Initially, food intake in the ID group was lower than controls. At this time animals fed low iron diet had markedly lower fecal iron output than controls. Both ID_{7-17} and ID_{10-20} males had similar iron input and output responses showing negative iron balance from 8-9 and 11-12 weeks of age respectively (Figure 2.4). Likewise ID_{7-17} and ID_{10-20} females had negative iron balance at 10 and 12-13 weeks respectively (Figure 2.4). Although negative iron balance was observed in all age and gender groups receiving dietary iron restriction at some point during the five week period of iron turnover monitoring period, average iron balance did not differ significantly as a result of gender or within treatment matched groups (Figure 2.4).

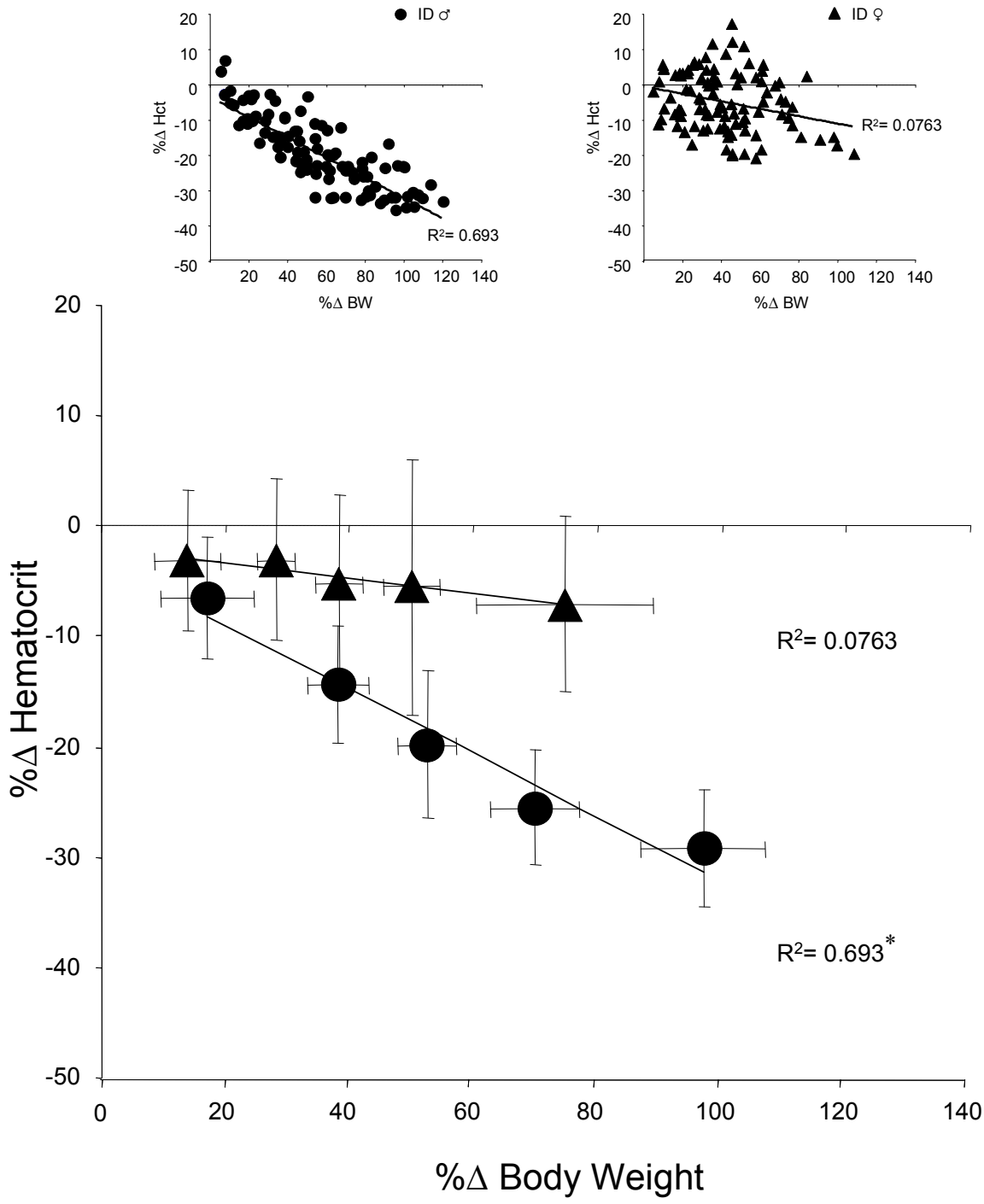

Figure 2.3 The relationship between % change(Δ) in hematocrit and body weight in males and females. Hematocrit lowering in males (●) but not females(▲) varies linearly with body weight. Data are presented as binned mean ± SD (20 data points per bin from scatter plots inset above). *p<0.01 for the linear regression analysis.

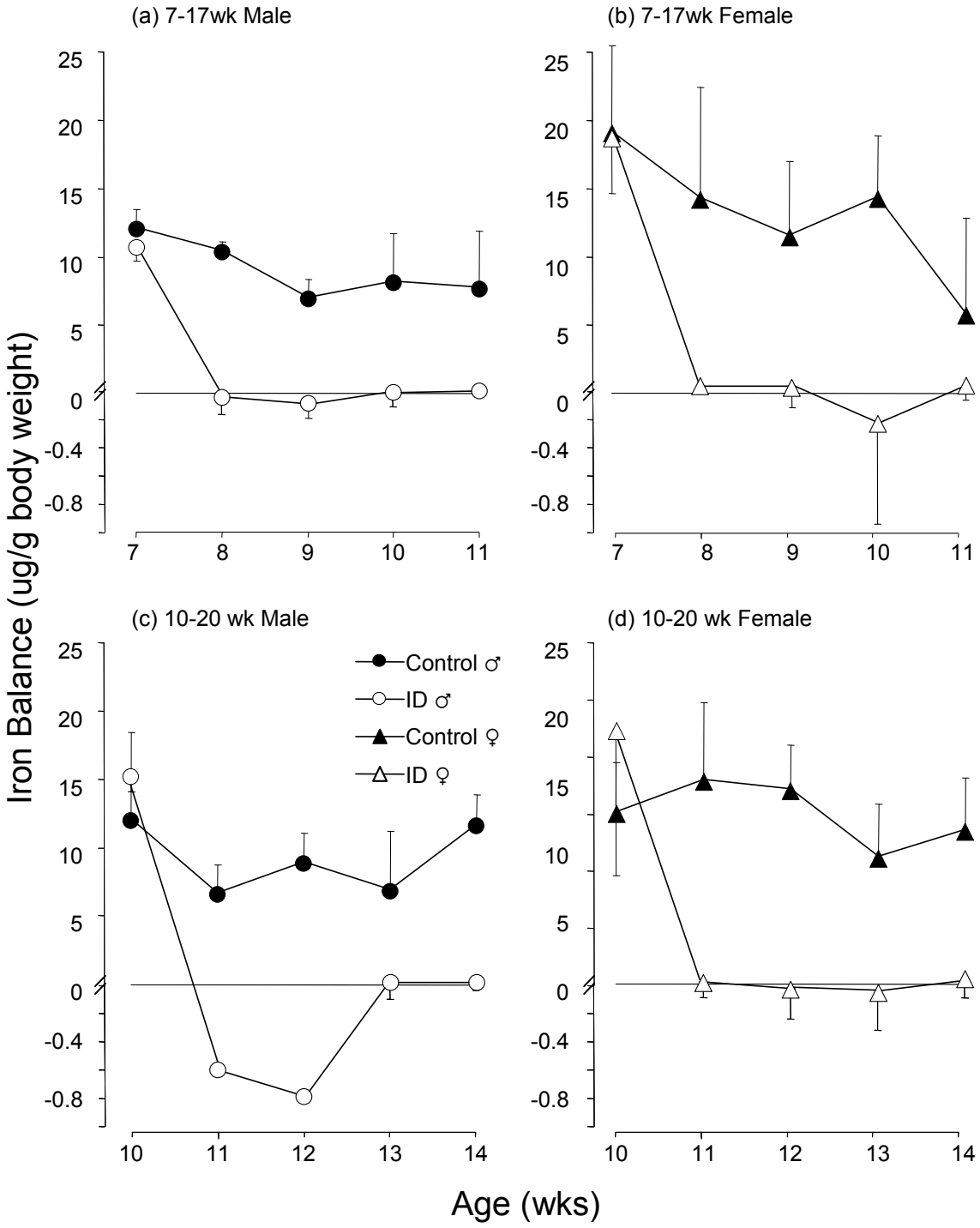

Figure 2.4 Iron balance in 7-17 week and 10-20 week males (a,c) and females (b,d). Iron balance was measured as the difference between iron input (ug/g body weight) – iron output (ug/g body weight). Data are presented as mean ± SD.

4. Tissue Total Iron:

Dietary iron restriction resulted in a dramatic depletion of total iron in the liver, and kidney cortex but little or no change in the kidney medulla (Figure 2.5). The most marked response to dietary iron restriction was observed in the liver where total iron decreased an average 88% ($p<0.001$). Significant lowering of tissue total iron also occurred in the kidney cortex where total iron decreased an average 72% across groups ($p<0.001$). Although total iron was lowered an average 38% in the kidney medulla, the changes that occurred in response to dietary iron restriction were varied. Unlike the liver and kidney cortex, kidney medulla total iron did not decrease significantly from control as a result of dietary iron restriction. In fact, in ID_{7-17} females there was an increase in kidney medulla total iron levels in comparison to age matched female controls ($p<0.001$). Comparison of animals fed control iron diet indicated greater liver and kidney cortex total iron levels in control females (C_{7-17} and C_{10-20}) compared to males ($p<0.001$). Total iron did not change with age in control males although C_{10-20} females had greater liver, kidney cortex and kidney medulla total iron than C_{7-17} females (Figure 2.5).

5. Tissue Non-Heme Iron Responses to ID:

Changes in non-heme iron paralleled changes in total iron in all organs examined. Liver and kidney cortex non-heme iron were significantly lowered in both age groups of male and female ID animals in comparison to controls ($p<0.001$). In the kidney medulla, non-heme iron was increased in ID_{7-17} ($p<0.001$) and was not changed in comparison to controls in all other groups (Figure 2.6).

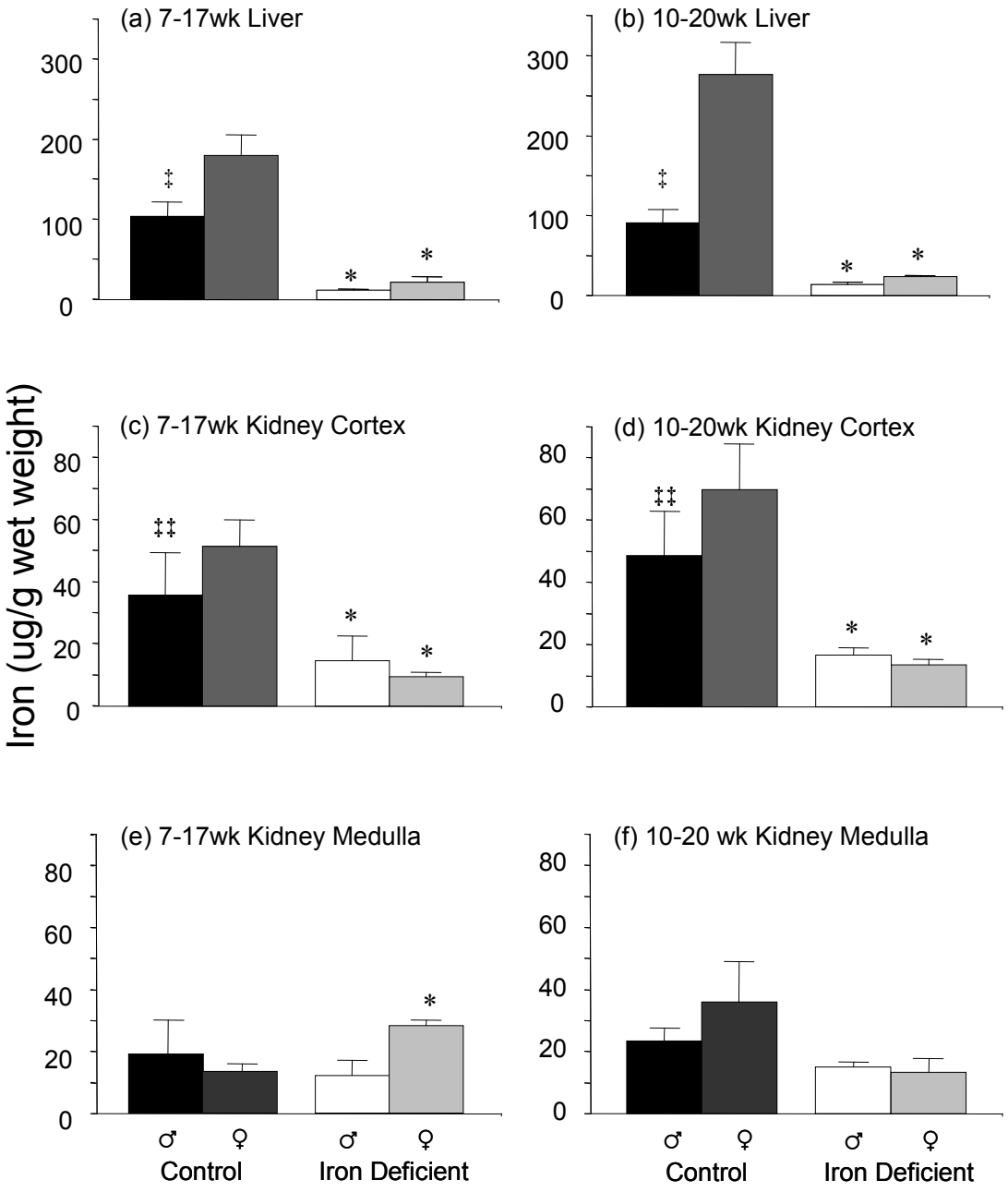

Figure 2.5 Total iron in liver (a,b), kidney cortex(c,d) and kidney medulla(e,f), of animals fed control iron (270ppm) or low iron (3ppm) diet from 7-17 or 10-20 weeks of age. Data are presented as mean ± SD. *p<0.001 versus controls. ‡-p<0.001 between male and female controls. ‡‡-p<0.05 between male and female controls.

6. Tissue Heme Iron Responses to ID:

Heme iron levels were greater in the liver than kidney cortex and kidney medulla however, no significant differences in heme iron were observed between kidney cortex and kidney medulla (Figure 2.6). Heme iron did not change as a result of dietary iron restriction in all tissue examined. Likewise, no differences were observed between 7-17 and 10-20 week age groups or between males and females.

7. Pregnancy Specific Iron Changes:

Pregnancy lowered hematocrit in both control and ID animals to a similar extent (Control: 44.8 ± 1.4%→37.5 ± 2.2%; ID: 36.6 ± 4.0% →28.6 ± 4.4%, $p<0.01$). However, there were significant differences in the tissue iron changes in control animals in response to pregnancy in comparison to iron restricted animals. In the liver, pregnancy decreased non-heme iron by 52 % ($p<0.05$) in both $C_{7\text{-}17}$ and $C_{10\text{-}20}$ animals but had no effect on $ID_{7\text{-}17}$ and $ID_{10\text{-}20}$ animals (Figure 2.7). Heme iron did not change as a result of pregnancy. Tissue iron changes as a result of pregnancy appear to be localized to the liver as there were no significant changes in the kidney cortex or medulla (Figure 2.7).

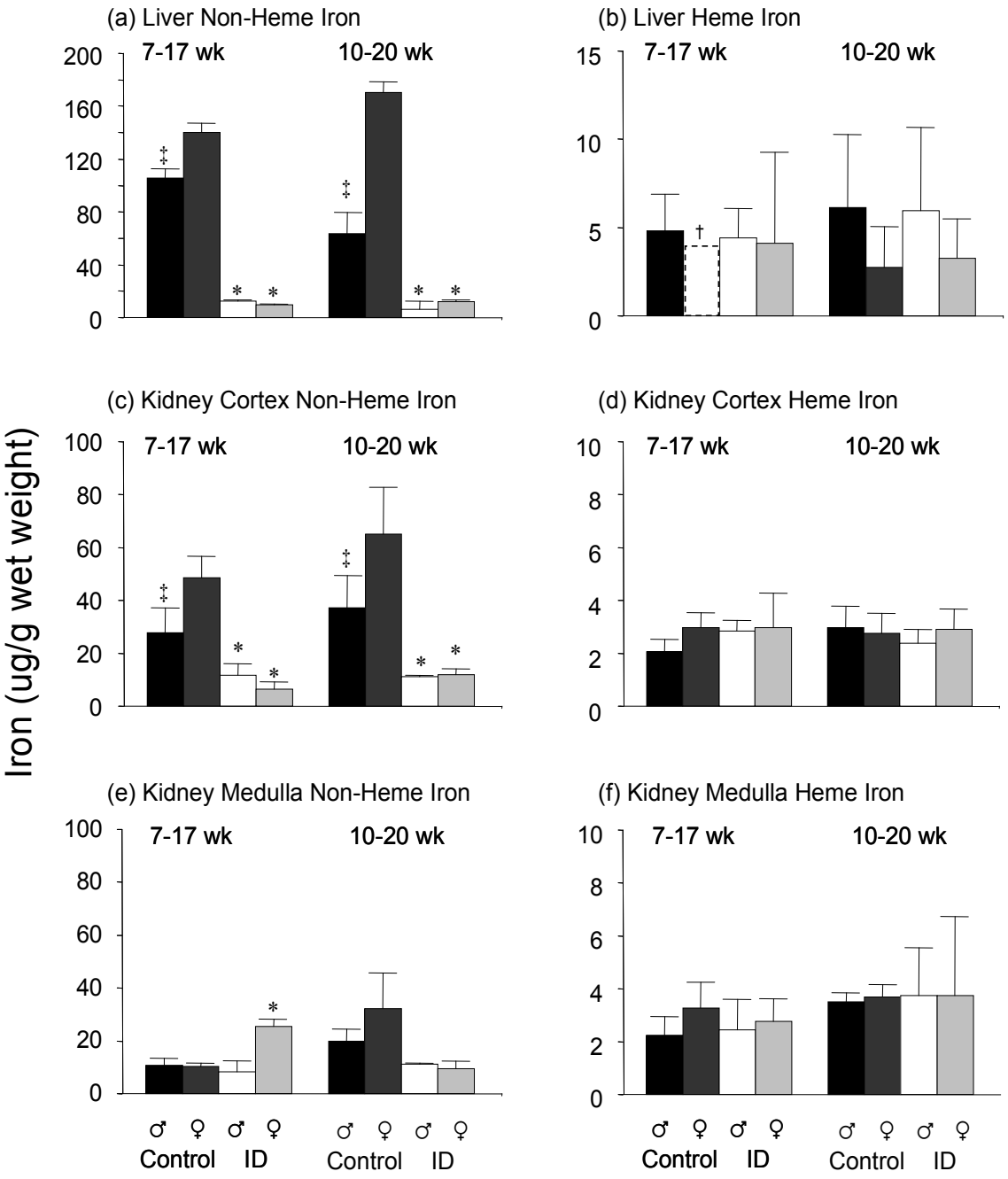

Figure 2.6 Non-heme (left column) and heme iron (right column) in liver, kidney cortex and kidney medulla for male and females fed control and iron deficient diet from 7-17 and 10-20 weeks of age. Non-heme iron follows similar trend to total iron and heme iron is spared with iron deficiency. †- sample was lost. Data are presented as mean ± SD. *$p<0.001$ versus controls.‡-$p<0.01$ between male and female controls.

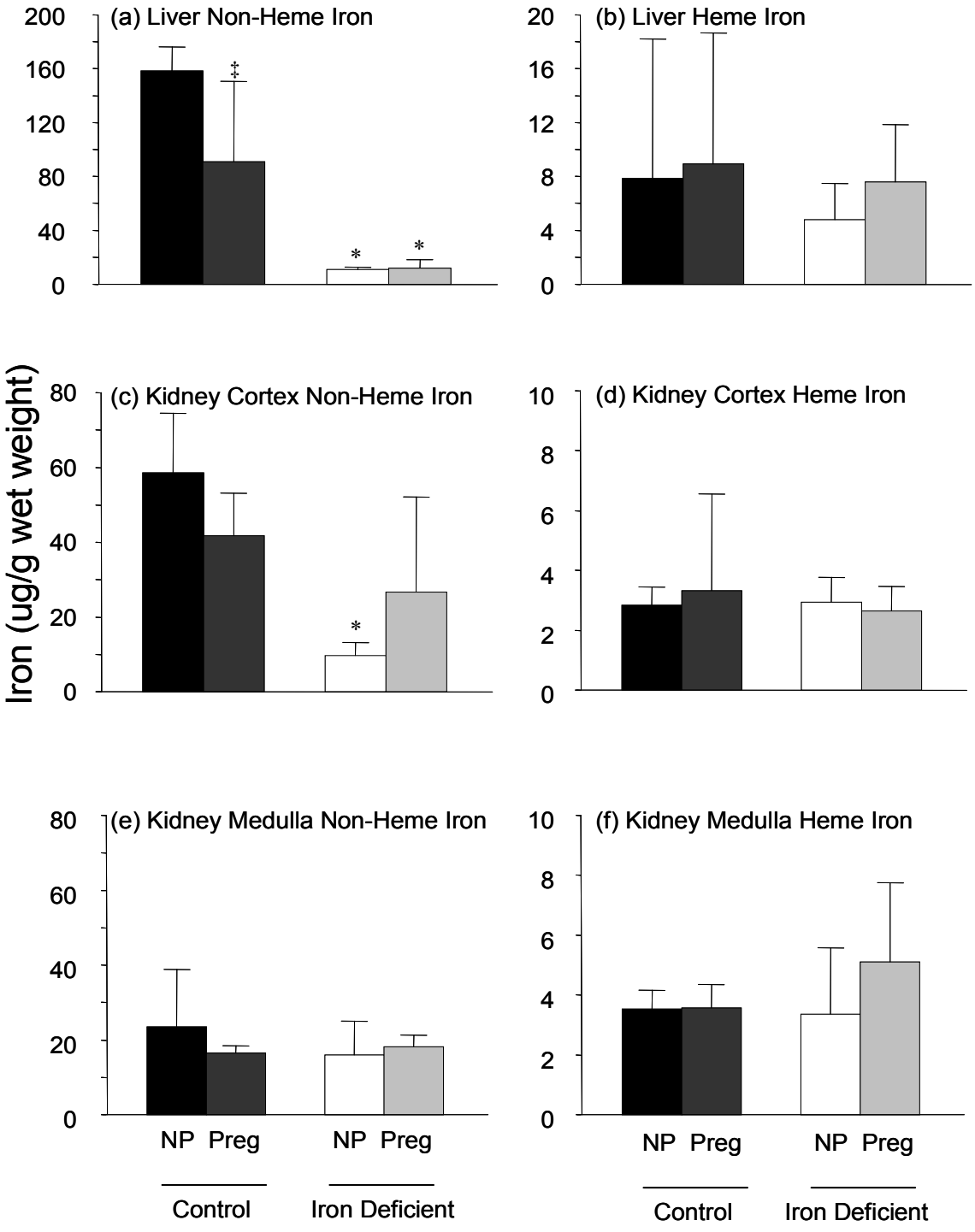

Figure 2.7 Liver, kidney cortex and kidney medulla non-heme and heme iron in non-pregnant (NP) and pregnant (Preg) control and ID females. Data is pooled for 7-17 and 10-20 wk groups. No significant change in non-heme iron was observed in ID groups in response to pregnancy, however, in control groups pregnancy caused a significant lowering of liver non-heme iron. Heme iron did not change. Data are presented as mean ± SD. *$p<0.001$ versus controls. ‡-$p<0.05$ control pregnant versus non-pregnant.

2.4 Discussion

The major findings of this study were that in response to prolonged dietary iron restriction (i) there was a greater negative impact on hematocrit in males than females, (ii) total and non-heme iron decreased to a greater extent in the liver than the kidney, (iii) depletion of non-heme iron stores was greater in the renal cortex than in the renal medulla and (iv) heme iron levels were completely spared in all treatment groups.

Collectively, the present findings confirm and extend observations reported from previous studies on ID in rats. Our findings confirm the results of previous studies which indicate that male rats are more susceptible to ID providing further evidence for these differences. In early studies by Otis and Smith[195,196], it was shown that female rats absorb dietary iron more efficiently than male rats. However, no evidence was provided to confirm the differences that they observed. In this study, following ten weeks of dietary iron restriction tissue iron levels were lowered to a similar extent in both males and females, however there was a smaller reduction in hematocrit in females. Similarly control females had greater liver and kidney cortex total and non-heme iron than control males and consumed more iron relative to their increase in body weight than males. Consistent with the findings of previous studies, these results confirm that females accumulate iron more readily than males[184,195-197]. However, it has been well established that males grow faster than females, such that the greater susceptibility to dietary iron restriction in males can be attributed, at least in part, to the greater iron requirement associated with rapid growth[24,210]. Although growth differences likely play role in the difference in susceptibility to ID observed between males and females, we have shown that while male hematocrit varies predictably with changes in body weight, female

hematocrit does not. This further emphasizes the fact that the differences between males and females in response to dietary ID might not be explained by differences in growth rate alone, but might also include other factors such as the ability to absorb dietary iron[184]. In support of this argument we found that females consumed more iron in their diet with respect to their body weight than males. However, this might also reflect the greater rate of growth in males, suggesting that because males grow much faster their relative (per gram body weight) intake of iron is significantly diminished in comparison to females. In addition to the difference in susceptibility to ID between males and females, the results of the present study indicate that there is a differential impact of ID on total and non-heme iron in the liver, kidney cortex and kidney medulla

Previous studies show that there are changes in the priority of iron supply to specific organs in response to dietary iron restriction[207-209]. However, the relative impact of ID on specific regions of the kidney has not previously been characterized. The disproportionate non-heme iron changes in response to ID in the tissues examined may in part be explained by differences in tissue specific iron requirements. For example, the liver is known as a major storage and distribution site of iron in the body[206,211] and contains much larger storage iron pools than other organs such as the kidney. In control animals we showed that the renal cortex and renal medulla have 1/3 and 1/5 the tissue non-heme iron levels of the liver, respectively. However, in response to dietary iron restriction tissue non-heme iron decreased such that levels were comparable in the liver, kidney cortex and kidney medulla. While non-heme iron decreased significantly in the liver and renal cortex as a result of ID, in the renal medulla levels were similar in control

and ID animals. These observations can be interpreted to mean that the liver and kidney cortex have non-heme iron reserves that can be mobilized in response to lowered tissue iron supply. Likewise, this would indicate that the renal medulla does not have such a reserve and therefore must maintain its iron levels in response to ID. Therefore, the relative sparing of non-heme iron in the renal medulla in response to ID anemia might occur because the kidney medulla cannot lose non-heme iron without causing functional tissue damage. Alternatively, iron supply to the kidney medulla might be maintained at the expense of iron supply from other areas of the body (i.e. kidney cortex or liver)[30]. The marked lowering of tissue non-heme iron in liver and kidney cortex is consistent with previous studies indicating that ID has the greatest impact on iron-sulfur (Fe-S) or non-heme iron dependent proteins as these tissues had greater non-heme iron levels than the kidney medulla[193,198,199,202]. However, given the limited change in kidney medulla non-heme iron levels, it is possible that there is preferential sparing of non-heme iron proteins in the kidney medulla. Although the mechanism for this preferential sparing is not known, given the importance of the renal medulla in sodium and water balance, and in turn, its role in the regulation of the long term level of arterial pressure, it possible that iron may play an important factor in the functional roles of the renal medulla [9,201].

In contrast, despite significant depletion of tissue total and non-heme iron as well as circulating iron, heme iron was completely spared in all tissues examined. Given the vital role of hemoproteins in many different organs and tissues from vascular control to energy supply, it is not surprising that tissue heme iron is preferentially spared in response to ID[24,40,229,230,269-273]. Although the sparing of heme iron in this study was

unequivocal, varied changes in the pattern of expression of specific hemoproteins in response to ID have been reported[24,189,193,198, 200,202]. The major focus regarding the impact of ID on hemoproteins has been on expression levels of specific hemoproteins such as cytochrome c (cyt c) in the heart skeletal muscle and brain[24,193,198,200,202]. However, few studies have examined hemoprotein expression in the kidney in response to ID. The studies that have been done on the kidney indicate that ID upregulates both endothelial and inducible nitric oxide synthase (NOS) and in addition alters the levels of cyt c and catalase[188,204]. Although these studies have indicated the effect of ID on expression of specific hemoproteins in the kidney, they did not examine the impact of ID on tissue heme supply or total tissue heme levels.

Previous studies in our lab have demonstrated that there is significant *de novo* synthesis of tissue heme, in particular in the vasculature[212]. Given the importance of iron in the process of heme synthesis, it is therefore reasonable to anticipate that altering iron supply in a highly vascularized organ such as the kidney may impact on tissue heme content[40]. In fact, it has been proposed recently that tissue heme deficiency may cause cellular damage in cases where ID has not progressed to the point of ID anemia[202]. Our findings indicate that dietary ID does not impose tissue heme deficiency in the liver or kidney prior to signs of anemia as tissue heme levels did not decrease despite pronounced anemia[135,191].

Taken together, our findings demonstrate that there is preferential tissue iron allocation within the kidney in response to extended dietary iron restriction. Despite the

development of ID in our animals there was limited change in kidney medulla iron. These findings are consistent with similar responses to iron restriction reported in the brain[191] in which iron is spared in isolated regions. In this study, the sparing of heme iron in the liver, kidney cortex and kidney medulla, in both males and females, regardless of age, as well as following pregnancy in females, despite the development of ID anemia, further emphasizes the importance of maintaining tissue heme supply and hemoprotein function at the expense of non-heme iron. Collectively these findings agree with the concept of micronutrient distribution by triage proposed by Ames (2006), who suggests that the body preferentially protects organ and tissue functions which are most vital in response to micronutrient shortage[213]. By analyzing global tissue iron changes in response to dietary iron restriction, we have shown that ID anemia has important implications in the kidney and in particular in the kidney medulla. Further studies are required to characterize the specific hemoprotein changes which occur in the various regions of the kidney in response to ID anemia in order to determine whether these changes are linked mechanistically to alterations in renal function. These studies may provide further insight into the role of iron, in particular in the kidney medulla, in the mechanisms responsible for controlling renal sodium excretion and fluid balance.

2.5 Perspectives

Our findings demonstrate that there is preferential protection of tissue heme iron over non-heme iron in non-erythroid tissues as part of the response to ID in the rat. Similarly, we have shown that ID has a greater impact on non-heme iron levels in the kidney cortex as compared to the kidney medulla. Collectively, these findings draw

attention to the fact that the regulation of tissue iron in the kidney deserves further study. In particular, the impact of ID on renal hemodynamics and fluid and sodium balance should be further characterized.

Chapter 3: The Impact of Iron Deficiency on Hemodynamics and Renal Function

3.1 Introduction

Maternal iron deficiency (ID) during gestation has been shown to cause the development of hypertension in the offspring of rats as early as six weeks of age[116,153-156]. Although this has been demonstrated in numerous studies, the mechanisms responsible for the development of hypertension in response to ID remain to be elucidated. Hypertension has often been associated with alterations that occur within the kidney[218-220]. In fact, in all cases of hypertension it has been shown that there is a shifting or blunting of the pressure-natriuresis relationship (pressure induced sodium excretion within the kidney), such that a greater pressure is required to achieve the same level of sodium excretion[221-223]. Likewise, alterations in the mechanisms responsible for sodium handling within the kidney have been shown to cause salt-sensitivity of blood pressure[224-226]. There is a strong correlation between salt sensitivity and hypertension[115,227,228]. It has been shown that approximately 51% of those who have salt sensitivity eventually develop hypertension[91]. We have shown recently, that in addition to programming of hypertension, ID during gestation causes salt sensitivity of blood pressure (Unpublished data, Stephane Bourque). Despite the established connection between hypertension, salt-sensitivity, and ID, it remains to be determined how these factors interrelate.

Given the importance of the kidney in the long term regulation of pressure and changes within the kidney associated with salt sensitivity, the kidney provides a logical starting point for determining the relationship between ID and hypertension[9,243-245]. It has also been shown previously that with both hypertension and salt sensitivity there are alterations in heme iron dependent nitric oxide (NO) production and cytochrome P450

(CYP450) mediated metabolism of arachadonic acid (AA) within the kidney [93,95,102,112-114,229,230]. In previous studies, salt sensitive blood pressure has been associated with alterations of NO production in the kidney[95,102.241,243]. Similarly, disruption of NO production by administering nitric oxide synthase (NOS) inhibitors such as L-NAME has been well established as a robust means of inducing hypertension in the rat[112-114]. Likewise, impairments in CYP450 expression have been shown to alter sodium channel regulation and subsequently pressure-natriuresis in the kidney[93,229,230,244]. Given the association between NO, CYP 450 mediated AA metabolism and blood pressure regulation, in addition to the fact that heme iron is an integral part of both the NO production and CYP 450 mediated AA metabolism, it is possible that alterations in these systems may represent a point of association between ID, hypertension and salt sensitivity[115]. Similarly, it is reasonable to predict that if alterations in iron supply in response to ID alter either NO production or CYP 450 mediated AA metabolism, there will likely be associated blood pressure changes. Very few studies have characterized the impact of ID on the expression hemoprotein in the kidney, and to our knowledge only one study has examined the impact of ID on NOS[188,204].

In order to determine the connection between ID, hypertension and salt sensitivity, it is necessary not only to consider the systems that might be altered as a result of ID, but also how the time frame of ID exposure influences the impact of ID. That is, despite the association between gestational ID, hypertension and salt sensitivity, in order to characterize the mechanistic link between ID, potential tissue alterations and hypertension, it is necessary to determine the effects of ID following the gestational

period (i.e. connection between circulating and tissue iron changes and functional outcomes). This will further elucidate the association between the tissue iron alterations in response to ID and the development of hypertension. Chronic anemia has invariably been associated with reduced systemic resistance and increased cardiac output[47,214-217]. Likewise, hemodilution studies in animal models have been shown to cause similar circulatory alterations[231-233]. These effects seem paradoxical given the impact of ID during the developmental period[153-156]. However, altered renal function may be a link between these factors because chronic anemia has been associated with renal impairments[234-236]. It is not known whether chronic ID will alter renal function and subsequently if it will cause salt sensitive blood pressure, an indicator of renal function. Likewise, it is still not clear how the induction of salt sensitivity in the offspring of ID mothers is related to ID during the developmental period. In this regard, it remains to be established whether renal changes can be induced by postnatal ID and whether ID can limit the supply of iron to hemoproteins in the kidney associated with the development of salt-sensitive blood pressure [93,95,102,112-114,229,230]. Therefore, given the collective evidence, we hypothesized that ID would alter the supply of iron to key hemoproteins within the kidney, and would thus, render animal blood pressure sensitive to dietary salt manipulations.

The present study was designed to test whether ID, postnatally, would alter salt sensitivity. Animals were age and treatment matched to the previous study on tissue and circulating iron responses to ID (Chapter 2) in order to determine the impact of tissue iron changes on hemodynamics. In this study the impact of ID on renal function was

assessed indirectly by measuring hemodynamic responses to dietary salt manipulation[224-226]. To this end, animals were implanted with radio-telemetry blood pressure transducers at six weeks of age and given subsequent dietary salt challenges in conjunction with dietary iron restriction. Hemodynamic parameters were monitored continuously in response to dietary salt and iron manipulation.

3.2 Methods

1. Animals:

Sixteen adult male Wistar rats were obtained at five weeks of age (~175g body weight) from Charles River Canada (*Montreal, QC, Canada*). Rats were housed in individual cages at 22^{o}C to 24^{o}C and maintained under a 12-hour light/dark cycle. All animals received access to food and water *ad libitum* until eight weeks of age. Animals were cared for in accordance with the principles and guidelines of the Canadian Council on Animal Care. The experimental protocol was approved by the Queen's University Animal Care Committee.

2. Blood Pressure Acquisition:

Multiple blood pressure parameters were monitored continuously using a radio-telemetry data acquisition system (*Data Sciences International, St. Paul, Minnesota, USA*). Before implantation Model TA11PA-C40 radio-transducers (*Data Sciences International*) were verified to be within ± 4 mm Hg of zero. One week of acclimatization to the new facility was allowed before performing radio-transducer implantation surgeries[249]. At the time of surgery animals were approximately six weeks

of age, their weight ranging from 177.5→ 227g. Animals were anesthetized with ketamine/xylazine (70 mg. $kg.^{-1}$ by intraperitoneal injection) and isofluorane (dosed to effect by inhalation). A fluid filled telemetric catheter was introduced into the lower abdominal aorta, such that it was positioned approximately one centimeter below the left renal artery. The body of the transducer was sutured to the muscular layer of the abdominal wall to prevent device movement. All animals were allowed to recover for ten days before recording baseline hemodynamic parameters[249]. All rats were housed in individual cages, placed on Model RA1010 and RPC-1 receivers (*Data Sciences International*), which convert the radio-telemetric waveform into a digital signal to give online readings of hemodynamic parameters. This information was then transmitted via a BCM100 (*Data Sciences International*) consolidation matrix to a computer based Dataquest IV, version 2 acquisition system (*Data Sciences International*) located in an adjacent room.

3. Hemodynamic Measurements:

The hemodynamic parameters heart rate (HR), diastolic pressure (DBP), mean arterial pressure (MAP), systolic pressure (SBP), and pulse pressure (PP) as well as animal activity were measured at 150 Hz. Data samples were performed every four minutes for 15 seconds, in all animals, starting at approximately eight weeks of age. Twenty four hour average, 12-hour night average and 12-hour day average were calculated using the HemoDynamic statistics spreadsheet produced by Dr. Bruce Van Vliet (*Memorial University, Newfoundland, 2006*) which automatically filtered blood pressure parameters falling outside the threshold 50-200mmHg[252,254]. Hemodynamic

parameters were measured continuously given the above collection protocol from 7.5-20 weeks of age.

4. Dietary Salt Manipulations:

All animals received control iron diet (Formula AIN-93G, Iron content- 225ppm ferric citrate, Salt content-1108ppm sodium chloride, *Research Diets Inc. New Brunswick, NJ, USA*), until eight weeks of age. All animals received three dietary salt challenges at 8, 13, and 17 weeks of age respectively. Each dietary salt challenge lasted a total of 20 days, during which hemodynamics and daily consumption were measured. Each dietary salt challenge consisted of four parts: i) five day pre-challenge control period (Pre-LS) animals followed by ii) low salt (LS) diet for five days, followed by iii) high salt (HS) diet for five days, followed by iv) control salt diet for five days (NS). All diets regardless of salt and iron content were variations of the AIN-93G formula from *Research Diets Inc.*. The LS diet consisted of sodium chloride (NaCl) content 108.5ppm while HS diet consisted of control chow, NaCl content 1108ppm in addition to 1% NaCl (w/v) replacing the animal's regular drinking water. 1% NaCl drinking water was replaced with tap water following the five day HS diet in order to return animals to normal salt levels. The initial salt challenge was started at eight weeks of age. This was performed as a control in order to allow direct association of potential hemodynamic changes observed during the salt challenges performed at 13 and 17 weeks of age respectively, to changes induced by dietary iron restriction.

5. Dietary Iron Manipulations:

Dietary iron manipulations were started at ten weeks of age, immediately following the initial dietary salt challenge. At this time half of the animals (8 of 16) were placed on a low iron diet (ID group:Formula AIN-93G, Iron content- 3 ppm ferric citrate, Salt content-1108ppm NaCl, *Research Diets Inc. New Brunswick New Jersey, USA, Dyets Inc. Pennsylvania, USA*) while the remaining animals stayed on control iron diet (Control group: Formula AIN-93G, Iron content- 225 ppm ferric citrate, Salt content-1108ppm NaCl, *Research Diets Inc. New Brunswick New Jersey, USA, Dyets Inc. Pennsylvania, USA*). All animals remained on their respective control or low iron diets from ten weeks of age onward, for the duration of the study. During the second and third dietary salt challenge periods, (~13-15 and ~18-20 weeks of age respectively), salt content was altered but iron content was kept at 3 ppm and 225 ppm for the low and control iron groups respectively. By matching diet and age to those tested previously, it was anticipated that circulating and tissue iron changes would be similar (10-20 week old males, Chapter 2).

6. Renal Function Assessments:

Food, water and animal weight were measured every 24 hours starting during each dietary salt challenge starting at the Pre-LS control period and continued daily until the end of the NS period. This corresponds to the 20 day salt challenge description mentioned above. Sodium intake in each animal during the i) Pre-LS, ii) LS, and iii) NS periods was determined by multiplying the appropriate dietary salt content (LS-108.5ppm, Pre LS & NS-1108.5ppm) by 24 h food consumption. During the HS period,

in addition to sodium intake from 24h food consumption, additional NaCl consumption from 24h 1% NaCl (w/v) drinking water intake was added. Subsequently, average NaCl intake was determined throughout the LS, HS, NS periods and expressed as a function of body weight. The log NaCl intake as a function of body weight, log (ug/g body weight) was then determined for each animal. This was performed for each of the three successive dietary salt challenges. In order to create renal function curves, MAP was also determined for the respective LS, HS and NS periods. MAP for each of the respective dietary salt manipulations was calculated by determining: i) the lowest 24h average MAP during the LS period, ii) the highest 24h average MAP during the HS period, and iii)the average 24h MAP for the 4^{th} and 5^{th} days during the NS period. Change in MAP (ΔMAP) was calculated for the LS→ HS transition period in each animal for each period of dietary salt challenge. This was determined by: [(average MAP from the HS period)- (average MAP LS period)] ,LS and HS values corresponding to the time period just described in i) and ii) in this section. Renal function curves were created for each animal by plotting the corresponding: a) log (ug/g body weight) sodium intake during each of the LS, HS, and NS periods as a function of the corresponding b) average MAP during the LS, HS and NS periods (Figure 3.7). Renal function curves were also created for both ID and control groups by plotting the corresponding: c) group average log (ug/g body weight) NaCl intake for each group during each of the LS, HS, and NS periods as a function of the corresponding d) average group MAP during each of the LS, HS and NS periods. The slope of the renal curves for each animal between the LS→HS, HS→NS, and LS→NS transitions was determined. Slope for the LS→HS transition was determined as follows:

(sodium intake HS - sodium intake LS) / (MAP HS – MAP LS). The same pattern was followed for HS→NS and LS→NS transitions, respectively.

7. Blood Collection:

Blood was collected via toe-clip immediately following each dietary salt challenge at 10, 15, and 20 weeks of age, respectively. Blood was collected from only 6 of 16 animals (three control, three low iron) at both 10 and 15 week of age immediately following dietary salt challenge #1 and #2, respectively. This was done in order to track the lowering of hematocrit and hemoglobin in response to dietary iron restriction. Following dietary salt challenge #3, blood was collected from *all animals* in order to assess the final impact of dietary iron restriction on circulating blood parameters. Blood samples were not taken from all animals at 10 and 15 week of age because the primary objective of this study was to determine the hemodynamic changes. Hemodynamics have been shown to be altered for up to five days following anesthesia[253]. By taking blood from only a select group we were able to track hematocrit and hemoglobin changes without compromising the characterization of the progressive impact of dietary iron restriction on hemodynamics. In order to ensure that the general anesthetics did not alter blood pressure measurements, ten days were allowed following blood collection before hemodynamic parameters were included in hemodynamic assessments. Nevertheless, the ten days allotted for blood pressure normalization following exposure to general anesthetic did not overlap with any of the dietary salt challenges during which hemodynamics was assessed. Animals were anaesthetized using isofluorane (dosed to effect by inhalation). The cuticle of each animal was clipped, and a small volume of

blood (<150uL) was collected in a micro-hematocrit capillary tubes (*Fisher Scientific, ON, Canada*) for both hematocrit and hemoglobin determinations. Two tubes were taken for hematocrit measurements and one tube for hemoglobin measurements.

8. Hematocrit Measurements:

The first and last micro-hematocrit tubes of blood taken from each animal were used for hematocrit measurements while hemoglobin measurements were performed using the second of three micro-hematocrit tubes. The tubes obtained for hematocrit measurements were sealed with crito-seal, a vinyl plastic putty (*Fisher Scientific, ON, Canada*), and centrifuged at 11 500 x g for 14 minutes to separate cells from plasma. Hematocrit was determined visually as the percentage of packed cell volume over total volume of blood collected, using a ruler to measure each component.

9. Hemoglobin Measurements:

The micro-hematocrit tube obtained for hemoglobin measurement was emptied manually into a 1.5ml pre-weighed eppendorf tube, containing 1000ul Drabkins reagent, a constituent mixture of sodium bicarbonate, potassium ferricyanide and potassium cyanide (*Sigma Aldrich, Oakville, ON*). The tubes containing Drabkins reagent were pre-weighed and blood was rapidly transferred from micro-hematocrit tube to Drabkins reagent preserving it in the form of hemoglobin cyanide for measurement of hemoglobin concentration at a later time[263-268]. Sample blood volume for hemoglobin determinations was also determined using this method by subtracting i) initial tube + Drabkins reagent weight from ii) post collection tube + Drabkins reagent + blood volume weight. The concentration of hemoglobin cyanide was later determined by measuring absorbance at

540nm (*Beckman Instruments, Fullerton, CA*) following appropriate dilution (as determined by blood volume collected). Standard curves were prepared over the concentration range [HbCN] 0.5 ug/ml to 8 ug/ml using HbCN standard, stock concentration 8 ug/ml (*Sigma Aldrich, Oakville, ON, Canada*).

10. Statistical Analysis:

Hemoglobin, hematocrit and animal weight are presented as mean ± SD. Hemodynamic parameters DBP, MAP, SBP and ΔMAP are presented as mean ± SEM. A Student's unpaired t-test was used to determine change in hematocrit and hemoglobin between control and ID animals. ΔMAP as a result of dietary iron restriction and salt manipulation were determined using a Student's unpaired t-test. Changes in hemodynamic parameters (DBP, MAP, SBP, PP, HR) between ID and control groups during each dietary salt challenge were assessed as group averages using a Student's unpaired t-test. Changes in hemodynamic parameters (DBP, MAP, SBP, PP, HR) to determine the impact of iron restriction over time (from the beginning of the study) were assessed using two-way analysis of variance, followed by one-way analysis of variance coupled with Newman-Keuls post-hoc test for a statistically significant F statistic. Changes in salt sensitivity of blood pressure (used as an index of renal function) between ID and control groups were assessed by comparing average slope of the renal function curves between NS→LS, HS→and LS→dietary transitions (Δlog sodium intake/ ΔMAP) for each dietary salt challenge using a student's unpaired t-test.

3.3 Results

1. Impact of Dietary Iron Restriction on Hemoglobin and Hematocrit:

Hematocrit decreased in a progressive manner throughout the ten weeks of dietary iron restriction, showing a significant lowering from controls at 20 weeks of age (Control 10wks: 42.5±2.7%→ Control 20wks: 44.8±1.4% ; ID 10wks: 42.2±2.3%→ ID 20wks: 38.6±4.3%). Hematocrit in the control group remained constant between 10 and 20 weeks of age. Although hemoglobin appeared to follow a similar trend to hematocrit, changes in hemoglobin observed over the same time frame did not differ between animals fed control (225ppm) and low (3ppm) iron diets (Figure3.1).

2. Hemodynamic Profiles:

There was a progressive increase in diastolic blood pressure (DBP), mean arterial pressure (MAP), systolic blood pressure (SBP) and pulse pressure (PP) over time in both ID and control groups ($p<0.0001$) (Figure 3.2a-d). There was also a progressive decrease in heart rate (HR) over time in both ID and control groups ($p<0.0001$)(Figure 3.2e). Although the increases in DBP, MAP, and SBP appeared to be greater in the control compared to ID group, there was no statistical difference between groups (Figure 3.2 a-c). Similarly the progressive increase in PP and progressive decrease in HR was not significantly different in control and ID groups (Figure 3.2d). There were no notable changes in activity throughout the study period in either control or ID groups (Figure 3.2f).

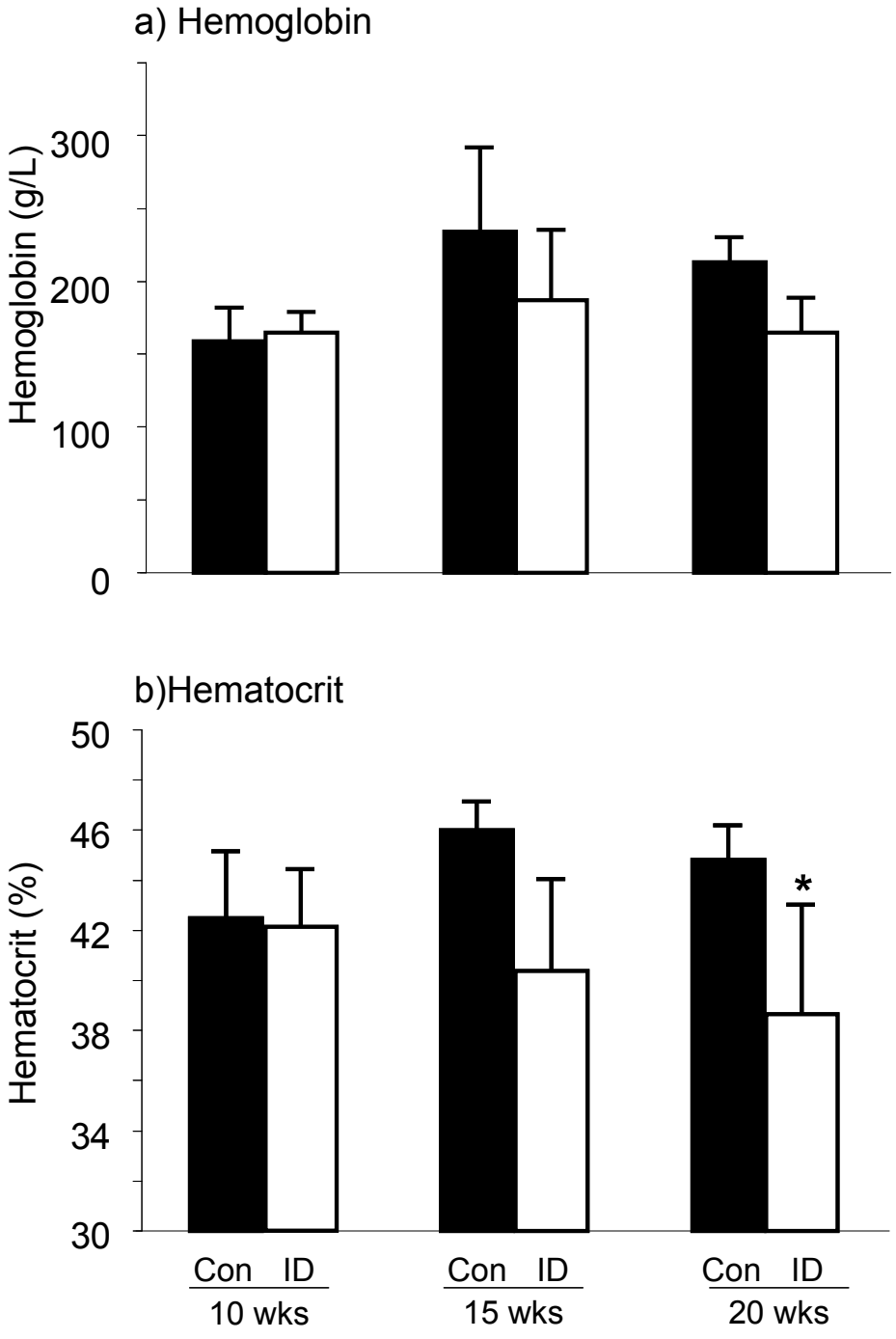

Figure 3.1 Hemoglobin and hematocrit following dietary salt challenge #1 (age 10 wks, n=3), dietary salt challenge #2 (age 15 wks, n=3) and dietary salt challenge #3 (age 20wks, n=7). Data are presented as mean ± SD. *$p<0.05$ from controls.

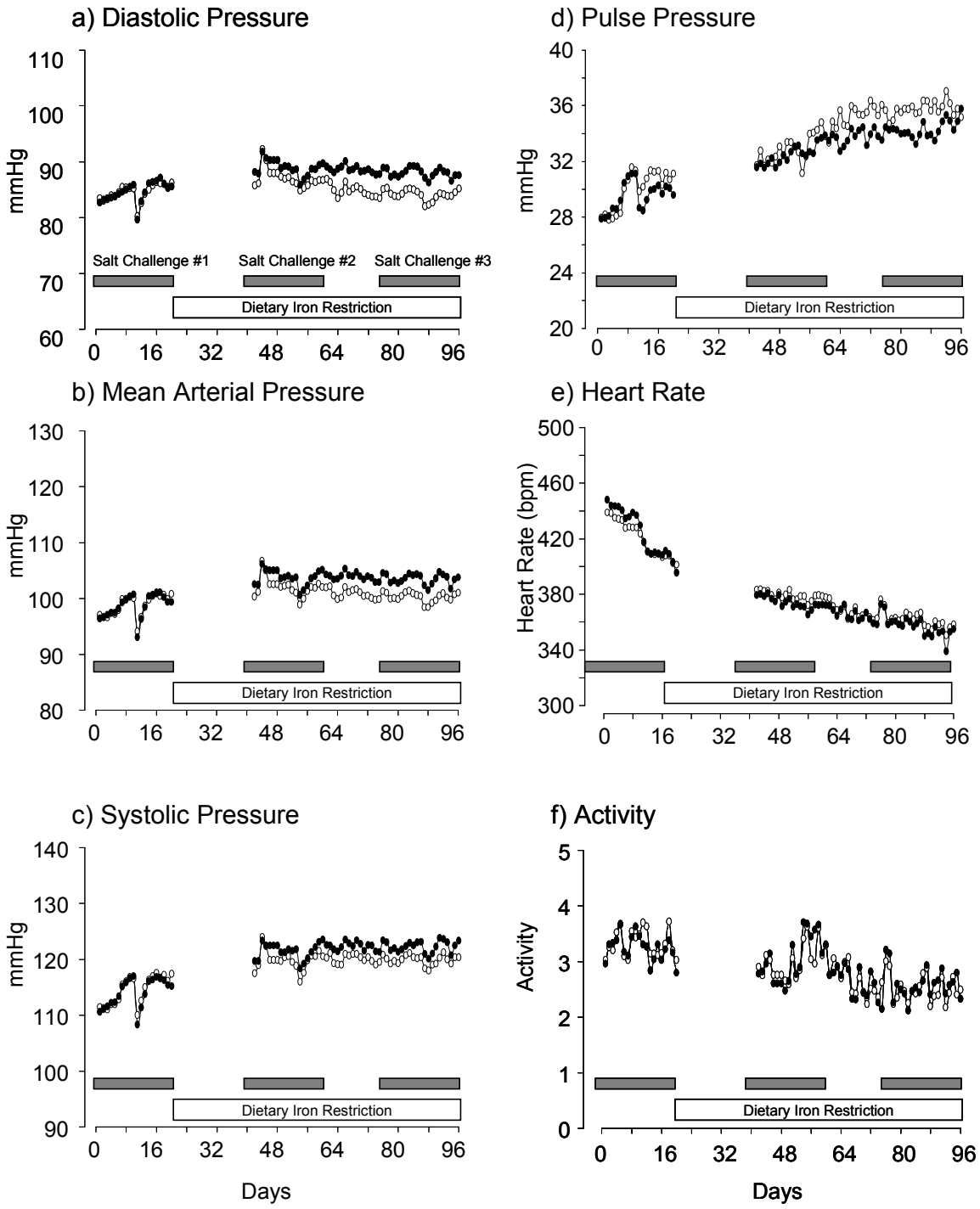

Figure 3.2 Comparison of 24 hour average a) diastolic pressure, b) mean arterial pressure, c) systolic pressure, d) pulse pressure, e) heart rate and f) locomotor activity in control(●) and ID (○) groups from 8-20 weeks of age. Shaded bars indicate periods of dietary salt challenge.

3. Hemodynamics During Dietary Salt Challenges #1-3:

During the initial dietary salt challenge (dietary salt challenge #1), hemodynamic parameters DBP, MAP, and SBP did not differ between ID and control groups. The initial dietary salt challenge was performed prior to the ID group starting on low iron diet (Figure 3.3a). Unexpectedly, during this dietary salt challenge, DBP, MAP and SBP increased while animals were on low salt (LS) diet. This was followed by a drastic decrease in DBP, MAP and SBP accompanied by a threefold increase in water consumption from 40 ± 10 ml to 123 ± 46 ml over the first 24 hours of the transition to high salt (HS) diet. DBP, MAP and SBP increased gradually during the HS period to levels slightly greater than that of the LS period. Small decreases in blood pressure were observed upon transition to normal salt (NS) diet (Figure 3.3a).

During the second dietary salt challenge (dietary salt challenge #2) DBP, MAP and SBP decreased during LS period, increased while animals received HS diet and normalized following transition to NS diet, although changes were not significant (Figure 3.3b). It can be seen that DBP begins to decrease in ID in comparison to controls at the beginning of the LS period. These changes, although variable, appear to persist throughout the HS and NS salt transitions. DBP was significantly lower ($p<0.05$) in ID animals compared to controls during the NS period when averaged for the dietary period (Mean ± SD: Control: 88.3±6.2 mmHg ; ID: 85.6±6.7mmHg). Changes in MAP and SBP were less pronounced (Figure 3.3b).

At the beginning of dietary salt challenge #3, average DBP for each dietary salt transition was significantly lower ($p<0.05$) in ID animals compared to control fed animals (Mean ± SD: Control: 87.8±6.2 mmHg ; ID: 84.3±6.3mmHg). However, there were no differences in MAP and SBP between groups (Figure 3.3c). As observed during the previous dietary salt challenge DBP, MAP and SBP decreased very little during the LS period, increased to a small extent during the HS period and normalized to pre-salt challenge pressures following three days on NS diet. The greatest difference in DBP between ID and control animals occurred during the HS period ($p<0.01$, Figure 3.3c) causing a small average increase in PP (Mean ± SD: Control: 34.3±3.6 mmHg ; ID: 35.8± 5.1mmHg) in the ID group ($p=0.055$).

4. Animal Growth:

Animals grew at similar rates in both control and low iron groups, doubling in body weight over the first six weeks of the study, from 6-12 weeks of age (Figure 3.4). At 20 weeks of age control animals averaged 572.7 ± 58g, whereas low iron fed animals averaged 560.3 ± 47g.

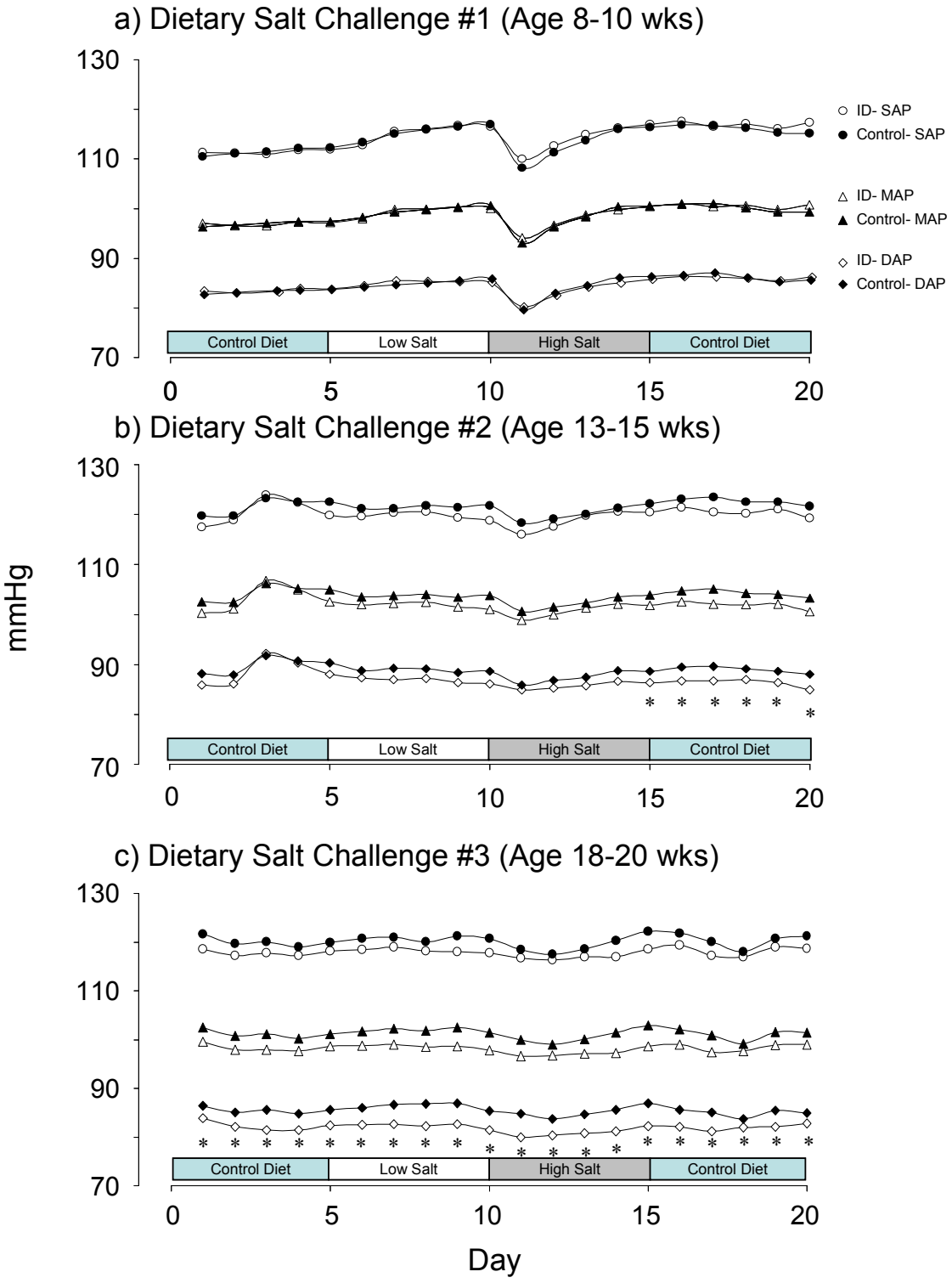

Figure 3.3 Continuous systolic, mean and diastolic pressures during dietary salt challenge #1-3(a-c) respectively. 24 h group means are presented. Solid symbols denote control, open symbols iron deficient animals. Symbols for DBP,MAP and SBP are indicated in a). *p<0.05 ID compared to controls.

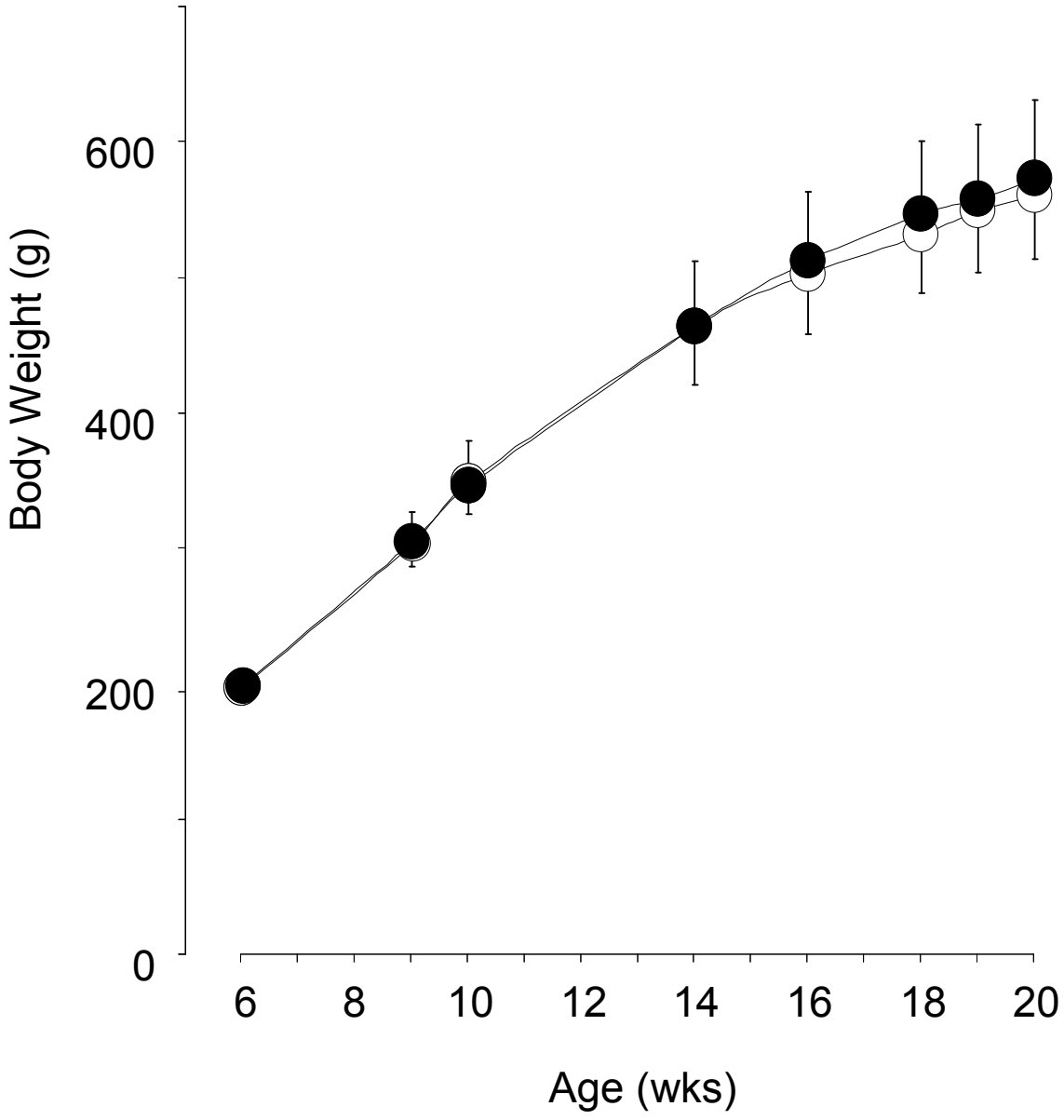

Figure 3.4 Growth profile of control (●) and iron deficient (○) males from 6-20 weeks of age. Iron deficient group was started on low iron diet at 10 weeks of age. Data are presented as mean ± SD.

5. Effects of Dietary Salt Challenge on Mean Arterial Pressure:

24 hour MAP averages during LS, HS, and NS periods showed no variation in response to dietary salt challenge (Figure 3.5). Small variations in MAP occurred in parallel with dietary salt manipulations although the changes were only significant during the period of HS in dietary salt challenge #3. At this time MAP was significantly lower in ID compared to control animals ($p<0.05$, animal age= 19-20 weeks). This difference was not statistically significant during the preceding, LS ($p=0.075$) or NS dietary regimen ($p=0.11$).

6. Low Salt to High Salt Diet Transition:

Change in MAP (ΔMAP) was greatest during the transition from LS to HS diet of each dietary salt challenge (Figure 3.6). However, during this transition the ΔMAP was still small, not exceeding 3 mmHg (Figure 3.6a). The ΔMAP in response to LS to HS transition decreased progressively in ID animals throughout the study period, but was variable in the control group. The greatest difference in ΔMAP between control and ID groups occurred during dietary salt challenge #3 but the changes were not statistically significant ($p=0.139$). There was no predictable correlation ($R^2 =0.01$) between ΔMAP and the level of hematocrit (Figure 3.6b). LS to HS changes in DBP paralleled changes in MAP. Although ID animals had significantly lowered DBP by the end of the second dietary salt challenge compared to controls ($p<0.05$)(Figure 3.3b), there were no significant differences in ΔDBP over time between control and ID animals.

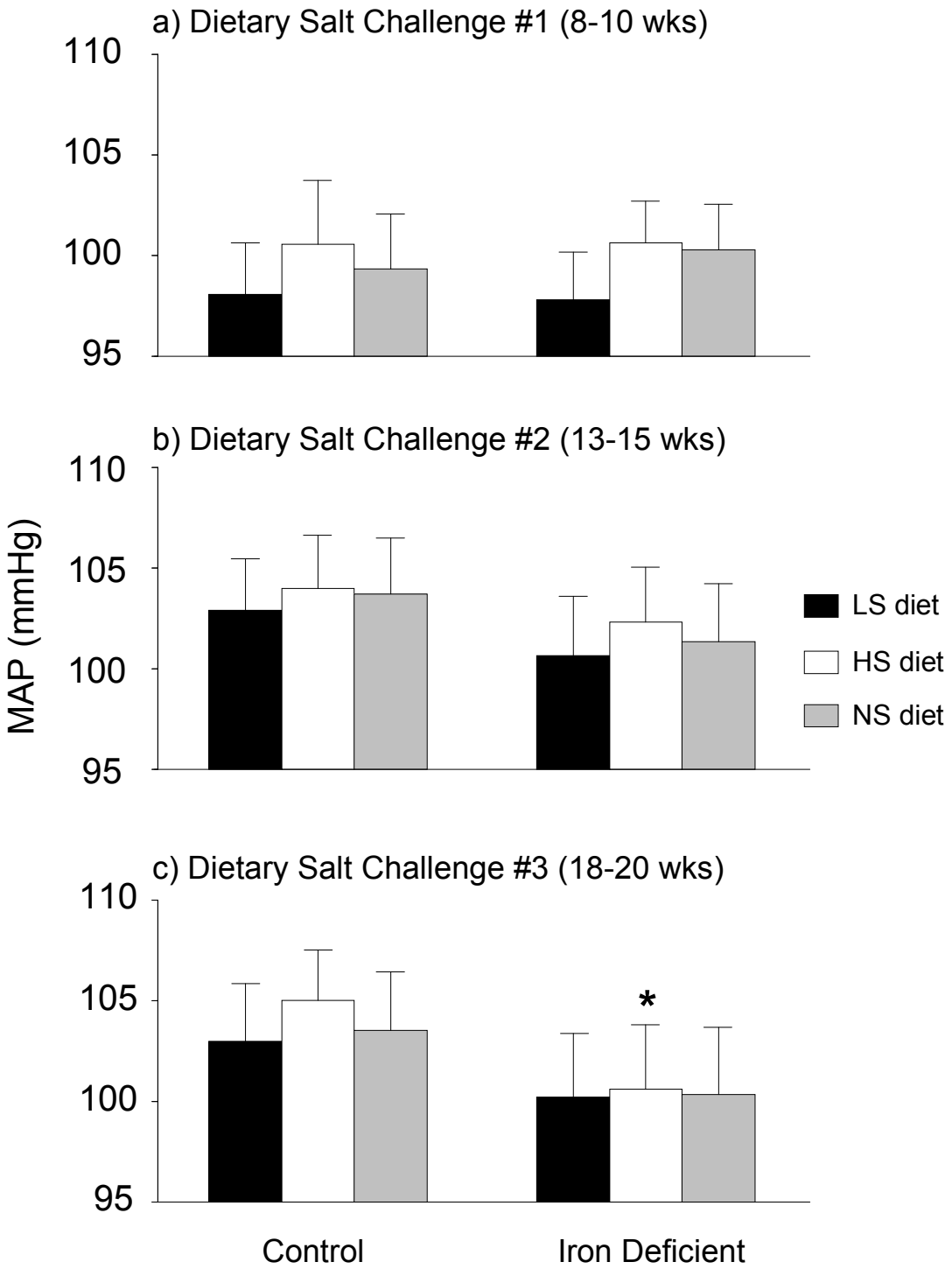

Figure 3.5 Mean arterial pressure in response to low (LS), high (HS) and normal (NS) salt diets. Data are presented as mean ± SEM. *$p<0.05$ iron deficient versus control fed animals.

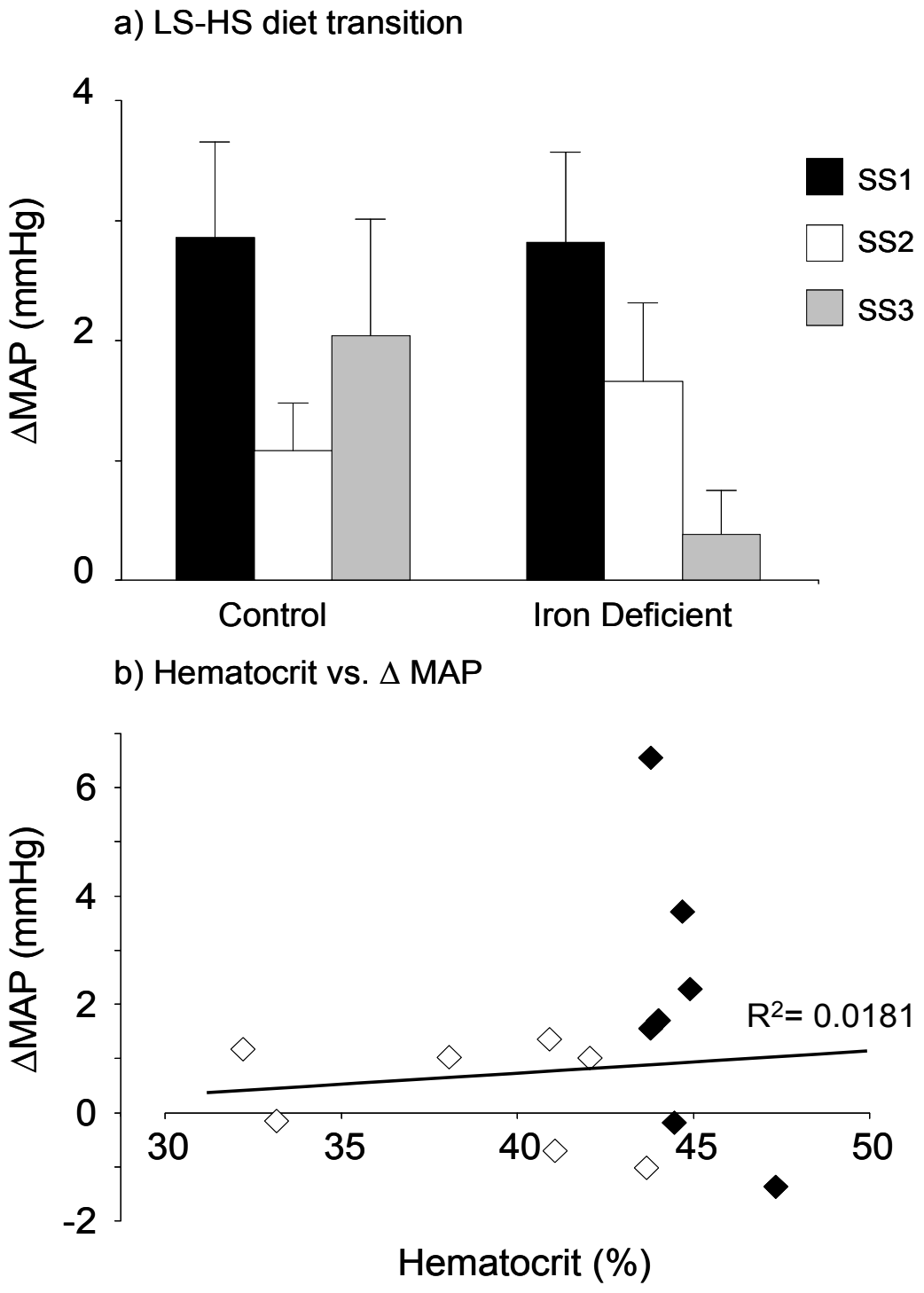

Figure 3.6 a) Change in mean arterial pressure (ΔMAP) from low to high salt diet during dietary salt challenge #1 (SS1), #2(SS2) and #3 (SS3). Data are presented as mean ± SEM. b) Comparison of ΔMAP and hematocrit following 10 weeks of dietary iron treatment shows no correlation. Solid symbols denote control and open symbols ID animals.

7. Renal Function Curves:

There were no changes in the indices of renal function as a result of dietary iron restriction as indicated by minimal change in the overall slope of the dietary salt to MAP relationships generated by dietary salt challenges #1 - #3 (Figure 3.7). Likewise, these relationships highlight the fact that there were only minimal changes in MAP over time between control and ID groups.

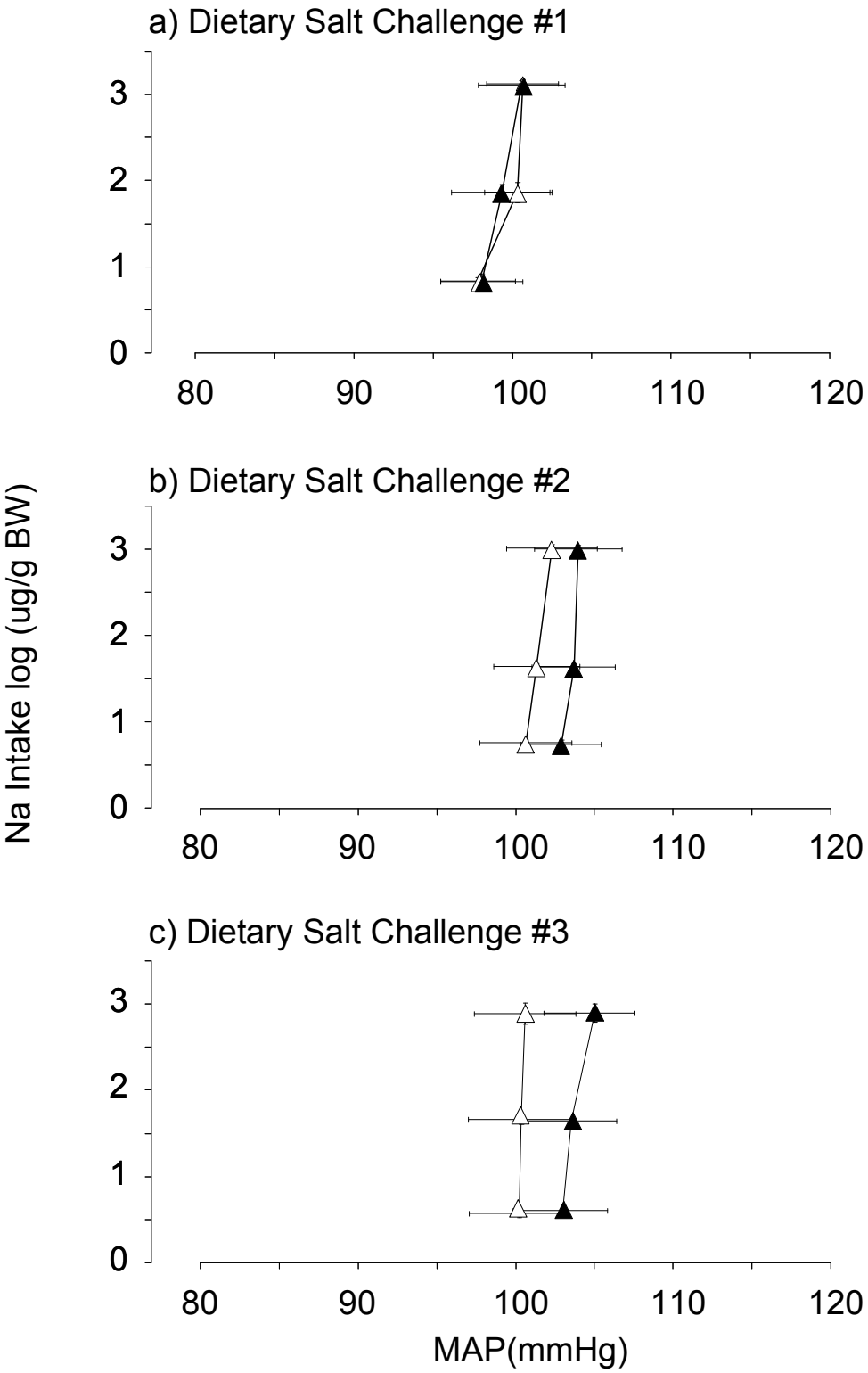

Figure 3.7 Renal function curves for a) dietary salt challenge #1 (animal age ~8-10 weeks), dietary salt challenge #2 (~13-15 weeks) and dietary salt challenge #3 (~18-20 weeks).(△-iron deficient; ▲- control). Data are presented as mean ± SEM.

3.4 Discussion

The major findings of this study were that although dietary iron restriction causes (i) a substantial lowering of hematocrit the impact on the circulation was minimal including a moderate lowering of diastolic pressure (DBP), but more limited changes in mean arterial pressure (MAP), systolic pressure (SBP) and pulse pressure (PP), but (ii) without causing marked changes in renal function.

It has been shown previously that chronic anemia has marked effect on hemodynamics, decreasing blood viscosity, peripheral vascular resistance, and blood pressure in addition increasing pulse pressure and cardiac output[47,214-217], changes consistent with the development of a hyperkinetic circulation[216,217]. It is understood that accompanying these effects are proportional decreases in DBP, MAP and SBP[217]. The present finding of a progressive lowering of DBP is consistent with the circulation becoming hyperkinetic [47,214]. In support of this finding, blood pressure lowering in response to acute anemia (i.e. lowest hematocrit achieved in <2 weeks) has been shown to be directly proportional to change in hematocrit[231-233]. Given that we have shown only a mild change in hematocrit, it is not surprising that we see only a small change in blood pressure. The lack of correlation between individual hematocrits and blood pressure may be the result of differences between acute and chronic studies. That is, in the present study only a moderate lowering of hematocrit was obtained whereas in previous studies the association with blood pressure was much stronger when the relative lowering of hematocrit was much more pronounced[233]. It is not surprising that we did not see a correlation between the lowering of blood pressure and hematocrit because, in previous

studies the relationship between hematocrit and blood pressure became linear only over a large range of hematocrit values (25% → 65%)[233].

The mechanisms responsible for the hyperkinetic changes induced by anemia are not completely understood, however, substantial evidence indicates that these changes are mediated in part by changes in peripheral vascular resistance resulting from increased basal production of nitric oxide (NO)[205]. Similarly, in response to ID it has been shown that inhibition of the cytochrome P450 (CYP 450) mediated metabolism of arachadonic acid (AA) alters the pressure-natriuresis relationship by preventing mobilization of sodium channels in the proximal tubule of the kidney[229,230]. Although our data does not test the effect of ID on these pathways directly, it is possible to predict the indirect effects because functional alterations in both NO production and CYP 450 mediated AA metabolism have been shown to cause salt-sensitivity of blood pressure[93,244-248]. The present data reveal overall that blood pressure changes in response to manipulations of dietary salt content were not different. Although we have shown previously that the offspring of ID mothers develop salt-sensitive blood pressure, the results of the current study suggest that the level of ID induced in this study likely does not have a marked impact on renal NO production or AA metabolism (Unpublished data, Stephane Bourque). In fact, if anything, the hemodynamic findings (i.e. decrease in DBP and trend indicating progressive lowering of MAP and SBP) indicate that NO production may be increased. In line with these results Ni *et al* . (1997) show an increase in renal nitric oxide synthase (NOS) expression in response to ID[204]. Although increases in NOS expression are not always predictive of increased NO production, the gradual decrease in DBP

suggests that there may be a decrease in peripheral vascular resistance, consistent with increased basal NO production[241-243]. The lowering of DBP in ID animals was first observed following five weeks of dietary iron restriction. At this time, hemoglobin and hematocrit were not significantly lowered. Therefore, an ID-induced decrease in DBP at this time period suggests that circulatory changes to dietary iron restriction may occur even before observable changes in circulating iron. Consistent with previous work, the present finding also suggests that a decrease in DBP might be an early adaptation to the development of ID, preceding other hemodynamic changes. In fact, during the HS period of the final dietary salt challenge #3, MAP in ID animals had become significantly decreased compared to controls.

Hemodynamic changes caused by anemia can be reversed by correcting anemia[205, 236-239]. Similarly, in support of our finding that change in DBP was greater than change in MAP and SBP, it has been shown that DBP is more responsive to anemia correction (increase hematocrit from 20-40%) than other hemodynamic parameters[240]. Furthermore, this fits well with the proposed concept that the lowering of DBP is an initial indicator of anemia-induced hyperkinetic circulation. General lowering of blood pressure associated with chronic anemia has been shown to be a natural protective mechanism, in chronic renal failure to prevent further glomerular damage[240]. Likewise, it is possible, even with mild ID, that protective adaptations may occur in anticipation of more severe ID.

In this study, the changes in hematocrit following ten weeks of dietary iron restriction were less than those of our previous study (Chapter 2). These data confirmed that although we have induced ID in this study, it was not likely as severe as in our previous experiment. Although the reason why there was a lesser degree of iron depletion is not obvious, it may be related to the decreased frequency of hematocrit measurements, differences in pre-study diet, or a natural variation within this strain of rats. In our previous study, hematocrits were taken weekly throughout the period of dietary iron restriction. Although the weekly blood loss as a result of hematocrit measurement was small, it is possible that the lowering of circulating iron may have been accelerated as a result, in our previous study (Chapter 2). Hematocrit was not taken weekly in this study because the primary objective was to measure progressive changes in hemodynamics in response to the induction of ID. Anesthesia was required in order to take hematocrit, and has been shown to alter hemodynamics for up to five days[253]. As a result of these considerations, hematocrit assessments were done more sparingly. It is also possible that the animals in this study had greater tissue iron stores at ten weeks of age as a result of differences in pre-study diet. Animals in this study were obtained from the supplier at five weeks of age and received formula AIN-93G diet (*Research Diets Inc.*) for five weeks prior to dietary iron restriction. However, animals in our previous study were obtained at eight weeks of age, and thus received diet from the breeding facility up until that time period. Whether the pre-study diet difference is responsible for the differences observed in the severity of ID remains to be established. Nevertheless, it is evident that an extension of the dietary iron restriction period is required in this study in order to achieve ID, of comparable severity to the previous study.

Blood pressure responses in rats to dietary salt manipulation can be quite heterogeneous, in that they do not always follow the dietary salt content. [261]. In this study the unexpected increase in blood pressure on low salt diet and decrease in blood pressure upon transition to high salt diet during the first challenge may in part be explained by this heterogeneity to dietary salt manipulation in this strain of rat. In addition, the age of the animals might be a factor since in this study the radiotelemetric transducers were implanted at six weeks of age, whereas in previous studies, units were implanted at 12 weeks of age or older[250-252]. Similarly, few studies have monitored blood pressure responses in younger rats to dietary salt challenge[254, 262]. A factor known to be altered in response to changes in dietary salt and in addition often altered in response to changes in blood pressure is NO production[258,260].

To further assess these findings, it has been shown that NO production does not always parallel NOS expression in response to dietary salt manipulation[241,243]. Although the majority of studies[255-259] show increased NOS expression in response to low salt and decreased NOS expression in response to high salt, there is evidence which suggests that the actual production of NO is increased by high salt diet and decreased by low salt diet[260]. This may in part explain the increase in blood pressure in response to low salt diet in these animals. As indicated previously, as soon as animals were transferred to high salt diet there was an average 300% increase in water intake in all animals accompanied by a drastic ~5mmHg drop in MAP. This hemodynamic response was not expected. In fact, in response to an increase in water intake the normal physiological response should be an increase in blood pressure[219,221]. There is substantial evidence for heterogeneity in blood

pressure responses following dietary salt manipulation although the mechanisms linking divergent responses have not been clearly established. That is, the altered role of the various local, neural and humoral factors would require substantial further investigation that is beyond the scope of the present study. Evidence that age was an important part of the responses was suggested when a similar profile was not observed during the second (~age 13-15 weeks) and third (~age 18-20 weeks) salt challenges.

3.5 Perspectives

Collectively, these findings indicate that dietary ID, started in rats following weaning, has little impact on renal function as indicated by limited blood pressure fluctuation in response to dietary salt challenge. Although the severity of ID was mild, the data indicates the development of the classic hemodynamic symptoms of chronic anemia[47,214]. ID should be further induced in order to track the impact of more severe hematocrit lowering on the indices of renal function and hemodynamics.

Chapter 4: General Discussion and Future Directions

Iron deficiency (ID) is a problem of staggering proportions worldwide, affecting nearly one third of the world's population[1]. Hypertension is also a major health concern, accounting for almost half of the current global cardiovascular morbidity and mortality[275]. In animal studies, the offspring of ID mothers have been shown to develop hypertension as early as six weeks of age[116,153-156]. Despite the magnitude of these problems individually, in addition to their established association, the mechanism by which ID and hypertension interrelate is not known. As mentioned previously, the kidney is an important regulator of the long term level of arterial pressure[9]. Likewise, changes in heme- iron dependent mechanisms have been shown to impair the kidney's regulatory control of blood pressure and thus, have been associated with the development of hypertension[93,244-248]. However, despite these associations few studies have shown the impact of ID directly on the kidney[204]. The aim of the studies contained in this thesis was firstly to determine the relationship between circulating and tissue iron levels in response to ID with specific focus on the tissue iron changes in the kidney. Secondly, studies were designed to determine whether changes in tissue iron in response to ID are associated with changes in renal function and circulating hemodynamics.

The mechanisms responsible for sensing iron requirement and in turn initiating adaptive changes in response to altered iron supply are still not entirely understood[30]. The tissue and circulating iron responses to ID characterized by this study confirm those of previous studies demonstrating that susceptibility to ID is mediated primarily by iron requirement[30,39,73]. Accordingly, our animal model confirmed that factors such as gender and pregnancy are important determinants of susceptibility to ID[32,137-139,170]. Although

this study did not focus on controllers of the relationship between iron requirement and iron distribution, insight has been provided into the important relationship between tissue heme, and non-heme iron and circulating iron in response to ID- integral factors in iron metabolism[30,40]. Perhaps most importantly, from gender, age and pregnancy comparisons of the susceptibility to ID it was shown that in response to ID, tissue heme iron is preferentially spared. This occurred despite depletion of non-heme iron stores, marked lowering of circulating iron levels, and pregnancy. Therefore, when describing the relationship between circulating and tissue iron during the development of ID, this study has shown that the iron pool is an important consideration[58,67,72]. In this regard, these findings contribute to the mechanistic understanding of the adaptations of tissues such as the kidney in response to limited dietary iron supply.

Although there have been many studies detailing specific alterations in hemoprotein expression, this study addressed the question of the impact of ID on relative tissue heme supply. It had recently been proposed that the erythroid demands imposed by ID may cause heme deficiency in non-erythroid tissues, even before the development of detectable ID[202]. Instead, the findings of this study suggest that heme deficiency is not a concern with ID, at least not in the liver and kidney. However, in order to be certain that tissue heme sparing occurs in all tissues in response to ID, it is necessary to further analyze tissues such as the heart, spleen, brain, and skeletal muscle. Nevertheless, these findings demonstrate that non-erythroid heme supply is maintained even at the expense of vital circulating iron levels in response to ID in the liver and kidney.

In addition to the preferential protection of heme iron there was also a differential impact of ID on non-heme iron stores- significant depletion of non-heme iron occuring in the renal cortex but not renal medulla in response to ID. These findings demonstrate that the body has the ability to protect iron by location (i.e. renal medulla > renal cortex> liver). However, it remains to be determined how the preferential protection of iron occurs. It is possible that, as has been shown in the liver, tissues can readily mobilize iron for use elsewhere in the body in response to changes in iron supply[71,73]. Therefore, in this study, the sparing of iron observed in the kidney medulla may occur as a result of iron shuttled from other areas of the body. In order to determine the mechanism for iron sparing in the kidney medulla in response to ID, it is necessary to determine whether iron is shuttled from other areas of the body to supply the renal medulla (i.e. the renal cortex), or whether iron is held by the renal medulla in response to ID. An understanding of the relative iron distribution parameters in the kidney in response to ID would further elucidate the renal mechanisms responsible for controlling iron distribution in response to ID.

In characterizing the tissue iron changes in the kidney, we have shown that mild ID did not alter renal function. Despite significant lowering of hematocrit, there were minimal fluctuations in blood pressure in response to dietary salt manipulation. Likewise, the progressive lowering of diastolic, mean, and systolic blood pressures that emerged with continued dietary iron restriction were consistent with the symptoms of progressively developing chronic anemia[47.215-217]. It has also been shown that in response to chronic anemia there is an increase in peripheral nitric oxide (NO) production which in

turn facilitates oxygen delivery by reducing peripheral vascular resistance[47]. Few studies have characterized the progressive changes in hemodynamics in response to the progressive development of anemia[231,233]. Unfortunately, because the severity of ID induced was much milder than anticipated from previous tissue iron analysis (Chapter 2), there are limitations in the conclusions that can be made from this study. In order to expand upon these findings it is necessary to further extend the duration of dietary iron restriction.

Given the dependence on heme iron in renal NO production and cytochrome P450 (CYP 450) mediated arachadonic acid (AA) metabolism and the importance of these systems in the regulation of the long term level of arterial pressure within the kidney, it is reasonable to predict that alterations in iron supply as a result of ID may cause functional impairments in these systems[241,243-245]. Collectively, our findings demonstrate indirectly that ID, to the degree of severity induced in this study, did not sufficiently alter such regulators of sodium and fluid balance within the kidney[115]. This conclusion is based on the findings that ID did not cause significant blood pressure fluctuations in response to dietary salt challenge, that there were limited changes in renal medulla iron, and that there was a preferential sparing of heme iron over non-heme iron in all tissues examined. Further, given the fact that blood pressure profile showed a downward trend in response to ID our findings suggest that NO production might alternatively be increased in response to ID. This interpretation concurs with the limited evidence available on the impact of ID on the renal expression of nitric oxide synthase (NOS)[204]. In order to provide direct evidence for the mechanistic connection between ID and hemodynamic

change, in future studies it will be necessary to measure directly, the changes in NOS, CYP 450 and their key products NO, 20-hydroxyeicosatetraenoic acid (20-HETE) and epoxyeicosatrienoic acid (EET)[245]. Nevertheless, our findings serve as an effective tool for predicting the impact of ID at both a tissue and functional level, providing evidence to narrow the focus of future studies on the specific time frames and forms of iron that might link ID and blood pressure regulation.

This study has provided unique insight into the relationship between ID, hypertension, and the kidney specifically. The data suggest that the connection between these factors may be limited to changes that occur with exposure to ID *in utero*. In contrast to previous studies indicating that the offspring of ID mothers develop hypertension and salt sensitivity[116,153-156], the present studies show that by allowing the animal to establish tissue iron stores before restricting iron supply (i.e. inducing ID following birth), that prolonged ID has little global circulatory changes. It remains to be established whether the causal link between gestational ID, hypertension and salt-sensitivity is the lowering of tissue and circulating iron supply specifically, or whether the impact of ID *in utero* is attributable to changes that occur in conjunction with ID[116]. Given the protection of heme iron observed in this study in response to ID, in addition to the well established role of hemoproteins in the kidney's regulation of the long term level of arterial pressure, it is reasonable to speculate that ID *in utero* may impact on heme iron levels which are protected in response to ID following birth[158]. Further studies should address the specific effect of gestational ID on heme iron levels. It is possible that during the perinatal period tissue heme iron is more susceptible to change[148,19,152]. Further

studies are required to assess the nature of tissue specific changes in iron associated with ID during gestation so that they can be contrasted with those of the current study which were imposed following birth. This will further elucidate the mechanistic association between ID and hypertension.

Overall, the studies carried out in this thesis have provided significant evidence for the relationship of tissue iron, circulating iron depletion and the impact that changes in these components have on the kidney at both a biochemical and functional level. We have provided mechanistic evidence which suggests that the relationship of ID to alterations in renal function and the development of hypertension are specific to the effects of ID during the gestational period. Demonstration of the preferential protection of heme has provided a unique focus for further mechanistic studies which would help to characterize the impact of gestational ID on tissue heme and non-heme iron components and the important cellular processes in which they are involved. The studies outlined in this thesis show that ID has little impact on the circulatory system over the short term. Despite the staggering global prevalence of ID, and the chronic nature of the problem, little is known about the long term impact of ID with age. Further studies should address the impact of ID over more prolonged periods of time and in addition should continue to characterize the long term impact of ID during the critical period of development.

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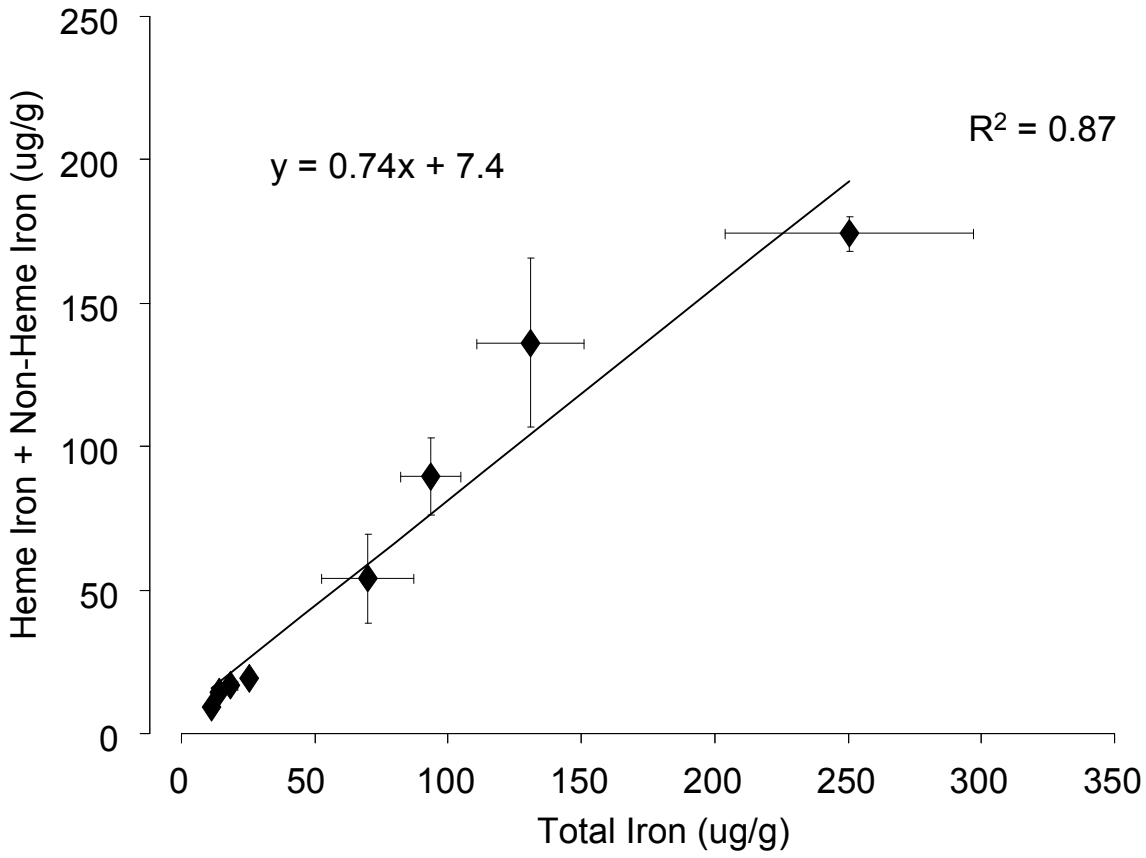
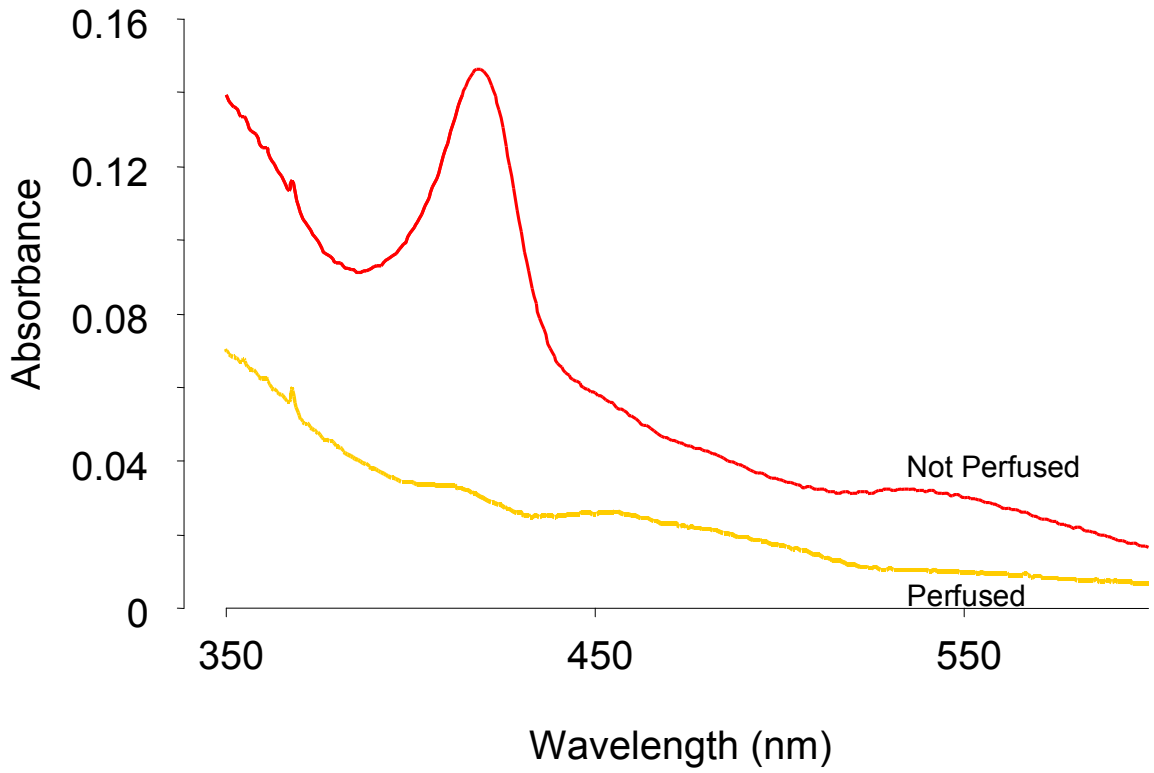
Appendix A
Tissue Heme and Non-heme Iron Measurements Are Additive With Total Iron



The figure above demonstrates that the individual heme and non-heme iron assays used in this thesis are additive to total iron. The values shown in the figure above are for liver tissue from both male and female, control and iron deficient animals. The R^2= 0.87 and $p<0.001$, determined from linear regression analysis verifies that the tissue iron analyses used in this thesis are effective indicators of relative levels of tissue heme and non-heme iron.

Appendix B
Tissue Perfusion Eliminates Blood Iron Contamination



This figure shows the absorbance spectrum of liver tissue perfused bi-directionally through the aortic arch with 0.1% saline solution compared with tissue that has not been perfused. It can be seen that in non-perfused tissue there is a large absorbance peak in the soret region (~ 420 nm) attributable to the presence of hemoglobin. Perfusion eliminates the Soret band absorbance peak, indicating that the perfusion techniques used in these studies have effectively removed blood from the tissue samples. Given this evidence, combined with the proof from Appendix A that heme, non-heme and total iron assays are additive, it can be concluded that the techniques used are an effective indicator of the relative heme and non-heme tissue iron components in both control and iron deficient tissues.