STUDIES ON IRON BIOFORTIFIED MAIZE

by Mercy Gloria Lung'aho

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Iron deficiency is an important public health problem that is estimated to affect over one-half the world population. Improving the nutritional quality of staple food crops such as maize, by developing varieties with high bioavailable iron represents a sustainable and cost effective approach to alleviating iron malnutrition. The aim of our study was to use a Caco-2 cell / quantitative trait loci (QTL) / and poultry approach to discover the genes that influence maize grain iron concentration and amount of bioavailable iron. After several breeding cycles guided by measurements of iron bioavailability with the Caco-2 cell model, we identified two maize lines with identical grain iron concentration (20 µg/g), but one line contained double the amount of bioavailable iron ($p < 0.001$). Three QTL were found to influence the bioavailability of iron in the two maize lines. Measurement of iron status in a poultry model confirmed the results of the Caco-2 cell model. Some of the lines screened using the Caco-2 cell / QTL / and poultry approach have been grown at multiple locations and data show a strong genotype × environment (GxE) interaction. We conclude that conventional breeding can significantly improve iron bioavailability in maize grain and that the GxE effect plays a major role in iron bioavailability. Human feeding trials should be conducted to determine the efficacy of consuming the high bioavailable iron maize.

BIOGRAPHICAL SKETCH

Mercy Gloria Lung'aho was born and raised in Kenya. Mercy attended Jomo Kenyatta University of Agriculture and Technology and graduated with her Bachelor of Science in Food Science in April 2003. She joined Cornell University for her Master of Science in Food Science in August 2005. In August 2007, she began her Ph.D. program in Food Science at Cornell University under the supervision of Dr. Raymond P. Glahn.

Dedicated to my precious son, Preston.

With Jehovah's blessing,

may all your dreams come true.

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LIST OF ABBREVIATIONS

Dcytb	Duodenal cytochrome *b*
DMT1	Divalent metal transporter 1
DNA	Deoxyribonucleic acid
DW	Dry weight
Fe, $Fe^{2+,}$ Fe^{3+}	Iron, ferrous iron, ferric iron
Fp	Ferroportin
G×E	Genotype by Environment interaction
HFE	Human hemochromatosis protein
HIV	Human immunodeficiency virus
Hp	Hephaestin
IBM	Intermated B73 × Mo17
ID, IDA	Iron Deficiency, Iron Deficiency Anemia
IREs, IRPs	Iron regulatory elements, Iron regulatory proteins
mRNA	Messenger ribonucleic acid
NIL	Near isogenic lines
ppm	Parts per million
QTL	Quantitative trait loci
RIL	Recombinant inbred lines
SEM	Standard error of mean
Tf, TfR	Transferrin, Transferrin receptor
UN	United Nations
UTR	Untranslated region
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

The problem

Iron deficiency (ID), which affects more than two billion people globally, is by far the most widespread micronutrient deficiency in the world. Its prevalence in women of childbearing age and young children, especially those in developing countries, is high and often due to multifactorial causation (Zimmermann & Hurrell, 2007; World Health Organization [WHO], 2001). Iron deficiency develops after iron stores in the liver, bone marrow, and spleen are depleted and erythropoiesis is compromised or diminished. If not corrected, the resulting depletion of storage iron and reduction in transport iron typically lead to a reduction in hemoglobin concentration, which is an indicator of iron deficiency anemia (IDA; see Figure 1.1, Beard, Dawson & Piñero, 1996).

Nutritional IDA often results when an individual's dietary iron supply cannot meet the physiological requirements for the synthesis of functional iron compounds such as hemoglobin. Such a condition can be caused by either low levels of iron intake or poor bioavailability of dietary iron, particularly in populations that consume monotonous plant-based diets with little or no intake of iron-rich foods such as liver, pork, or other meat products. Non-nutritional factors such as malaria, HIV infection, and other chronic diseases are also associated with anemia. Helminth infections also contribute to anemia, principally by increasing iron losses and further exacerbating any existing iron deficiency (Underwood, 2001; Allen & Casterline-Sabel, 2001).

The major consequences of IDA include impaired growth, retarded psychomotor development and poor cognitive development in children, a damaged immune mechanism associated with increased morbidity and mortality rates in all age

groups, and reduced work capacity in adults (WHO, 2001; Neumann, Gewa & Bwibo, 2004).

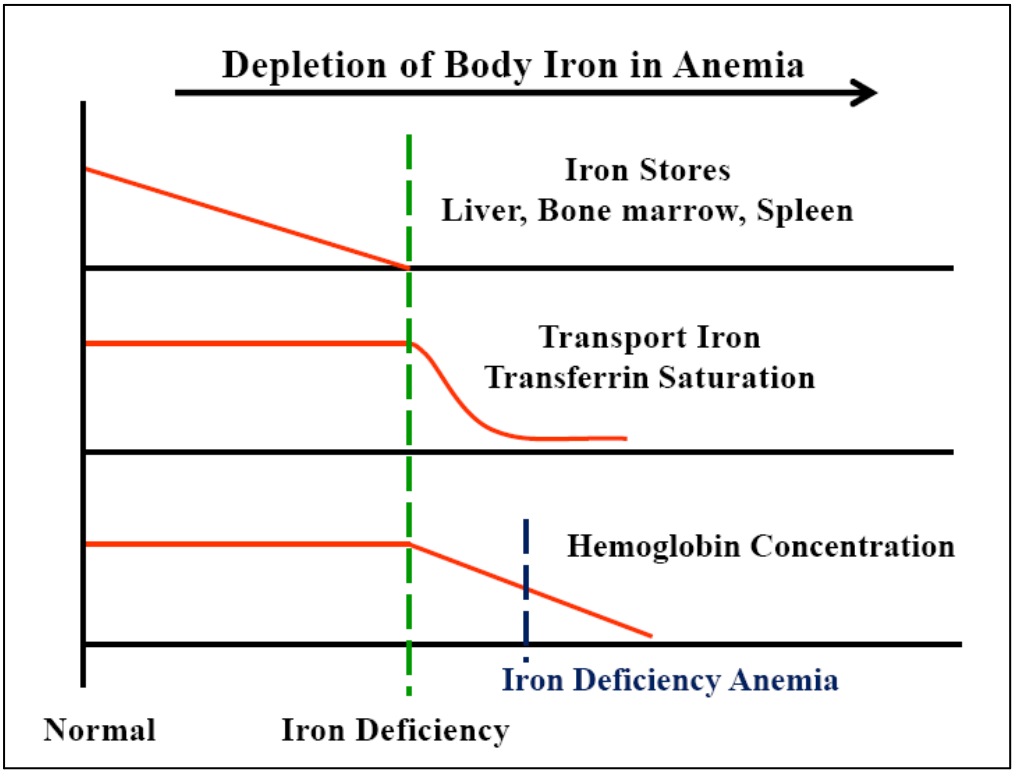

Figure 1.1: Depletion of body iron resulting in iron deficiency and anemia

This overview chapter will briefly discuss the main strategies for correcting ID, focus attention on why biofortification is needed, highlight aspects of iron homeostasis in the plant and animal kingdom, and lastly consider our research interests and objectives.

Strategies for addressing nutritional iron deficiency

Traditionally, three main strategies for correcting ID have been employed in various populations. These include:

1. *Dietary diversification and modification*: The monotonous plant diet, and low iron absorption from such diets have been identified as a major cause of nutritional iron deficiency in many populations. The goal of dietary diversification and modification is thus to improve dietary iron intake and bioavailability. This strategy promotes intake of foods low in iron inhibitors and the use of iron-rich foods like meats in conjunction with other foods such as orange juice or lime juice that are rich in ascorbic acid, which is known to enhance iron bioavailability. Properly designed dietary modification also encourages reduced consumption during meals of phenolic-rich foods or beverages such as teas and coffee that are known to inhibit iron bioavailability (Ruel & Levin, 2002; Hallberg & Rossander, 1982a, 1984). Although dietary diversification and modification is an ideal approach, it is difficult to achieve the desired results without a strong educational component. It is difficult, that is, to change traditional dietary behaviors or preferences and doing so may be costly in many poor resource areas where meat is expensive (Zimmermann & Hurrell, 2007).

2. *Iron supplementation*: Iron supplementation is a public health intervention targeted at groups at high risk of developing IDA, providing high doses of iron in the form of tablets for adults or drops for children. Supplementation programs can be cost effective, but constraints such poor infrastructure, poor public health management policies, and ineffective institutions may diminish their effectiveness and outreach success, particularly in developing countries. Poor compliance at the individual level (as a result of side effects such as nausea and stomach pain) may also limit the success of supplementation programs. In addition, recent studies show that untargeted iron supplementation in children living in malaria-endemic regions may carry increased risk of morbidity and mortality (Zimmermann & Hurrell, 2007; Sazawal et al., 2006).

3. *Iron fortification*: Iron fortification - adding iron to foods - is a prophylactic approach that is probably the most practical and cost-effective long-term solution to

IDA, but its effectiveness depends on the quality of the iron fortificant and the frequency of intake of the fortified food. The quality of an iron fortificant depends on its bioavailability and compatibility with the food vehicle of choice. Iron salts such as NaFeEDTA, ferrous sulphate, and ferrous fumarate have been shown to exhibit reasonable bioavailability. These salts have also been shown to retain the taste and appearance characteristics of food vehicles by not causing off-flavors or color changes when used in appropriate concentrations (United Nations [UN], 2001). As for frequency of use, in many developing countries the use of fortified foods depends mainly on socioeconomic status. Resource-poor households that are most frequently afflicted by IDA lack both market and economic access to fortified products. More importantly, in these countries the major constraint on the success of food fortification is the need for central processing facilities, as poor populations seldom purchase processed foodstuffs, instead growing and consuming their own food (Zimmermann & Hurrell, 2007).

Based on this overview of the conventional approaches to addressing iron deficiency, it is clear that a new and complementary strategy that provides more iron at an affordable cost and does so principally via the agricultural system is needed to further reduce and better address nutritional ID among resource-poor populations worldwide.

Biofortification of staple foods

Because agriculture is the principal source of most micronutrients, it has been postulated that micronutrient malnutrition as it exists today is the consequence of malfunctioning food systems that fall short of delivering sufficient nutrients to meet human requirements. Thus efforts are now being dedicated to addressing these

deficiencies via agricultural interventions aimed at improving the micronutrient quality of foods (Graham et al., 2007).

Biofortification of staple foods as lead by HarvestPlus® focuses on increasing the pro-vitamin A, iron, and zinc content of crops using plant breeding strategies (Nestel et al., 2006). Research has shown that the enrichment traits available for iron and zinc within the genomes of staple crops allow for sizeable increases in the micronutrient content of these foods without diminishing yields. Moreover, micronutrient-dense seeds can increase crop yields even when planted in micronutrient-poor soils. And if the enrichment traits appear to be relatively stable across various soil types and climatic environments, it is possible for biofortification to target resource-poor populations around the globe while complementing and enhancing other nutrition interventions (Welch & Graham, 2002).

The advantage of biofortification lies in its ability to target both the root causes of nutritional deficiencies (dysfunctional food systems) and at-risk populations (rural poor who grow and consume their own foods). In the case of iron deficiency, iron biofortification of staple foods that dominate the diets of resource-poor households can help increase dietary iron intake and/or absorption in such households, whose members form the bulk of those suffering from nutritional iron deficiency and its consequences (Nestel et al., 2006). The success of biofortification as an agricultural intervention will depend on the extent to which such iron-biofortified lines can maintain or improve the iron status of iron deficient populations. Its effectiveness will be influenced by: i) iron concentration of staple food, ii) iron bioavailability, iii) amount of staple food consumed, and iv) potential of biofortified food to increase iron status. Hence, our goal is to determine explicitly whether nutritionally enhanced crop lines significantly improve the amount of dietary iron absorbed from iron-biofortified foods.

Iron Nutrition

Iron is an abundant mineral element in the earth's crust that is vital to both plants and animals. In plants, iron is involved in redox reactions, it facilitates carbon, sulfur, and nitrogen assimilation, and it is critical for cholorphyll formation, photosynthesis, and respiration (Taiz & Zeiger, 2006). Iron uptake is therefore tightly regulated to prevent iron toxicity and, yet provide sufficient iron to enable proper function (Theil & Briat, 2004). We cannot over-emphasize the importance of iron in the human diet. It is an essential mineral for life, largely because of its fundamental role in oxygen transport and energy metabolism. As a result of the role iron plays in human health and disease, intestinal iron absorption and its regulation have been the focus of intense research for several decades (Yip, 2001).

Plant iron homeostasis

Iron homeostasis in plants is a dynamic process resulting from the coordinated regulation of a series of processes beginning with iron uptake from the rhizosphere and proceeding through iron storage in various vegetative organs. This process depends on a plant's genotype and nutritional status as well as on other environmental conditions such as soil pH and soil inorganic matter concentration (Theil & Briat, 2004; Grusak, 2001).

A plant's ability to absorb iron from the soil will often be limited by its availability at the surface of the root. In response to deficiencies, plant roots solubilize and absorb iron using one of two strategies (Hirsch & Sussman, 1999):

Iron uptake in strategy-I: Strategy-I, which is characterized by an increase in the reducing capacity of roots, is evident in dicotyledonous plants such as beans and non-graminaceous monocotyledonous plants such as coconut (Hirsch & Sussman, 1999). The first step in strategy-I is soil acidification. Roots first solubilize Fe^{3+} by

acidifying the rhizosphere with protons and small organic acids in particular citric and malic acids, which increases ferric iron solubility and availability (Taiz & Zeiger, 2006). The next step is the reduction of ferric iron to the more soluble ferrous form. Root plasma membranes contain iron-chelating reductase enzymes that have the capacity to alter the redox state of iron prior to membrane influx (Taiz & Zeiger, 2006). For example, in *Arabidopsis*, ID induces the synthesis of *FRO2*, a ferric-chelate reductase that reduces ferric iron to ferrous iron (Robinson et al., 1999). The last step in strategy-I is iron uptake, in which Fe^{2+} is absorbed from the rhizosphere into the cytoplasm via a transporter. In *Arabidopsis*, for example, an iron-regulated transporter 1 (IRT1) transports Fe^{2+} into the cytoplasm.

Iron uptake in strategy-II: Strategy-II, which is present in grasses, is characterized by the secretion of ferric chelating compounds like mugeneic acids (MA). These compounds are also known as phytosiderophores (Hirsch & Sussman, 1999). The first step in strategy-II involves the biosynthesis of MA from nicotianamine in the roots. The second step is the secretion of MA into the rhizosphere. This is followed by the chelation of Fe^{3+} by MA (Sugiura & Nomoto, 1984), and the resulting MA–Fe^{3+} complex is then taken up into the cytoplasm via a plasma-membrane transporter (Hirsch & Sussman, 1999; Römheld & Marschner, 1986).

Roots can also excrete iron chelators such as malic acid, citric acid, or phenolics to form complexes with Fe^{3+}, which are then absorbed by the plant. For example, in grasses such as corn or barley, roots secrete a siderophore, which forms highly stable complexes with Fe^{3+} that are then taken up by the root via the Fe^{3+} - siderophore transport system (Taiz & Zeiger, 2006).

In addition to physiological adaptation, plants can undergo root morphology changes so as to increase the root surface area and thus absorb more iron. In

Arabidopsis, for example, iron deficiency induces the formation and elongation of root hairs; in red clover (*Trifolium pretense L.*) it increases lateral root length; and in sunflowers it results in swelling at the root tips (Jin et al., 2008; Schmidt, 1999; Landsberg 1996).

A study of iron uptake in plants, focusing in particular on the presence of ID, shows complex, highly regulated interactions between plant roots and the rhizosphere (Schmidt, 1999), revealing the formidable challenges involved in breeding for increased iron in food crops. More information is required to further comprehend iron homeostasis in plants. Fortunately, ongoing research on various crops such as *Arabidopsis*, maize and rice continues to reveal the molecular mechanisms governing iron homeostasis in plants (Krämer, Talke & Hanikenne, 2007). This new found knowledge will be instrumental in guiding plant breeding strategies for iron-biofortified staple foods.

Human iron homeostasis

Iron Function: Iron-containing compounds in the body can be classified into two groups: functional iron in the form of hemoglobin, myoglobin, and cytochromes; and storage iron in the form of ferritin and hemosiderin - a water insoluble degradation product of ferritin (Stipanuk 2000; Yip, 2001; Institute of medicine [IOM] 2001).

About two-thirds of the iron in the body is present in the erythrocytes as hemoglobin, a molecule composed of four units each containing one heme group and one protein chain. In hemoglobin iron serves as a carrier of oxygen from the lungs to the tissues. Myoglobin is an oxygen storage protein located in the cytoplasm of muscle cells with a structure similar to that of hemoglobin, but it has only one heme unit and one globin chain. In myoglobin, iron controls the rate of oxygen diffusion from capillary red blood cells to the cytoplasm and mitochondria of muscle cells.

Cytochromes include several iron-containing enzymes that have one heme group and one globin protein chain. These enzymes act as electron carriers within the cell. Their role in oxidative metabolism is to transfer energy within the cell and, more specifically, in the mitochondria. Other key functions of iron-containing enzymes include the synthesis of steroid hormones and bile acids; detoxification of foreign substances in the liver; and signal controlling in some neurotransmitters, such as the dopamine and serotonin systems in the brain (Stipanuk 2000; Yip, 2001; IOM 2001).

Storage iron comprises 20-30% of total body iron and is especially important in young children and women of childbearing age as a reservoir during emergency blood loss. Ferritin and hemosiderin are stored mainly in the liver, spleen, and bone marrow and are used primarily for the production of hemoglobin as well as for meeting other cellular iron needs (Stipanuk 2000; Yip, 2001; IOM 2001).

Iron Absorption: The capacity of iron to either accept or donate electrons and readily interchange between Fe^{2+} and Fe^{3+} makes it an essential micronutrient for life, as it plays a crucial role in a number of processes (Yip, 2001). Nevertheless, this redox activity can also result in the production of oxygen-free radicals, which can damage various cellular components. Thus iron levels must be tightly controlled so as to provide for cellular needs without developing the toxicity caused by an excess of iron. Since the body lacks a discrete mechanism for the active excretion of iron, its levels are regulated at the point of absorption, primarily in the proximal small intestine (Frazer & Anderson, 2005).

There are two pathways for the absorption of iron in humans. One supports the uptake of heme iron derived from hemoglobin and myoglobin in meats, while the other mediates the absorption of non-heme iron (Yip, 2001). This discussion will focus mainly on the absorption of non-heme iron.

The process of nonheme iron transport across the intestinal epithelium can be divided into three steps: (a) uptake from the intestinal lumen, (b) mucosal intracellular transport, and (c) transfer to systemic circulation (see Figure 1.2).

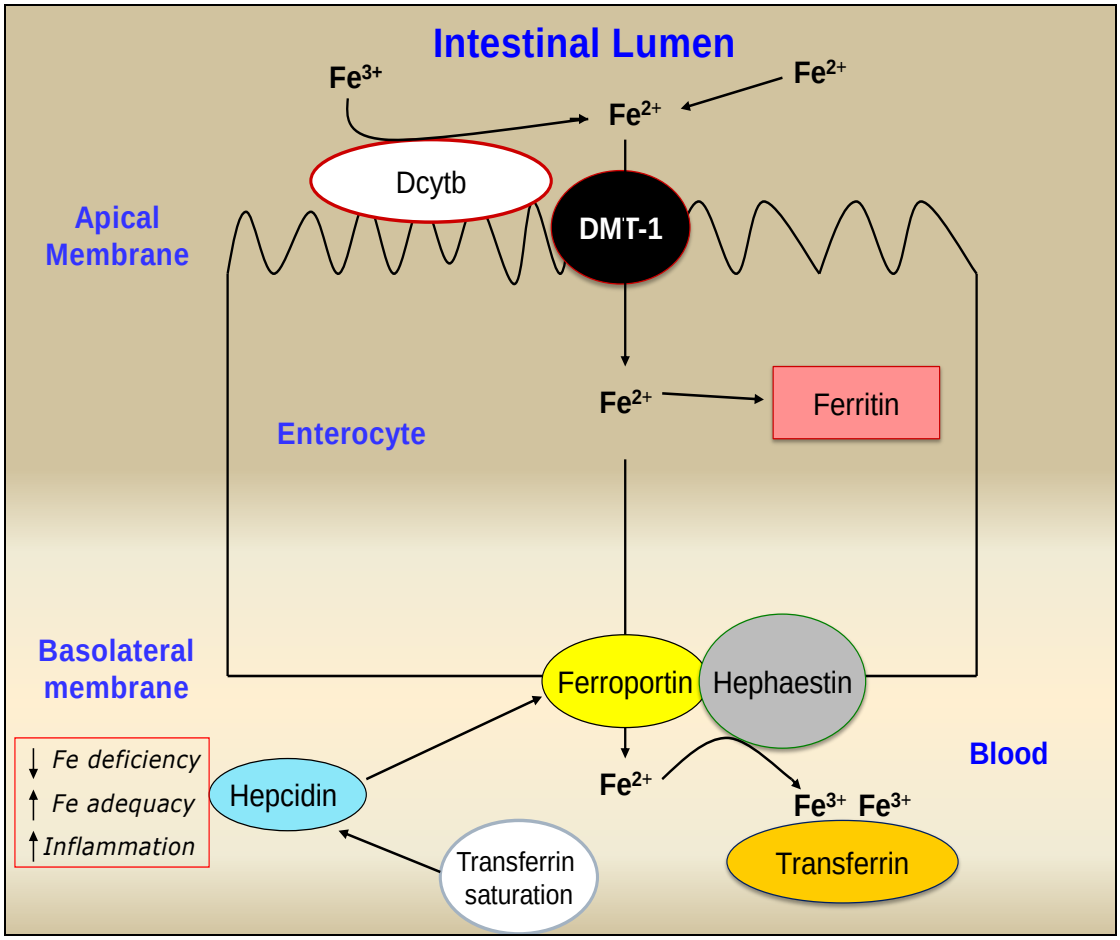

Figure 1.2: Schematic diagram of nonheme iron uptake at the enterocyte.

(a) *Iron uptake from the intestinal lumen*: Most absorption of dietary iron is carried out by mature villus enterocytes of the duodenum and proximal jejunum. The uptake of iron from the lumen of the intestine across the apical membrane and into the enterocyte is mediated by the brush border iron transporter divalent metal transporter 1 (DMT-1), which transports iron in ferrous form. Because much of the iron that enters

the lumen of the duodenum is in ferric form it is reduced, likely enzymatically, by a brush border ferric reductase known as duodenal cytochrome *b* (Dcytb; see Frazer & Anderson, 2005).

(b) *Mucosal intracellular iron transport*: The intracellular movement of iron from the brush border membrane to the basolateral membrane is not completely understood. Some suggest that intracellular iron may be bound to chaperone molecules to maintain its solubility, but to date none have been identified—although DMT-1 has been proposed as one of the potential carrier proteins (Ma et al., 2002, Yeh et al., 2008). Iron not transferred to the body is incorporated into the iron storage molecule ferritin and is lost in the feces when the cell is ultimately sloughed at the villus tip (Frazer & Anderson, 2005).

(c) *Iron transfer to systemic circulation*: The transfer of iron across the basolateral membrane and into systemic circulation is believed to be mediated by the iron transport proteins ferroportin and hephaestin. Ferroportin (Fp) is the only known cellular iron exporter in vertebrates that is not only necessary for the release of iron from the enterocyte, but is also known to be involved in the export and recycling of iron from other cell types, including macrophages (Domenico et al., 2008). Hephaestin (Hp), a ceruloplasmin homologue, is thought to interact with ferroportin to facilitate the movement of iron across the membrane. Its role as a ferroxidase is to re-oxidize ferrous iron to form ferric iron, thus facilitating Fe^{3+} binding to transferrin (Tf), a circulating iron carrier in the blood (Vulpe et al., 1999).

Iron Transportation in the body: Once in circulation (bound to transferrin in the plasma), iron is transported to specific peripheral body tissue sites, where the transferrin-iron complex is sequestered by transferrin receptor proteins and, after undergoing receptor-mediated endocytosis, which internalizes the entire complex into clathrin-coated vesicles, the iron is eventually released from transferrin into the cell

cytoplasm and the transferrin is recycled back to the blood stream (Goswami, Rolfs & Hediger, 2002). In the absence of bleeding, iron is fairly well conserved by the body and only about 1mg iron/day may be lost through excretion (Bothwell et al., 1979).

Regulation of iron absorption in the body: Early models of iron absorption regulation were based on the programming of crypt cells. As previously mentioned, mature absorptive enterocyte cells constitute the major site of iron absorption. These villus enterocytes differentiate from crypt cells during migration from the crypts to the apex of the villus. The early models of the regulation of iron absorption suggested that in normal, healthy individuals the sensing of bodily iron status by duodenal crypt cells might effect a change in the iron absorptive activity of daughter enterocytes. These models propose that the crypt cells of the distal duodenum sense bodily iron status through the uptake of Tf-bound iron. The human hemochromatosis protein (HFE) associates with transferrin receptor (TfR) proteins on the basolateral surface, resulting in the internalization of Tf-bound iron into the crypt cells. When Tf saturation is low, less iron is transferred via DMT1 into the cytosol, resulting in a relatively iron-deficient state in the crypt cells. Consequently, binding of iron-regulatory proteins (IRPs) to the 3' untranslated region (UTR) of iron-regulatory elements (IREs) increases, resulting in increased mRNA stability for iron transport genes—DMT1, Dcytb, Fp and Hp—expressed on differentiation of these cells to villus absorptive enterocytes. On the other hand, when Tf saturation is high, IRPs bind to the 5' UTR of IREs, blocking mRNA translation for these iron transport genes and thus reducing their abundance in the resulting mature villus absorptive enterocytes (Fleming & Britton, 2006; Pietrangelo, 2002; Philpott 2002). The crypt cell hypothesis is supported by results from a mouse study conducted to test the hypothesis that the HFE gene is involved in the regulation of iron homeostasis (Zhou et al., 1998). In this study, researchers studied the effects of a targeted disruption of the murine homologue

of the HFE gene on iron homeostasis. Even on a standard diet, the HFE-deficient mice showed profound increases in transferrin saturation and hepatic iron compared with what occurs in the wild type, suggesting that, in crypt cell programming, HFE is the main protein involved in the regulation of iron transfer into the blood.

With the discovery of hepcidin, however, a new model for the regulation of iron absorption emerged. Hepcidin, a hepatic bactericidal protein, has been shown to affect the function, distribution, and concentration of ferroportin and is thought to regulate iron efflux from enterocytes and macrophages by binding to Fp and inducing its internalization and degradation (Nemeth et al., 2004). Thus reduced levels of hepcidin may lead to tissue iron overload, while overproduction of hepcidin could result in hypoferremia and the anemia of inflammation. Hepcidin production is regulated by high iron levels, inflammatory stimuli, and demand for erythropoiesis (Beutler, 2004; Hentze, Muckenthaler, & Andrews, 2004). On this contemporary model of iron absorption, hepcidin controls plasma iron by modulating iron absorption in the gut, the release of recycled hemoglobin iron by macrophages, and the movement of stored iron from hepatocytes. Evidence from a rat study conducted by Frazer et al. (2002) supports the role of hepcidin over crypt cell programming as the process of regulation of iron absorption in the epithelial cell. In their study adult rats were switched from an iron-replete to an iron-deficient diet and the expression of Dcytb, DMT1, Fp, and Hp in their duodena, as well as hepcidin in their livers, was studied over a 14-day period. The researchers also analyzed the effect of the ID diet on iron absorption and iron status. The switch from an iron-replete to an ID diet resulted in a rapid change in gene expression in both the duodenum and liver. Increases in Dcytb, DMT1, and Fp expression in the duodenum were observed, but not of Hp. On the other hand, hepcidin expression in the liver decreased almost simultaneously. Crypt cell migration is thought to take about three to five days, so the rapid nature of these

changes (within one day) is thought to support the proposed role of hepcidin in signaling the bodily iron requirements to the epithelial cells. Frazer et al. also noted that the changes in gene expression occurred before changes in both hematologic and storage iron were evident, but were correlated precisely with a change in transferrin saturation, which suggests that Tf saturation may be a regulatory factor for hepcidin regulation and iron absorption.

Iron Bioavailability

Iron absorption in humans is influenced by many factors, including dietary iron content, iron bioavailability, and bodily storage iron status (Yip, 2001). In human nutrition terms, bioavailability is commonly defined as the proportion of a nutrient in a food or meal that is absorbable and utilizable by the person eating the food or meal. In the case of iron, it is characterized as the percentage of dietary iron that is absorbed and utilized by an individual (Benito & Miller, 1998). Thus the total concentration of a nutrient in a food or diet does not necessarily reflect the consumer-available nutrient supply within a food system, and this is especially true with regard to micronutrients such as iron. Therefore in the case of iron biofortified foods it is essential to determine if the amount of bioavailable iron in the enriched lines can improve the iron status of the targeted populations (King, 2002; Welch & Graham 2002).

Dietary iron occurs in two forms: heme iron and non-heme iron. In the human diet the primary sources of heme iron are hemoglobin and myoglobin from meat, poultry, and fish. Non-heme iron is obtained from cereals, pulses, legumes, fruits, and vegetables. It occurs in plants in various forms in differing proportions with varied chemical properties according to food source (Hallberg, 1981). Bioavailability of heme iron averages about 25 percent, compared with 2-8 percent from non-heme iron in plant foods. This difference between non-heme and heme iron in terms of

bioavailability may be explained by the fact that heme iron is thought to be absorbed intact by receptor mediated endocytosis and its absorption is only slightly influenced by other constituents of the diet. As for non-heme iron, its chemical form significantly affects its absorption, independently of other dietary compounds that could further alter dietary iron absorption (West & Oates, 2008; Hallberg, 1981).

Iron bioavailability in plant foods is influenced by dynamic factors and the interactions that make iron bioavailability complex. Some factors increase the absorption and utilization of iron and these are referred to as enhancers or promoters of iron bioavailability. On the other hand, other factors inhibit iron absorption and utilization and these are known as inhibitors of iron bioavailability or antinutrients. Some of these factors are dietary while others are non-dietary.

Non-dietary factors that influence iron density/content in crops include genetic selection, certain agronomical practices, soil pH, and fertility, while characteristics such as age, sex, ethnicity, nutritional status, or the disease status of an individual can influence iron absorption and utilization in the body. In addition, food processing or preparation methods may increase iron bioavailability; milling, fermentation, and cooking are but a few such processes known to improve iron bioavailability in plant foods (Ruel & Levin, 2002; Graham, Welch & Bouis, 2001; Benito & Miller, 1998; Hallberg, 1981).

In the case of dietary factors that influence iron bioavailability, it is difficult to cite a unifying concept to account for the many factors that may inhibit or promote the efficiency with which dietary iron is incorporated into the body. There are multiple interactions that occur between iron and other macronutrients, micronutrients, or plant substances in a single-meal or whole diet that may enhance or inhibit iron absorption by the gut. Studies show that phytate and polyphenols are the major compounds that inhibit iron bioavailability, especially from plant-based diets (Hu et al., 2006;

Davidsson, 2003; Hallberg, Brune & Rossander, 1989; Morck, Lynch & Cook, 1983; Hallberg & Rossander, 1982b; Disler & others, 1975). On the other hand, ascorbic acid, organic acids such as tartaric acid, malic acid, succinic acid, and fumaric acid, and meats are said to enhance iron bioavailability (Hurrell et al., 2006; Salovaara, Sandberg & Andlid, 2002; Yip, 2001; Hallberg, Brune & Rossander-Hulthén, 1987).

Measurement of iron bioavailability

As already mentioned, iron bioavailability is defined as the proportion of iron in the meal that is digested, absorbed, and ultimately utilized for normal body functions (Fairweather-Tait et al., 2007). This definition recognizes and encompasses the concept that iron bioavailability involves the entire process of iron transfer from food into the body—iron digestibility, uptake, efflux, retention, utilization, and storage. Historical and current methods have been developed over time to assess these different aspects of iron bioavailability in foods (Table 1.1).

In vivo iron models (human and animal studies) and *in vitro* iron models (for example, the Caco-2 cell assay) are used to assess iron bioavailability. Human and animal studies may be categorized as isotopic or non-isotopic, depending on whether iron isotope tracers are used. Iron isotopes- ^{54}Fe, ^{57}Fe, ^{58}Fe, are commonly used in stable isotope studies while ^{55}Fe, ^{59}Fe are generally used in radioisotope studies to determine iron bioavailability of foods in *in vivo* iron models (Wienk, Marx & Beynen, 1999).

Table 1.1: Historical & current methods used to assess aspects of iron bioavailability.

Assay/Technique	Assessment
Solubility Dialyzability	Assess iron release from food
Duodenal loops	Assess iron absorbed into the blood
Caco-2 cell assay	Assess iron availability from foods Ferritin formation by Caco-2 cells is used as an indicator of iron uptake
Isotope techniques	Assess quantity of absorbed iron retained in the body
Hemoglobin incorporation / repletion	Assess iron utilization for normal body functions

In vitro methods have also been used extensively to study food-based iron bioavailability. Until recently, solubility and dialyzability were measured to estimate iron bioavailability by determining the amount of iron dissolved from food by dilute acids or the extractability of ionizable iron from food by chelating agents. More recent *in vitro* methods are based on simulated digestion of food or test meals with pepsin, hydrochloric acid, and sometimes other digestive enzymes, followed by determination of the dialyzable or soluble iron released (Wienk, Marx & Beynen, 1999; Fairweather-Tait et al., 2007). A good example of this method is the *in vitro* digestion/Caco-2 cell model that has been adapted to assess iron availability from foods (Figure 1.3). The model employs the Caco-2 cell line in conjunction with *in vitro* digestion, whereby foods undergo simulated peptic digestion followed by pancreatic-bile digestion in the presence of Caco-2 cell monolayers. These cells exhibit remarkable morphological

and biochemical similarity to the small intestinal cells, the primary site for iron absorption in the human gastrointestinal tract. For example, the Caco-2 cells form monolayers when cultured and contain brush border microvilli, enzymes, and transport proteins. They also maintain distinct apical and basolateral membranes (Pinto et al., 1983). The Caco-2 cell model is a very useful screening tool that has shown enormous potential in addressing iron bioavailability issues (Glahn et al., 1998; Yun et al., 2004).

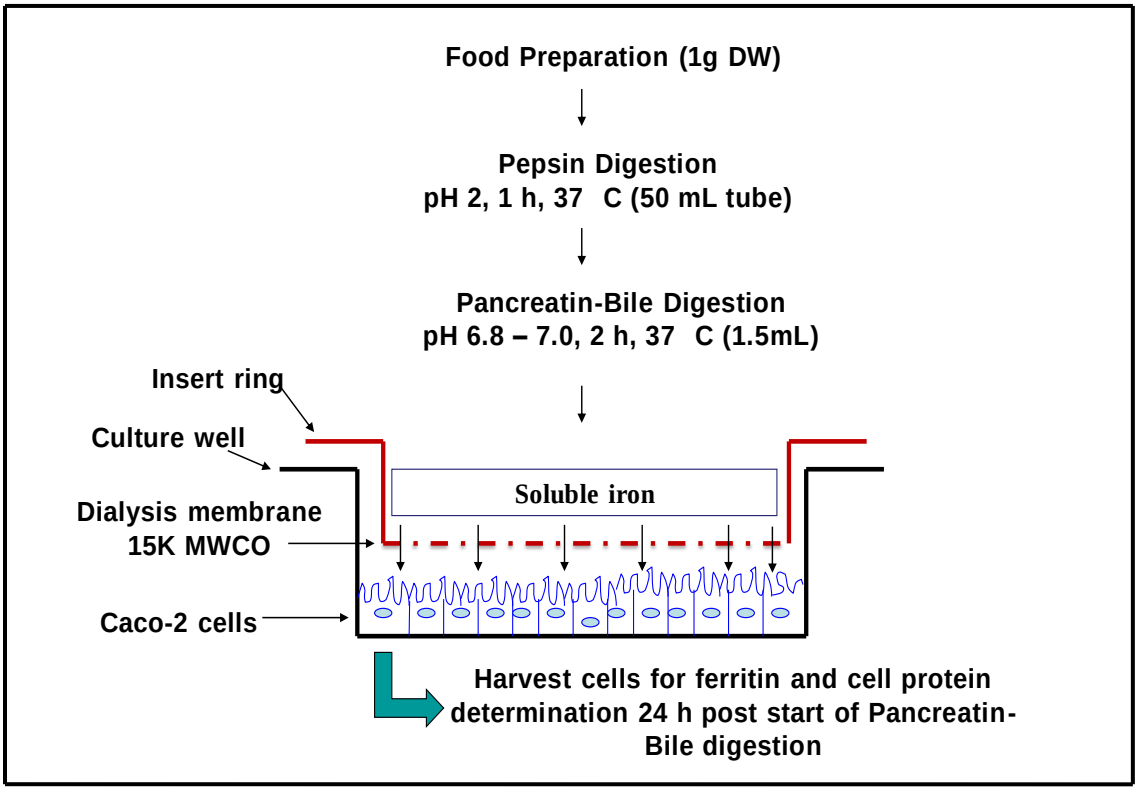

Figure 1.3: Schematic diagram of *in vitro* digestion/Caco-2 cell iron model.

Arithmetic models and algorithms have also been used to estimate food-based iron bioavailability. These models are based on existing data from iron bioavailability studies and are used to predict iron bioavailability from the diet (Wienk, Marx & Beynen, 1999; Au & Reddy, 2000). An analysis of six iron absorption prediction equations conducted by Beard et al. (2007) revealed, however, that these equations

exhibit a lack of agreement with each other and with the change in iron status as estimated by serum ferritin. Thus their validity in estimating iron bioavailability from foods remains questionable. A recent study by Rickard et al. (2009) has developed an algorithm used to assess intestinal iron availability for use in dietary surveys. The proposed algorithm is to be used to predict available iron in the gut and not iron absorption from the meal or diet. And although this algorithm by Rickard et al. seems to be an improvement from previous algorithms, further research is needed to evaluate its application beyond the population-level.

Study design, goals and objectives

The first step in breeding for improved nutrient density in crops is to identify the nutrient and the staple crop of interest. After this has been achieved, the goal of plant breeding in biofortification is to use available breeding methods to exploit the natural genetic diversity in the crop of interest so as to improve the nutritional quality of that crop. Previous iron biofortification efforts using conventional breeding have proved successful in rice (Haas et al., 2005) and iron biofortified beans have been tested in piglets with positive effects (Tako et al., 2009). The focus of our study was iron biofortification of maize using conventional breeding methods with the aid of a quantitative trait loci (QTL) model.

Experimental population: In genetic research programs, recombinant inbred lines (RIL) or near isogenic lines (NIL) are commonly used as experimental populations. RIL are produced by continually selfing or sib-mating the progeny of individual members of an F2 population until homozygosity is achieved (see Figure 1.4). NIL can be developed by backcrossing an RIL to the original parent (see Figure 1.4). There are some advantages to using NIL in research. Because the backcross derived lines are highly related to each other except for a small number of key

differences, the differences due to chance are relatively small or infrequent such that any detected differences are likely significant and related to the selected trait (Keurentjes et al., 2007).

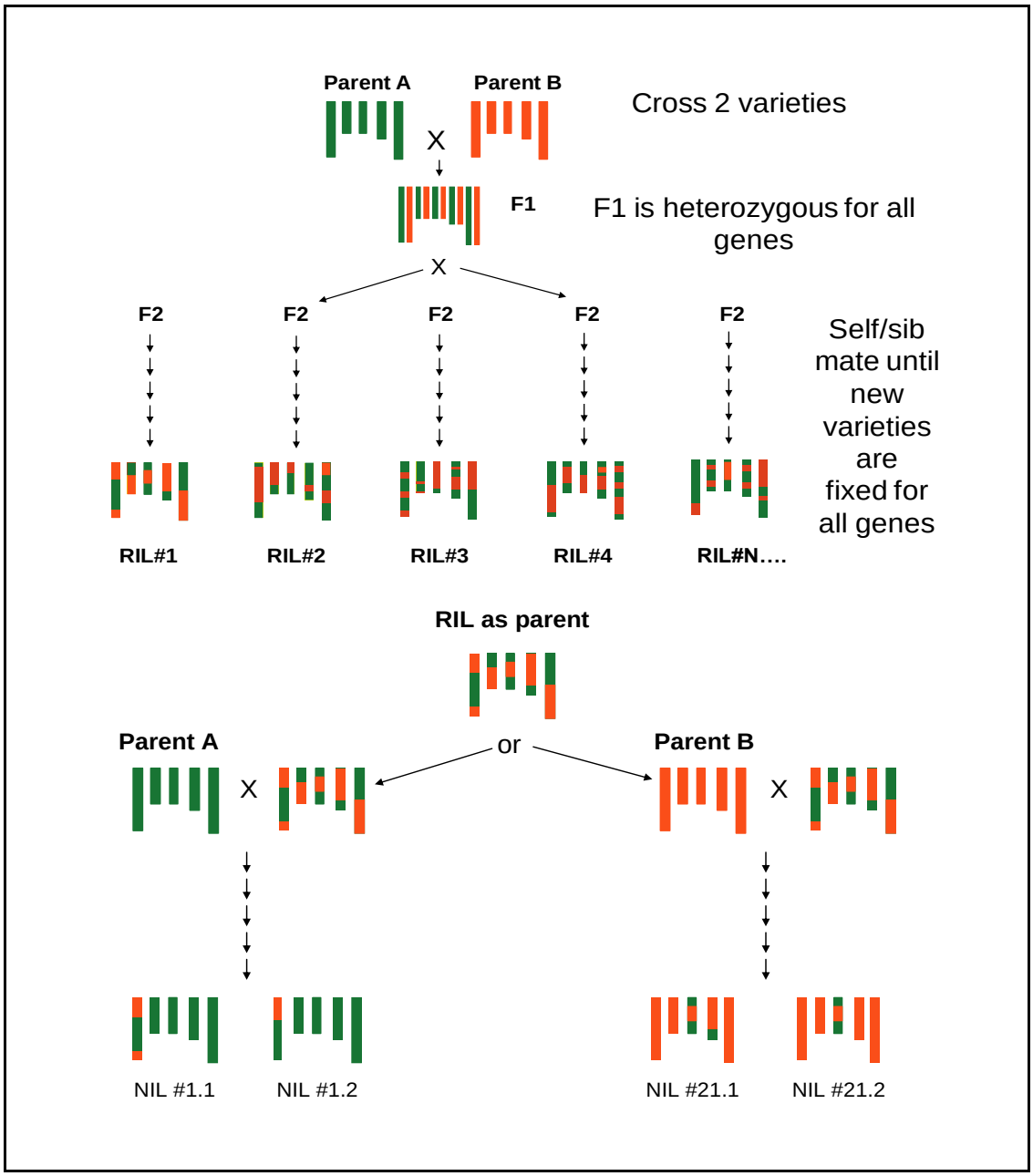

Figure 1.4: Schematic diagram of the production of recombinant inbred lines (RIL) and near isogenic lines (NIL).

RIL and NIL from the **I**ntermated **B**73 x **M**o17 recombinant inbred (IBM RI) population formed the experimental population in our study. IBM RI population was made from B73 (a temperate, stiff stalk) inbred and Mo17 (a temperate, non-stiff stalk) inbred, and is the best characterized mapping population in maize. IBM is a powerful resource for the analysis of quantitative traits and genetic mapping in maize (Lee et al. 2002; Sharopova et al. 2002; Falque et al. 2005)

Analysis of quantitative traits in iron biofortified maize: Many important agricultural traits, such as crop yield, are referred to as quantitative traits. Such traits exhibit a continuous distribution that can be measured and given a quantitative value. Quantitative traits are often affected by multiple genes and environmental factors. The quantitative traits of interest in our study were iron concentration and the amount of bioavailable iron in the IBM RI maize population.

Analysis of quantitative trait loci in iron biofortified maize: The alleles that control for quantitative traits occur in pairs and are located in loci on homologous chromosomes. The loci are therefore referred to as quantitative trait loci (QTL). The form of the gene that can exist at a single locus is determined by its DNA sequence and is referred to as an allele. Alleles can be either dominant or recessive. When different forms of an allele occur at a locus, it is heterozygous. When the alleles are the same it is homozygous, and can be either dominant or recessive (see Figure 1.5).

QTL analysis is a statistical method in plant research that allows breeders to link two types of information - phenotypic data (such as grain iron concentration or grain iron bioavailability) and genotypic data (usually molecular markers) - in an attempt to explain the genetic basis of variation in quantitative traits. In our study, QTL in the IBM RI maize population were analyzed to determine chromosomal regions that contain QTL that may influence grain iron concentration and the amount of bioavailable iron in our maize population.

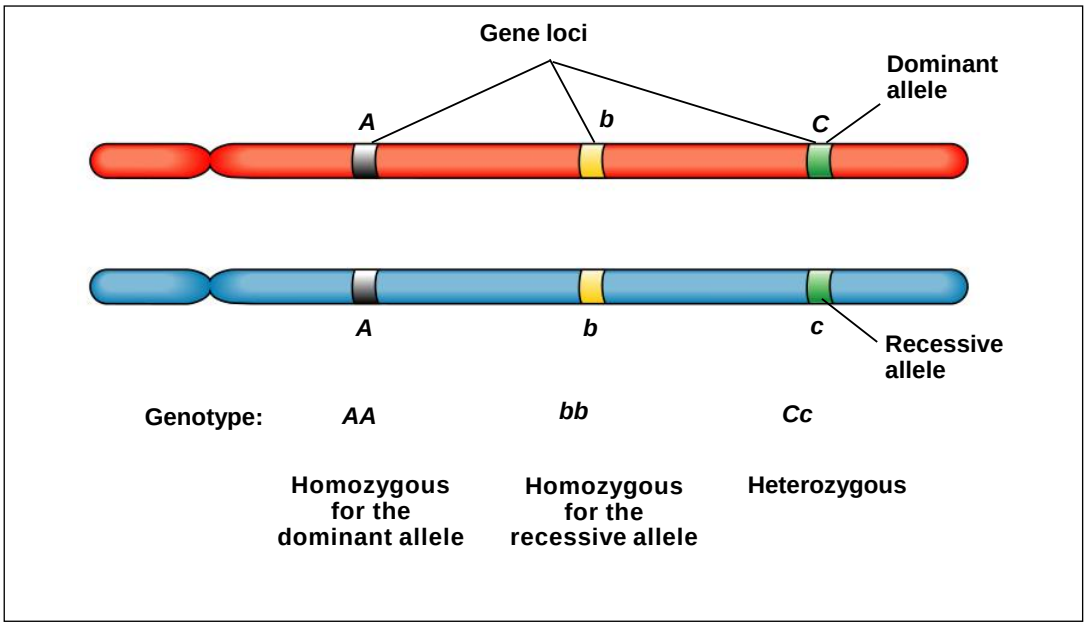

Figure 1.5: Schematic diagram of homologous chromosome

QTL model for iron biofortified maize: The main goal of characterizing quantitative trait loci in a segregating population is to determine how many genes are involved in the genetic control of the quantitative trait of interest and to estimate their location within the genome. The QTL model summarizes the genetic factors that contribute to the trait of interest, explaining where each of them are in the genome, and their relative contribution to the total phenotypic variance explained. The model is based on the fact that phenotypic differences for the trait of interest can be traced back to genetic markers located at specific positions on the chromosome (Koornneef, Alonso-Blanco & Peeters, 1997). The model can often be deduced from the statistical analysis of several segregating populations, and it helps researchers understand inheritance, variation in quantitative traits of interest, and predict future outcomes (Koornneef, Alonso-Blanco & Peeters, 1997; Gai & Wang, 1998). In our study a QTL model was employed to locate loci in the IBM RI maize population that influence iron concentration and iron bioavailability in the maize grain.

Analysis of iron content in iron biofortified maize: Mineral analysis was conducted using inductively coupled plasma-emission spectroscopy (ICAP; ICAP model 61E Trace Analyzer; Thermo Jarrell Ash Corporation, Waltham MA)

Analysis of iron bioavailability in iron biofortified maize: Analysis of the amount of bioavailable iron was done *in vitro* using the Caco-2 cell iron model and *in vivo* using a poultry model.

By targeting principally poor populations at risk of suffering micronutrient deficiencies, biofortification has the potential, as an agricultural intervention, to complement other approaches aimed at reducing or preventing micronutrient malnutrition. However, for biofortification to be successful the biofortified crops must prove to be beneficial to the populations that adopt and consume them. In the case of iron biofortified maize, (1) breeding efforts must improve iron density or bioavailability in maize, (2) the trait should be relatively stable across locations, soils and climatic regions it is adapted for, and (3) the nutritionally improved crop lines must improve the iron status of at-risk populations (Welch & Graham, 2002, see figure 1.6).

The overall goal of this research was to make a significant contribution toward breeding efforts for iron in maize. The specific objectives of the study were to (a) determine the quantitative trait loci that influence iron content and iron bioavailability in the maize grain, (b) examine environmental factors that may influence iron nutrition in maize, and (c) assess iron bioavailability from biofortified maize. To achieve these objectives, this research work was divided into three main studies that addressed specific research questions as listed:

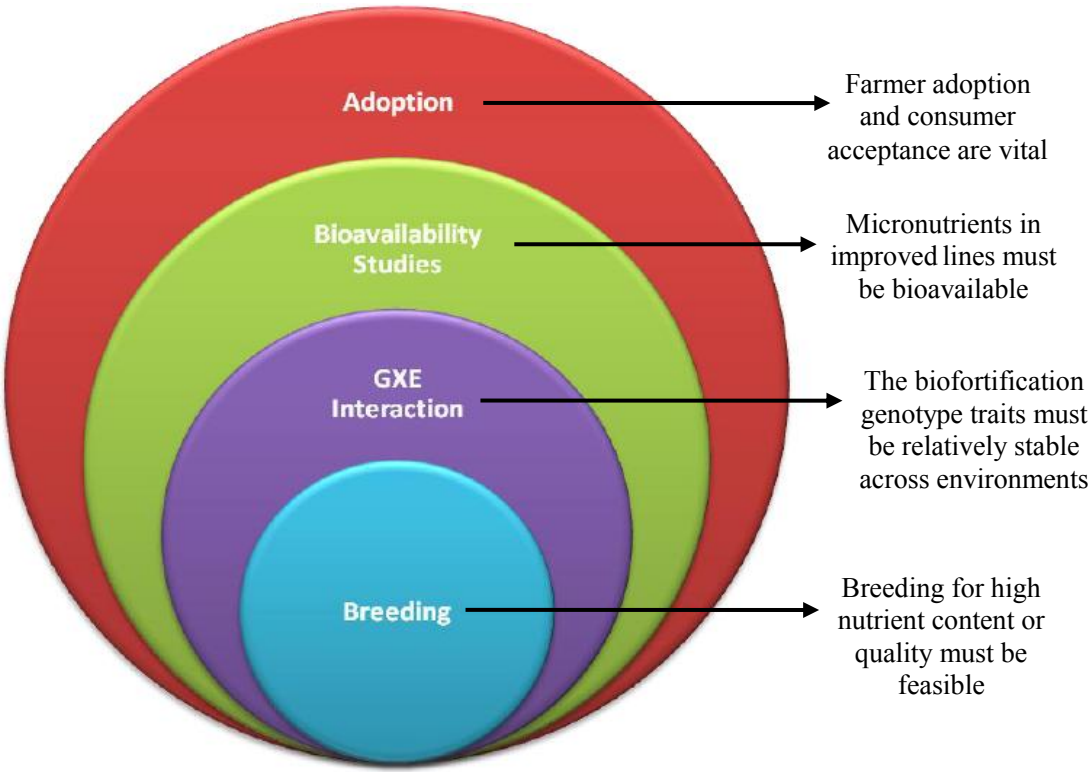

Figure 1.6: Tenets for biofortification success

Study 1: Enhancing iron bioavailability in maize using a Caco-2 cell/ QTL model.

- ♣ Is iron concentration in maize a genetically tractable trait?
- ♣ Is iron bioavailability in maize a genetically tractable trait?

Study 2: Evaluating Genotype x Environment interaction in biofortified maize.

- ♣ Does growing biofortified maize in different locations affect iron concentration and/or iron bioavailability in the maize lines?

Study 3: Assessment of iron bioavailability from iron biofortified maize.

- ♣ Are significant differences in iron bioavailability as measured by the Caco-2 cell *in vitro* model reflected in an *in vivo* model?

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CHAPTER 2

ENHANCING IRON BIOAVAILABILITY OF MAIZE USING A CACO-2 CELL / QUANTITATIVE TRAIT LOCI MODEL

Abstract

The aims of this study were to determine if there is a significant difference in grain iron concentration and grain iron bioavailability in intermated *B*73 x *M*o17 recombinant inbred maize populations grown in Aurora, New York in 2007 and 2008. Grain iron concentration was determined using emission spectroscopy and grain iron bioavailability was assessed using the Caco-2 cell bioassay. Using the Caco-2 cell/ QTL (quantitative trait loci) model, we identified two hybrid maize lines from our 2007 maize population with identical grain iron concentration (20 ppm), but one line contained double the amount of bioavailable iron ($P = 0.0064$). In the 2008 maize population, we found two inbred maize lines with similar grain iron concentration (25 ppm) but one line had 7 times more bioavailable iron than the other one had ($P < .0001$). These data can be explained either by the enhanced levels of a promoter or a suppression of an inhibitor through breeding, or a combination of both. Additional research is required to profile and identify the compounds. We conclude that conventional breeding using the QTL approach can significantly improve iron bioavailability in maize grain. The next step is to assess genotype-by-environment interaction, a process in which improved lines should be grown at multiple locations. *In vivo* bioavailability trials should be conducted to determine the efficacy of consuming the high bioavailable iron maize.

Introduction

Iron deficiency is an important public health problem that is estimated to affect over one-third of the world's population (Zimmermann & Hurrell, 2007). Improving the nutritional quality of staple food crops such as maize, by developing varieties with high bioavailable iron content, represents a sustainable and cost-effective approach to alleviating iron malnutrition (Welch & Graham, 2004). Biofortification is an agricultural intervention that seeks to improve human health by improving micronutrient levels in staple foods (Nestle et al., 2006). For biofortification to succeed, the first step is to ensure that the enhancement of nutritional quality is primarily under genetic control.

Rapid developments in maize genetics and genomics coupled with the need to integrate nutrition goals into agriculture has resulted in extensive interest and research in breeding for enhanced iron quality in maize varieties. One approach has sought to improve iron bioavailability in maize varieties by reducing iron inhibitors in maize. For example, to reduce phytic acid in maize, Raboy (2002) and others isolated low-phytic-acid mutations of maize and used these to show proof of principal that conventional breeding can be used to breed first-generation low-phytate maize varieties. Another approach is the use of transgenic breeding to improve iron nutrition in maize. For instance, to increase grain bioavailable iron in maize, Drakakaki et al. (2005) generated transgenic maize plants with endosperm-specific co-expression of *Aspergillus* phytase and soybean ferritin. In spite of their potential for success however, efforts to reduce phytate in crops and transgenic plants are mired in either scientific or political debate (Shamsuddin, 2008; Wainwright & Mercer, 2009). There is therefore a need to find a conventional breeding strategy that can improve the iron nutrition of maize and is 'perceived as safe' to both consumers and the environment.

Using conventional breeding, previous studies, such as Hoekenga et al. (manuscript submitted for publication), have sought to employ an integrated genetic and physiological analysis of iron nutrition in maize grain to determine the quantitative trait loci (QTL) that influence grain iron concentration and grain iron bioavailability in intermated B73 x Mo17 (IBM) recombinant inbred (RI) maize populations. From their studies, forty-two QTL for grain iron concentration (explaining 21 – 81% of the variance detected in a given year/location) were detected from samples collected over three years in Aurora, New York and one year in Clayton, North Carolina. Six out of the forty-two QTL were repeatedly detected, explaining 7 - 27% of the observed variance in grain iron concentration.

For grain iron bioavailability, loci associated with increased amounts of bioavailable iron were identified on 6 chromosomes and explained 54% of the observed variance in samples from a single year/location. Three of the largest iron bioavailability QTL were successfully isolated in near-isogenic lines (NILs). However, none of the identified QTL for grain iron content and grain iron bioavailability were co-located. In addition, grain iron bioavailability was not correlated with either grain iron concentration or phytate levels.

In regard to grain iron concentration, numerous genes may be involved in controlling iron absorption in the root-soil interface of the maize grain. These genes are located in QTL, so locating these QTL can facilitate the breeding process by providing important information about the location of iron nutrition genes in the maize genome, the number of genes that affect this nutrition trait, and the distribution of the genes in the maize genome. This information is essential to breeders because it can help them to distinguish between linkage and pleiotrophy and major genes and minor genes, as well as to clarify the mode of gene action. The results of the study by Hoekenga et al. lay the foundation for the follow-up research presented in this chapter.

The objectives of this study were: (i) to determine if there was a significant difference in grain iron concentration in IBM RI maize populations grown in Aurora, New York in 2007 and 2008, and (ii) to determine if there was a significant difference in grain bioavailable iron in these IBM RI maize populations.

Materials and Methods

Chemicals, enzymes, and hormones: Unless otherwise stated, all chemicals, enzymes, and hormones were purchased from Sigma Chemicals Co. To prepare reagents for cell culture, 18 MΩ water was used. Glassware and utensils used in the experiments were soaked in 1.2 M HCl for at least 4 hours and rinsed in deionized water prior to use.

Plant materials and field site details: The maize varieties tested are derived from **I**ntermated **B**73 x **M**o17 (**IBM**) recombinant inbred (RI) maize populations. The IBM RI population was selected for this study based on ready availability and the existence of advanced backcross families for rapid development of near-isogenic lines (NIL). One maize population was grown in Aurora, NY in 2007. It consisted of seventeen Mo17 background lines, thirty three B73 background lines and two hybrid lines. A second maize population, some of it derived from the 2007 maize population, was grown in Aurora NY, in 2008. It consisted of twenty-five Mo17 background lines and 43 B73 background lines The plots used for this study had a Lima Silt Loam soil, with average yield for maize of 120 bushels $acre^{-1}$ and water extractable soil pH of 7.7.

Maize sample preparation: Maize kernels (20g) were sorted to remove any debris or damaged seeds and then placed in a 50 mL centrifuge tube and covered with 25 mL of 18 MΩ water. Samples were then autoclaved at 121ºC and at a pressure of 115 kPa for 40 min, allowed to cool at room temperature, and then frozen overnight at –20 °C. Samples were then freeze-dried at 100 millTorr and a temperature of –50 °C

for 7 days, ground to a fine powder with a coffee mill (90 sec), and stored in 50 mL centrifuge tubes at 25°C. Tamale maize—used as a control in the Caco-2 cell bioassay—was prepared in an identical manner.

Mineral analysis for maize samples: A 0.3g dry ground maize sample was weighed into borosilicate glass test tubes and chemically digested using 4 mL of concentrated nitric acid at 120°C until the residue was light brown to yellow in color. Exactly 1.0 ml of a 50/50 mixture of concentrated nitric acid and perchloric acid was then added, and the temperature was increased to 180°C. After 2 hours, the temperature was further increased to 240°C until the digested samples were dry. After cooling, 0.25 mL of concentrated hydrochloric acid was added to dissolve the ash. One hour later, the sample was diluted with 10 mL of 5% nitric acid. The ashed sample was then mixed and transferred into 15 mL auto sampler tubes and analyzed on an axially viewed inductively coupled plasma (ICP) trace analyzer emission spectrometer (model ICAP 61E trace analyzer, Thermo Electron, Waltham, MA; see Rutzke, 2002).

Caco-2 cell screening: The *in vitro* digestion Caco-2 cell iron model was used as a screening tool to estimate the amount of bioavailable iron in the maize samples.

Cell Culture: Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) at passage 17 and used in experiments at passages 25–33. Cells were seeded at a density of 50,000 cells/cm^2 in collagen-treated six-well plates (Costar Corp., Cambridge, MA). The cells were grown in Dulbecco's Modified Eagle Medium (GIBCO, Grand Island, NY) with 10% v/v fetal calf serum (GIBCO), 25 mmol/L N-(2-Hydroxyethyl) piperazine-N'-2-ethane sulfonic acid (HEPES), and 1% antibiotic antimycotic solution (GIBCO). The cells were maintained at 37°C in an incubator with a 5% CO_2/95% air atmosphere at constant humidity, and the medium

was changed every 2 days. The cells were used in the iron uptake experiments at 13 days post seeding.

In Vitro Digestion: The preparation of the digestion solutions—pepsin, pancreatin, and bile extract—and the *in vitro* digestion was performed as previously published (Glahn et al., 1998). Briefly, 1.0 g of dry sample was used for each sample digestion. Ascorbic acid (AA) was added to enhance iron bioavailability using a 20:1 AA: Fe molar ratio. Then 10 ml of a pH 2 140 mM NaCl, 5 mM KCl buffer was added to the samples, and the mixture was pH re-adjusted to pH 2. Then 0.5 mL of pepsin solution was added to each of the samples prior to incubation. After a 1-hour incubation period, the sample pH was adjusted to pH 5.5 to 6.0 with 1.0 M $NaHCO_3$, and after the addition of 2.5 mL of pancreatin-bile solution, the sample pH was further adjusted to pH 6.9-7.0 with 1.0 M $NaHCO_3$. Sample volumes were then adjusted (by weight) to tube weight plus 15 g using the 140 mM NaCl, 5 mM KCl pH 6.7. Finally, 1.5 mL of the sample was transferred to appropriate inserts on the Caco-2 cell plates.

Harvesting: The harvesting of the Caco-2 cell monolayers was performed as previously published (Glahn et al., 1998). The cells were harvested after a 24-hour incubation period. First, growth media were carefully aspirated off the cells. The cells were then rinsed twice with 2 mL of 130mM NaCl, 5 mM PIPES, pH 6.7 buffer. To harvest the cells, 2 mL of 18.2 MΩ water was added to the cells, and the cells were placed in a sonicator (Lab-line Instruments, Melrose Park, IL) at 4°C for 15 minutes. The cells were then scraped off the plates, suspended in the 2 mL of 18.2 MΩ water, and transferred to pre-labeled 5 ml tubes in anticipation of protein and ferritin assays.

Cell Protein Analysis: Caco-2 cell protein was measured on samples that had been solubilized in 0.5 mol/L NaOH, using a semimicro adaptation of the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). A 25 µL sample of the

sonicated Caco-2 cell monolayer, harvested in 2 mL of water, was used for each protein measurement expressed in mg.

Ferritin Analysis: A one-stage, two-site immunoradiometric assay was used to measure Caco-2 cell ferritin content (FER-Iron II Ferritin Assay, RAMCO Laboratories, Houston, TX). A 10 µL sample of the sonicated Caco-2 cell monolayer, harvested in 2 mL of water, was used for each ferritin measurement expressed per unit of cell protein (ng ferritin/mg cell protein).

Statistical Analyses: Statistical analyses of the data were performed using the GraphPad Prism v4 (GraphPad Software, San Diego, CA) and *JMP* v7.0 (SAS Institute Inc., Cary, NC) software packages. In some cases, the data were log-transformed prior to analysis. Means were considered to be significantly different for P values ≤ 0.05.

Results

The objectives of this study were to determine if there was a significant difference in grain iron concentration and grain bioavailable iron in the IBM RI maize populations grown in Aurora, New York in 2007 and 2008. The maize populations were analyzed for grain iron concentration using emission spectroscopy and grain bioavailable iron using the Caco-2 cell bioassay.

(1a) Grain iron concentration – 2007 harvest: Table 2.1 shows a summary of grain iron concentration data for maize samples from the 2007 harvest. The maize populations consisted of two hybrid lines and 50 inbred lines. Seventeen of the inbreds were Mo17 lines, and 33 were B73 lines. The statistical model for grain iron concentration showed a significant difference in grain iron concentration in this IBM RI maize population (P = 0.0133). The grain iron concentration in the hybrid lines was

significantly different from that in the inbred lines. Grain iron concentration in the B73 maize lines was not significantly different from grain iron concentration in the Mo17 maize lines.

There was a significant difference in grain iron concentration across the samples in the three groups. Grain iron concentration was significantly different within the Mo17 lines (n = 17, P <.0001) and B73 lines (n = 33, P <.0001), respectively. However, a t-test revealed no significant difference in grain iron concentration between the two hybrid lines (P = 0.4407).

(1b) Grain iron concentration – 2008 harvest: Table 2.2 shows a summary of grain iron concentration data for maize samples from the 2008 harvest. The maize population consisted of 68 inbred lines. Twenty-five of the inbreds were Mo17 lines, and 43 were B73 lines. The statistical model for grain iron concentration showed a significant difference in grain iron concentration in this IBM RI maize population (P <.0001). Grain iron concentration in the B73 maize lines was significantly different from grain iron concentration in the Mo17 maize lines.

There was also a significant difference in grain iron concentration between the samples within the two maize lines. Grain iron concentration was significantly different within the Mo17 lines (P <.0001) and within the B73 lines (P <.0001), respectively.

Table 2.1: Grain iron concentration of maize lines grown in Aurora, NY in 2007.

	Grain iron concentration (2007 harvest)		
	Mean (ppm)	**Median (ppm)**	**Range (ppm)**
Hybrid lines (n = 2)	20.49	20.49	20.30 - 20.67
Mo17 background lines (n = 17)	22.37	23.04	15.29 - 27.47
B73 background lines (n= 33)	22.36	22.10	17.17 - 29.38
All IBM RI population	**22.29**	**22.61**	**15.29 - 29.38**

As previously mentioned, some maize samples harvested in 2008 were derived from one or two of the maize lines from the 2007 harvest. A comparison of grain iron concentration between the parental lines (2007 harvest) and daughter lines (2008 harvest) revealed no significant differences in grain iron concentration between the 2008 maize samples and either parent (parent 1, range = 17.17 - 27.47 ppm, P = 0.1635; parent 2, range = 23.12 – 24.81 ppm, P = 0.1726). This suggests that grain iron content is a genetically tractable trait in the IBM RI maize population.

(2a) Grain bioavailable iron – 2007 harvest: Figures 2.1 – 2.3 show a summary of grain bioavailable iron (as a percentage of the control) for the Mo17 lines, the B73 lines, and the two hybrid lines from the 2007 harvest. The statistical model for grain iron bioavailability showed a significant difference in grain bioavailable iron in the IBM RI maize population (P <.0001). Grain bioavailable iron in the B73 lines was significantly different from that in the hybrid and Mo17 lines. Grain bioavailable iron in the hybrid maize lines was not significantly different from that in the Mo17 maize lines.

There was a significant difference in grain bioavailable iron across samples within the three groups (P <.0001). Grain bioavailable iron was significantly different within the Mo17 lines (P <.0001) and B73 lines (P <.0001), respectively. A t-test also revealed a significant difference in grain bioavailable iron between the two hybrid lines (P = 0.0064).

Table 2.2: Grain iron concentration of maize lines grown in Aurora, NY in 2008.

	Grain iron concentration (2008 harvest)		
	Mean	**Median (ppm)**	**Range (ppm)**
Mo17 background lines (n= 17)	23.57	23.71	17.58 - 27.79
B73 background lines (n = 33)	20.32	19.96	15.52 - 26.86
All IBM RI population	**21.48**	**21.79**	**15.52 - 27.79**

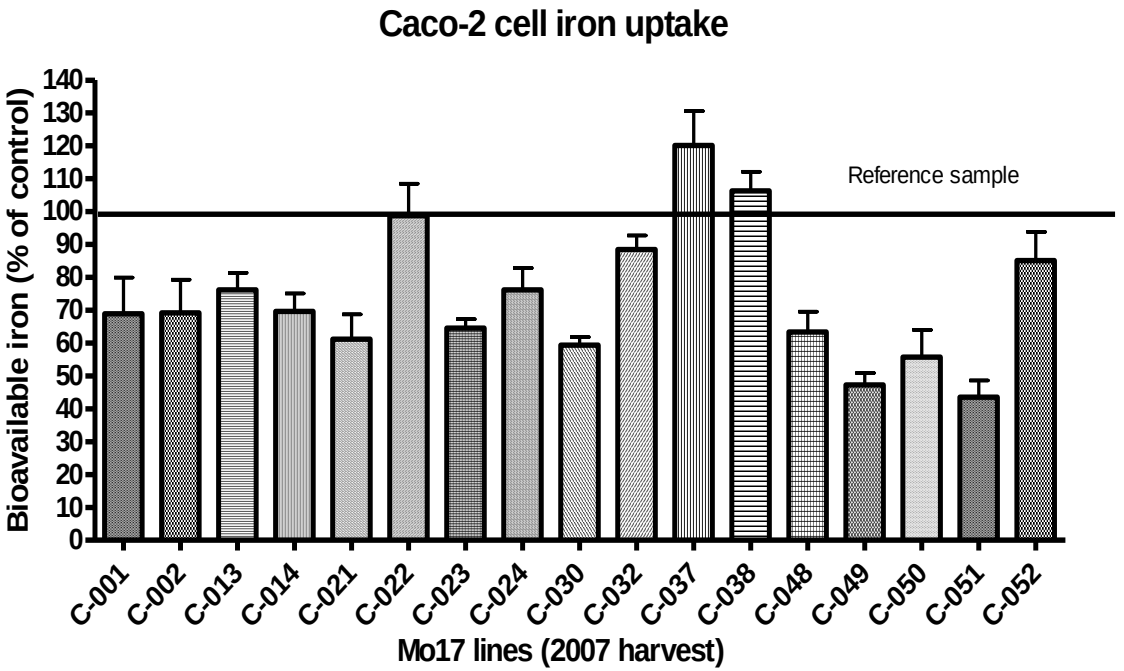

Figure 2.1: Caco-2 cell ferritin formation. The amount of bioavailable iron in the Mo17 lines was assessed using the Caco-2 cell model. Ferritin formation in the cells is an index of iron bioavailability. One gram (dry weight) of each sample was analyzed in the model. Bar values (mean ± SEM, $n = 6$).

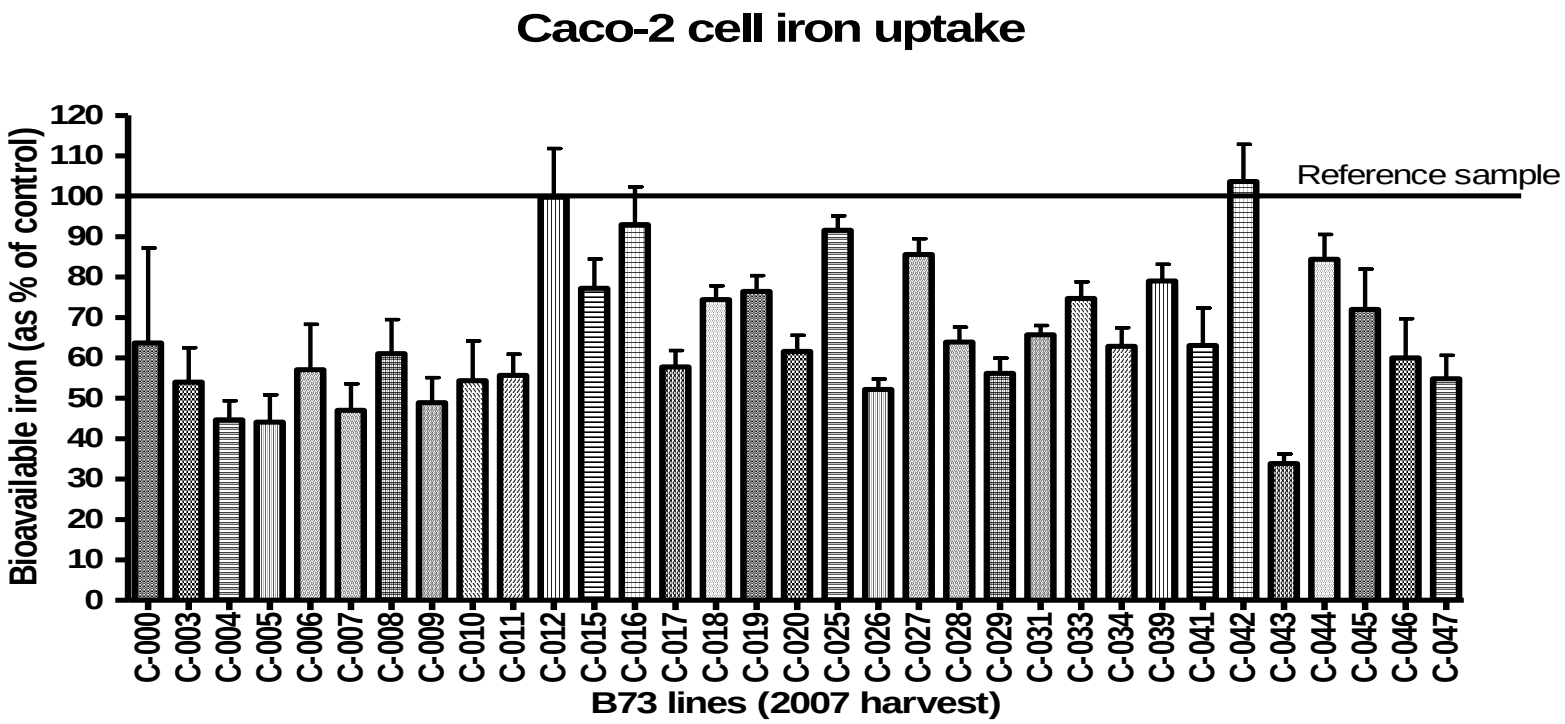

Figure 2.2: Caco-2 cell ferritin formation. The amount of bioavailable iron in the B73 lines was assessed using the Caco-2 cell model. Ferritin formation in the cells is an index of iron bioavailability. One gram (dry weight) of each sample was analyzed in the model. Bar values (mean ± SEM, $n = 6$).

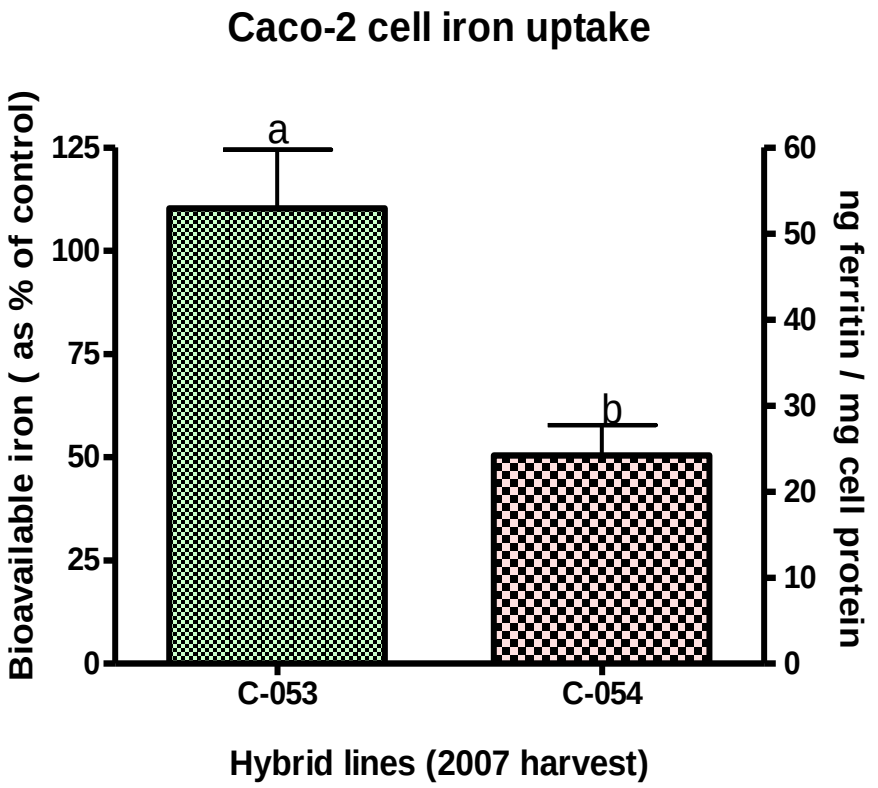

Figure 2.3: Caco-2 cell ferritin formation. The amount of bioavailable iron in the hybrid lines was assessed using the Caco-2 cell model. Ferritin formation in the cells is an index of iron bioavailability. One gram (dry weight) of each sample was analyzed in the model. Statistical analysis was performed by one-way ANOVA and student's t test. Column values (mean ± SEM, $n = 6$) with no letters in common are significantly different ($P < 0.05$).

(2b) Grain bioavailable iron – 2008 harvest: Figures 2.4 – 2.5 show a summary of grain bioavailable iron (as a percentage of the control) for the Mo17 and B73 lines from the 2008 harvest. The statistical model for grain iron bioavailability showed a significant difference in grain bioavailable iron in this IBM RI maize population. A student's t-test revealed a significant difference in grain bioavailable iron between the two groups—the Mo17 and B73 lines (P <.0001). There was a significant difference in grain bioavailable iron across samples within the two groups.

Grain bioavailable iron was significantly different within the Mo17 lines (P <.0001) and the B73 lines (P <.0001), respectively.

As mentioned earlier, maize samples harvested in 2008 were derived from maize lines from the 2007 harvest. A comparison of grain bioavailable iron between the parental lines (2007 harvest) and daughter lines (2008 harvest) revealed no significant differences in grain iron concentration between the 2008 maize samples and the parent 1 samples (P = 0.5688).

It is worth noting that the Caco-2 cell screening data from the 2007-2008 breeding cycle using the QTL approach isolated two hybrid maize lines from the 2007 maize population with identical grain iron concentration (20 ppm), but one line contained double the amount of bioavailable iron (P = 0.0064). In the 2008 maize population, we similarly found two inbred maize lines—sample 96 and sample 103—with similar grain iron concentration but one line had significantly more bioavailable iron (P < .0001). Grain iron nutrition details for the two inbred lines are shown in Table 2.3. These results further enforce our hypothesis that the breeding method employed is either enhancing levels of an iron bioavailability promoter, suppressing an iron bioavailability inhibitor, or a combination of both. Additional research is required to profile and identify the compounds.

Table 2.3: Grain iron concentration and bioavailable iron from inbreeds 96 and 103.

	Inbred line (2008 harvest)	
	96	**103**
Mean grain iron concentration (ppm)	25.14	24.75
Caco-2 cell ferritin formation (ng/mg; mean ± SEM)	4.0 ± 0.2	29.0 ± 1.2

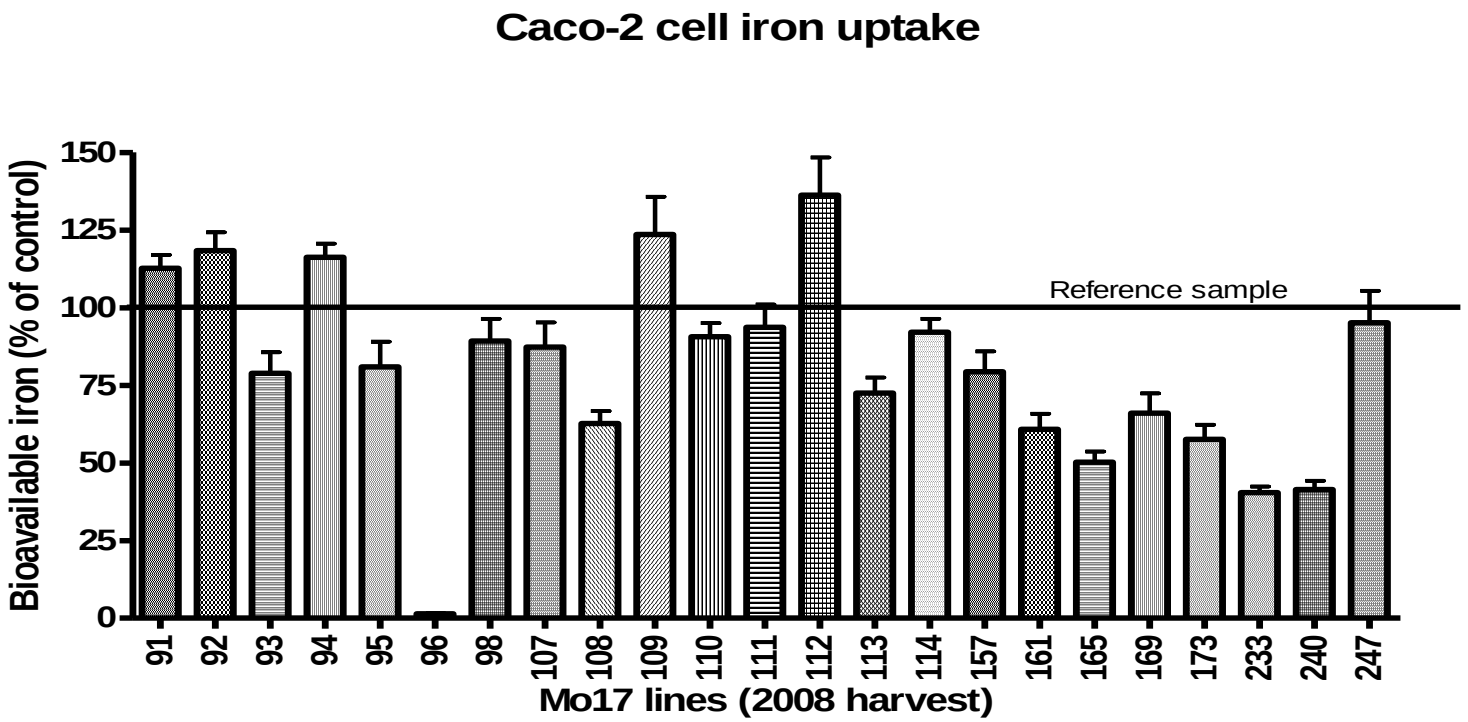

Figure 2.4: Caco-2 cell ferritin formation. The amount of bioavailable iron in the Mo17 lines (2008 harvest) was assessed using the Caco-2 cell model. Ferritin formation in the cells is an index of iron bioavailability. One gram (dry weight) of each sample was analyzed in the model. Bar values (mean ± SEM, $n = 6$).

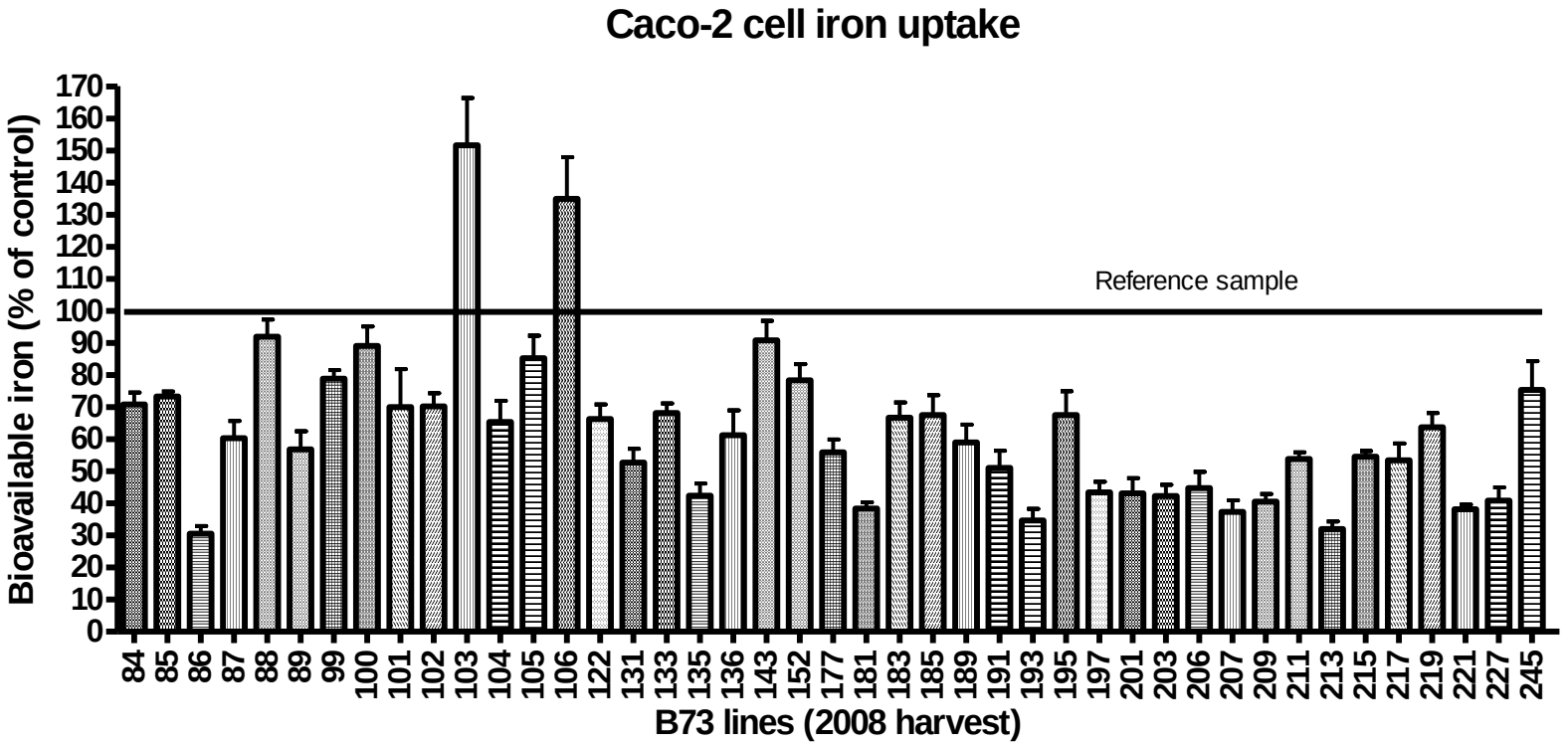

Figure 2.5: Caco-2 cell ferritin formation. The amount of bioavailable iron in the B73 lines (2008 harvest) was assessed using the Caco-2 cell model. Ferritin formation in the cells is an index of iron bioavailability. One gram (dry weight) of each sample was analyzed in the model. Bar values (mean ± SEM, $n = 6$).

Discussion

Studies on iron bioavailability have shown that simply increasing grain iron content may not automatically result in improved iron bioavailability in the grain. It is therefore a challenge when limited resources force breeders to choose between breeding for iron content and breeding for iron bioavailability. Our breeding work focused on breeding for iron bioavailability. The Caco-2 cell bioassay was used in this study as an *in vitro* screening tool for bioavailable iron. The *in vitro* iron model allows for rapid and cost-effective analysis of numerous genotypes and has produced iron bioavailability results that have been consistent with genetic data obtained through the QTL model.

The current breeding program that supports this study is based on the QTL model and has been ongoing for the past seven years. In 2002 - 2003, new genetic resources were created for a maize breeding program by Hoekenga (Owen Hoekenga, personal communication). The objective was to create maize lines from the IBM RI population that could be used for genetic analysis of multiple genetic traits in maize. The preliminary data indicated breeding for improved iron nutrition in maize was a complex task. Quantitative trait loci and marker-assisted analyses were carried out using both single-marker analysis (SMA) and composite interval mapping (CIM). The SMA that is less conservative identified ten QTL, while the more conservative CIM identified three QTL that influenced iron bioavailability in maize. The data also suggested that the iron biofortification breeding program should focus on grain iron bioavailability relative to grain iron concentration (Hoekenga, manuscript submitted for publication).

In 2002, the first cross between the B73 and IBM mapping lines was backcrossed to the original maternal parent (B73). The resulting maize material was self-pollinated (selfed) in 2005, producing about 600 sister maize lines that exhibited

either improved or diminished iron bioavailability potential. Due to limited resources only 37 of these lines that had the most promise for iron biofortification were evaluated using the Caco-2 cell assay. Analysis of grain iron concentration and grain iron bioavailability in these lines confirmed the 3 QTL predicted in the initial study and showed that iron traits were heritable and thus genetically tractable (Hoekenga et al., manuscript submitted for publication).

The information derived from the Caco-2 cell bioassay (grain iron bioavailability data) together with the information obtained from the QTL model (loci identity) was then used to generate new maize varieties. Improved maize lines exhibited superior QTL while the diminished lines exhibited inferior QTL (the three iron bioavailability QTL isolated using CIM). In 2007, new lines were cross-pollinated (improved vs. improved and diminished vs. diminished) to produce two hybrids with improved and diminished iron bioavailability potential, respectively. As seen in the results section, the hybrid pair had the same grain iron concentration but one line contained double the amount of bioavailable iron ($P < 0.001$). Based on these results, two new hybrid lines were created to produce a modest amount of grain that would be used to test iron bioavailability in a poultry model. The results of this study are presented in chapter 4 of this dissertation.

Also highlighted in the results section are two inbred maize lines—sample 96 and sample 103—from the 2008 maize population. These maize lines are similar to the previously highlighted results of the 2007 hybrid maize lines. They showed identical grain iron concentration (25 ppm) but one line had significantly more bioavailable iron. These data suggest the following: (1) grain iron concentration in this maize population is not significantly associated with grain iron bioavailability and (2) the iron biofortification approach used affects iron bioavailability in maize by either enhancing or suppressing secondary metabolites that affect iron bioavailability. It is

therefore plausible that the QTL approach is breeding for secondary compounds that enhance iron bioavailability in maize. It could be that the breeding method suppresses iron inhibitory compounds in the maize. Or that both scenarios play out at the same time, enhancing iron promoters while suppressing iron inhibitors. It is also possible that the QTL approach is breeding for secondary compounds that inhibit iron bioavailability in maize. Metabolic profiling of the hybrids and inbred lines is required to answer this question conclusively.

This study has yielded additional evidence that QTL information can be combined with iron bioavailability data from the Caco-2 cell bioassay to experimentally create or select candidate maize lines with either superior or inferior iron nutrition qualities. Multi-year breeding studies have shown that the Caco-2 cell/QTL method gives consistent results and can be used to breed for improved iron nutrition in maize provided that the resulting lines give the expected results *in vivo*. Further research is required to search for additional QTL that may influence iron bioavailability in maize and to determine the mode of gene action in the superior allele maize lines. The current research work has focused mainly on temperate maize varieties adopted for iron nutrition research. However, the target populations for iron biofortification grow and consume mainly tropical maize varieties. Concomitant iron biofortification efforts should therefore also focus especially on collaborating with maize breeders in Sub-Saharan Africa to transfer superior alleles into tropical and sub-tropical elite maize lines, assess bioavailability and genotype-by-environment interaction, and finally conduct *in vivo* trials to determine the efficacy of consuming such high bioavailable iron maize.

Conclusion

The IBM RI population selected for this study was based on easy availability and the existence of advanced backcross families for rapid development of near-isogenic lines. The alleles present in IBM RI may not represent the best alleles available for improved iron nutrition quality in maize grain. For optimal effect, the breeding program should therefore expand to identify any additional alleles that could be included in the breeding process. And although iron biofortification of maize via conventional breeding is a complex task, both grain iron content and iron bioavailability are genetically tractable traits. The Caco-2 cell/ QTL approach, coupled with *in vivo* testing can significantly improve iron bioavailability in maize grain. To make further progress, additional work in molecular genetics as well as metabolite testing is required. Collaboration with maize breeders in Sub-Saharan countries should also be pursued.

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CHAPTER 3

ASSESSMENT OF GENOTYPE X ENVIRONMENT INTERACTION IN IRON BIOFORTIFIED MAIZE

Abstract

This study was undertaken with two objectives: to evaluate the effects of interaction between genotype and environment (G×E) and to determine the association between soil iron status on grain iron concentration and the amount of bioavailable iron in six maize genotypes grown in five diverse locations. Grain iron concentration was determined using emission spectroscopy and grain iron bioavailability was assessed using the Caco-2 cell bioassay. The six maize genotypes tested were derived from the Intermated *B*73 x *M*o17 recombinant inbred maize population and grown in selected locations in New York, Missouri, North Carolina, Iowa, and Pennsylvania. Grain iron concentration ranged from 17.34 ppm – 36.32 ppm. Grain iron bioavailability ranged from 36.39% below to 75.26% above that of the reference control sample. The results indicated a significant G×E interaction for both grain iron concentration and grain iron bioavailability ($P < .0001$). Correlation data indicated a highly significant ($P < .0001$) negative correlation between total soil iron and grain iron bioavailability but no significant association between either soil extractable iron and grain iron concentration or grain iron bioavailability. We conclude that (G×E) interaction has an effect on iron trait expression.

Introduction

Maize (*Zea mays* L.) is one of the most important staple food crops in the world. In Africa and Latin America, maize kernels are processed into a variety of traditional food products such as pastes, gruels, porridges, tortillas and polenta. The rural poor in these geographical regions rely mostly on monotonous cereal-based diets to meet their nutrient requirements. Improving the nutritional quality of cereals such as maize can therefore have a significant impact on their nutritional status.

Biofortification is an agricultural intervention that seeks to improve human health by improving micronutrient levels in staple foods (Nestle et al., 2006). Using conventional plant breeding, the biofortification strategy can significantly contribute to alleviating micronutrient deficiencies such as iron deficiency anemia (IDA). To do so the first step is to ensure that the enhancement of nutritional quality is under genetic control. Next, possible interaction between genotypes and the environment (G×E) must be investigated. To succeed, the potential enhancement of iron nutritional quality in maize must be relatively stable across diverse environments (Welch & Graham, 2004). Recent studies have reported wide variations in grain iron concentration in maize (Pfeiffer & McClafferty, 2007). One study that evaluated 1,814 maize germplasms reported grain iron concentration ranging between 9.6 and 63.2 mg/kg. These variations in concentration were attributed to both genetic and environment effects (Bänziger & Long, 2000).

The expression of a phenotype of an individual plant is determined by both the genotype and the environment. However, these two effects are not always additive because of interaction between the genotype and the environment (G×E).

Equation 3.1: Phenotype = Genotype + Environment + (G×E) interaction

G×E interaction is a result of inconsistent genotype performance across environments caused by variations due to location and/or climatic zone. A significant G×E interaction results from changes in the magnitude of differences between genotypes in diverse environments or from changes in the relative ranking of the genotypes (Fernandez, 1991). To make any substantial progress in biofortification efforts, it is vital that breeders evaluate and understand factors that contribute to G×E interactions in their breeding programs, as this will reflect trait heritability, genetic variation, and potential genetic gains.

An increasing body of evidence suggests that the expression of iron in cereals such as maize can be subject to G×E interaction (Hoekenga et al., unpublished data). The goal of our study was to assess the G×E interaction in maize derived from the *I*ntermated *B*73×*M*o17 (IBM) maize population grown in diverse locations. The selected lines had been grown in Aurora, New York in 2007 and chosen for further study including the G×E evaluation study based on their iron bioavailability potential determined using the Caco-2 cell *in vitro* iron model. In 2008, the same lines were again grown in the New York location, and by our collaborators in selected locations in Missouri, North Carolina, Iowa, and Pennsylvania. The objectives of the study were: (i) to analyze grain iron concentration and amount of bioavailable iron from grain samples grown in our selected locations; (ii) to determine the association between soil iron status and both grain iron concentration and amount of bioavailable iron from grain samples grown in our selected locations; and (iii) to evaluate grain iron trait stability by analyzing the effects of genotype, environment and G×E interaction on grain iron concentration and amount of bioavailable iron.

Materials and Methods

Chemicals, enzymes, and hormones: Unless otherwise stated, all chemicals, enzymes, and hormones were purchased from Sigma Chemicals Co. To prepare reagents for cell culture, 18 MΩ water was used. Glassware and utensils used in the experiments were soaked in 1.2 M HCl for at least 4 hours and rinsed in deionized water prior to use.

Plant materials and field site details: The maize varieties tested are derived from the *I*ntermated *B*73 x *M*o17 (*IBM*) recombinant inbred (RI) maize population. These lines were selected for this study on the basis of their iron bioavailability potential, as indicated by the Caco-2 cell bioassay. Table 3.1 summarizes the genotype description of the maize varieties. Four derivatives from the IBM RI population were created to demonstrate significant differences in grain iron nutritional quality in maize. Near-isogenic lines were derived from IBM RIL #264 by back-crossing to the B73 parent (high iron nutritional quality B73 or "HB" and low iron nutritional quality B73 or "LB") and from IBM RIL #039 by back-crossing to the Mo17 parent ("HM" and "LM"). The four near-isogenic varieties (HB, LB, HM, and LM) and their respective hybrids (see Table 3.1) were initially grown in 2007 in Aurora, New York and analyzed for iron concentration and amount of bioavailable iron. Based on their iron bioavailability potential, the maize lines were grown at research farms owned by Cornell University (Aurora, NY), Iowa State University (Boone, IA), North Carolina State University (Clayton, NC), The Pennsylvania State University (Fairpoint, PA), and The University of Missouri (Columbia, MO) in the Summer of 2008.

Table 3.1: Genotype description of the maize varieties.

Maize sample number[1]	Description[2]	Iron bioavailability potential[3]	Genotype number
1/ 1a/1b*	IBM264 BC2 B73 S3	Low (LB)	**1**
2/2a/2b	IBM039 BC2 Mo17 S3	Low (LM)	**2**
3/3a/3b	LB×LM hybrid	Low	**3**
4/4a/4b	IBM264 BC2 B73 S3	High (HB)	**4**
5/5a/5b	1BM039 BC2 Mo17 S3	High (HM)	**5**
6/6a/6b	HB×HM hybrid	High	**6**

1. Maize varieties grown in 2008 in five selected locations; *some locations had replicate plots, hence the reference to a/b. Six genotypes were grown in each location.

2. BC = Backcross; S = Self.

3. Iron bioavailability potential is based on the Caco-2 cell analysis of maize grown in Aurora, NY in 2007. Low iron nutritional quality B73 and Mo17 lines are abbreviated LB and LM, respectively. High iron nutritional quality B73 and Mo17 lines are abbreviated HB and HM, respectively.

Table 3.2 gives a brief description of the selected locations where the maize trials were planted. The plots used in Aurora, New York had a Lima Silt Loam soil, with average yield for maize of 120 bushels acre^{-1} and water extractable soil pH of 6.7. The plots used in Boone, Iowa had a Webster Clay Loam soil, with average yield for maize of 210 bushels acre^{-1} and water extractable soil pH of 6.1. The plots used in Clayton, North Carolina had a Norfolk Loamy Sand soil, with average yield for maize of 106 bushels acre^{-1} and water extractable soil pH of 6.4. The plots used in Fairpoint, Pennsylvania had a Hagerstown Silt Loam soil, with average yield for maize of 135 bushels acre^{-1} and water extractable soil pH of 5.9. The plots used in Columbia, Missouri had a Leonard Silt Loam soil, with average yield for maize of 176 bushels acre^{-1} and water extractable soil pH of 6.3. These descriptions of soil conditions were obtained from the Web Soils Survey of the National Resource Conservation Service (http://websoilsurvey.nrcs.usda.gov).

Table 3.2: Brief description of selected locations where trials were planted.

Location[1]	Ecozones	Altitude (meters)	Annual Min/Max Temp (° C)	Mean annual rainfall (cm)
Ames, IA	Temperate grasslands /savanna and shrubland	304.80	3.4 - 16.7	92.20
Columbia, MO	Temperate broadleaf /mixed forest	243.84	6.4 - 19.5	102.31
Clayton, NC	Temperate broadleaf /mixed forest	106.68	8.3 - 22.2	116.08
Aurora, NY	Temperate broadleaf /mixed forest	125.88	3.9 - 14.2	93.93
State College, PA	Temperate broadleaf /mixed forest	384.05	4.2 - 15	100.99

1. IA = Iowa, MO = Missouri, NC = North Carolina, NY = New York, PA = Pennsylvania.

Maize sample preparation: Maize kernels (20g) were sorted to remove any debris or damaged seeds and then placed in a 50 mL centrifuge tube and covered with 25 mL of 18 MΩ water. Samples were then autoclaved at 121° C and at a pressure of 115 kPa for 40 min, allowed to cool at room temperature, and then frozen overnight at –20 °C. Samples were then freeze-dried at 100 millTorr and a temperature of –50 °C for 7 days, ground to a fine powder with a coffee mill (90 sec), and stored in 50 mL centrifuge tubes at 25 °C. A tamale maize sample, used as a control in the Caco-2 cell bioassay, was prepared in an identical manner.

Mineral analysis for maize samples: A 0.3g dry ground maize sample was weighed into borosilicate glass test tubes and chemically digested using 4 mL of concentrated nitric acid at 120 °C until the residue was light brown to yellow in color. Exactly 1.0 ml of a 50/50 mixture of concentrated nitric acid and perchloric acid was then added, and the temperature was increased to 180 °C. After 2 hours, the temperature was further increased to 240 °C until the digested samples were dry. After cooling, 0.25 mL of concentrated hydrochloric acid was added to dissolve the ash. One hour later, the sample was diluted with 10 mL of 5% nitric acid. The ashed sample was then mixed and transferred into 15 mL auto sampler tubes and analyzed on an axially viewed inductively coupled plasma (ICP) trace analyzer emission spectrometer (model ICAP 61E trace analyzer, Thermo Electron, Waltham, MA; see Rutzke, 2002).

Caco-2 cell screening: The *in vitro* Caco-2 cell iron model was used as a screening tool to estimate the amount of bioavailable iron in the maize samples.

Cell Culture: Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) at passage 17 and used in experiments at passages 25–33. Cells were seeded at a density of 50,000 cells/cm^2 in collagen-treated six-well plates (Costar Corp., Cambridge, MA). The cells were grown in Dulbecco's Modified Eagle

Medium (GIBCO, Grand Island, NY) with 10% v/v fetal calf serum (GIBCO), 25 mmol/L N-(2-Hydroxyethyl) piperazine-N'-2-ethane sulfonic acid (HEPES), and 1% antibiotic antimycotic solution (GIBCO). The cells were maintained at 37°C in an incubator with a 5% CO_2/95% air atmosphere at constant humidity, and the medium was changed every 2 days. The cells were used in the iron uptake experiments at 13 days post seeding.

In Vitro Digestion: The preparation of the digestion solutions—pepsin, pancreatin, and bile extract—and the *in vitro* digestion was performed as previously published (Glahn et al., 1998). Briefly, a 1.0 g dry sample was used for each sample digestion. Ascorbic acid (AA) was added to enhance iron bioavailability using a 20:1 AA: Fe molar ratio. Then 10 ml of pH 2 140 mM NaCl, 5 mM KCl buffer was added to the samples, and the mixture was pH re-adjusted to pH 2. Following this step, 0.5 mL of pepsin solution was added to each of the samples prior to incubation. After a 1-hour incubation period, the sample pH was adjusted to pH 5.5-6.0 with 1.0 M $NaHCO_3$, and after the addition of 2.5 mL of pancreatin-bile solution, the sample pH was further adjusted to pH 6.9-7.0 with 1.0 M $NaHCO_3$. Sample volumes were then adjusted (by weight) to tube weight plus 15 g using the 140 mM NaCl, 5 mM KCl pH 6.7. Finally, 1.5 mL of the samples was transferred to appropriate inserts on the Caco-2 cell plates.

Harvesting: The harvesting of the Caco-2 cell monolayers was performed as previously published (Glahn et al., 1998). The cells were harvested after a 24-hour incubation period. First, growth media were carefully aspirated off the cells. The cells were then rinsed twice with 2 mL of 130mM NaCl, 5 mM PIPES, pH 6.7 buffer. To harvest the cells, 2 mL of 18.2 MΩ water was added to the cells, and the cells were placed in a sonicator (Lab-line Instruments, Melrose Park, IL) at 4°C for 15 minutes.

The cells were then scraped off the plates, suspended in the 2 mL of 18.2 MΩ water, and transferred to pre-labeled 5 ml tubes in anticipation of protein and ferritin assays.

Cell Protein Analysis: Caco-2 cell protein was measured on samples that had been solubilized in 0.5 mol/L NaOH, using a semimicro adaptation of the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). A 25 μL sample of the sonicated Caco-2 cell monolayer, harvested in 2 mL of water, was used for each protein measurement expressed in mg.

Ferritin Analysis: A one-stage, two-site immunoradiometric assay was used to measure Caco-2 cell ferritin content (FER-Iron II Ferritin Assay, RAMCO Laboratories, Houston, TX). A 10 μL sample of the sonicated Caco-2 cell monolayer, harvested in 2 mL of water, was used for each ferritin measurement expressed per unit of cell protein (ng ferritin/mg cell protein).

Soil Analysis: Soil samples from the five growing locations were analyzed for organic matter and pH. The analyses were conducted by the Cornell Nutrient Analysis Laboratory (Ithaca, NY). In their tests, lab staff followed methods provided in the Soil Survey Laboratory Methods Manual developed by the National Soil Survey Center (National Resources Conservation Service, United States Department of Agriculture).

Statistical Analyses: Statistical analyses of the data were performed using the GraphPad Prism v4 (GraphPad Software, San Diego, CA) and *JMP* v7.0 (SAS Institute Inc., Cary, NC) software packages. In some cases, the data were log-transformed prior to analysis. Means were considered to be significantly different for P values ≤ 0.05.

Results

The goal of this study was to determine if grain iron concentration and grain iron bioavailability in the six maize genotypes were expressed consistently across all

five diverse locations. This is an important assessment because the success of iron biofortified maize in addressing iron malnutrition, especially in diverse regions such as Sub-Saharan Africa, will depend to a large extent on the stability of these iron traits.

(a) Grain iron concentration: The grain iron concentration of the six maize genotypes grown in diverse environments was analyzed using emission spectroscopy. Table 3.3 shows the grain iron concentration for each genotype by location. The grain iron concentration ranged from 17.34 ppm (Genotype 1b, Iowa) – 36.32 ppm (Genotype 4, New York). The mean grain iron concentration was 26.27 ppm.

Table 3.3: Grain iron concentration of maize varieties grown in five diverse locations.

Maize sample number[1]	Grain iron concentration by location[2] (ppm)				
	IA	**MO**	**NC**	**NY**	**PA**
1/ 1a	17.6 ± 0.4	23.4 ± 4.0	22.4 ± 1.2	19.1 ± 0.1	19.7 ± 0.6
1b*	17.3 ± 0.7	25.3 ± 0.1	25.8 ± 0.4	-	-
2/2a	25.2 ± 0.4	21.2 ± 0.2	24.0 ± 0.8	20.8 ± 0.3	21.1 ± 0.3
2b	21.3 ± 0.2	20.5 ± 0.2	23.6 ± 5.3	-	-
3/3a	21.3 ± 0.8	22.8 ± 0.7	28.6 ± 0.7	19.9 ± 0.2	19.3 ± 0.1
3b	19.0 ± 0.5	30.4 ± 0.3	19.1 ± 0.4	19.6 ± 0.5	-
4/4a	24.6 ± 0.8	20.8 ± 0.7	19.0 ± 0.4	36.3 ± 0.6	20.8 ± 0.1
4b	25.5 ± 0.2	27.2 ± 6.0	30.1 ± 2.3	-	-
5/5a	24.6 ± 0.1	30.1 ± 2.9	24.7 ± 0.6	27.5 ± 1.1	25.4 ± 0.5
5b	21.7 ± 0.6	21.6 ± 0.8	20.7 ± 0.5	-	-
6/6a	23.6 ± 0.2	23.5 ± 1.7	21.4 ± 0.4	25.6 ± 1.6	21.5 ±0.1
6b	22.9 ± 0.5	22.2 ± 1.0	23.7 ± 1.0	30.4 ± 0.4	-

1. Maize varieties grown in 2008 in five selected locations; *some locations had replicate plots, hence the reference to a/b. Six varieties (genotypes) were grown in each location.

2. Mean ± SD for; IA = Iowa, MO = Missouri, NC = North Carolina, NY = New York, PA = Pennsylvania.

A statistical analysis model was used to assess the effects of environment, genotype, and the G×E interaction for grain iron concentration. As seen in Table 3.4, there was a strong significant interaction term (G×E), and a main effect of genotype.

Table 3.4: Fixed-effects tests for grain iron concentration (*JMP* v7)

Source of variation	DF	F Ratio	Prob > F
Environment	4	3.9162	0.1266
Genotype	5	12.2342	<.0001
*Genotype*Environment*	20	6.1573	<.0001

Further analysis of the G×E interaction was performed using contrast testing across environments (Table 3.5). The data revealed significant differences in grain iron concentration for genotypes 1, 3, 4, and 6 across the five locations—those in Iowa, Missouri, North Carolina, New York, and Pennsylvania. The grain iron concentration for genotypes 2 and 5 was not significantly different across the five locations.

Table 3.5: Comparison of grain iron concentration across environments (*JMP* v7)

Source of variation: ***Environment***	DF	F Ratio	Prob > F
Genotype 1	4	8.2328	<.0001
Genotype 2	4	1.3043	0.2875
Genotype 3	4	5.6272	0.0012
Genotype 4	4	11.3467	<.0001
Genotype 5	4	1.5496	0.2095
Genotype 6	4	4.0832	0.0097

Assessment of the G×E interaction based on genotype (Table 3.6) indicated significant differences in grain iron concentration between the six genotypes in the Iowa, Missouri, and New York locations. The difference in grain iron concentration

between genotypes in the Pennsylvania location was significant after a bonferroni correction for multiple comparisons. In the North Carolina location, there was no significant difference in grain iron concentration across the six genotypes.

Table 3.6: Comparison of grain iron concentration between genotypes (*JMP* v7)

Source of variation: *Genotype*	**DF**	**F Ratio**	**Prob > F**
IOWA	5	7.5779	<.0001
MISSOURI	5	4.1447	0.0010
NORTH CAROLINA	5	1.1416	0.3425
NEW YORK	5	19.1962	<.0001
PENNSYLVANIA	5	2.3561	0.0445

(b) Grain Iron bioavailability: Because the six maize genotypes were chosen for the G×E evaluation study based on their iron bioavailability potential we analyzed the iron bioavailability data in two ways; 1) Group data analysis for all the six growing locations, and 2) Individual data analysis for the individual growing locations.

(1) Group data analysis: Amount of bioavailable iron was assessed using the Caco-2 bioassay (Figure 3.1). The mean amount of bioavailable iron ranged from 36.39% below (Genotype 1; Pennsylvania) to 75.26% above (Genotype 5b; Missouri) that of the reference control sample. The statistical analysis model assessed the effects of environment, genotype, and the interaction of the two (G×E) for grain iron bioavailability. As seen in Table 3.7, there was a strong significant interaction term (G×E; P <.0001) and a significant main effect of both genotype (P <.0001) and environment (P = 0.0025).

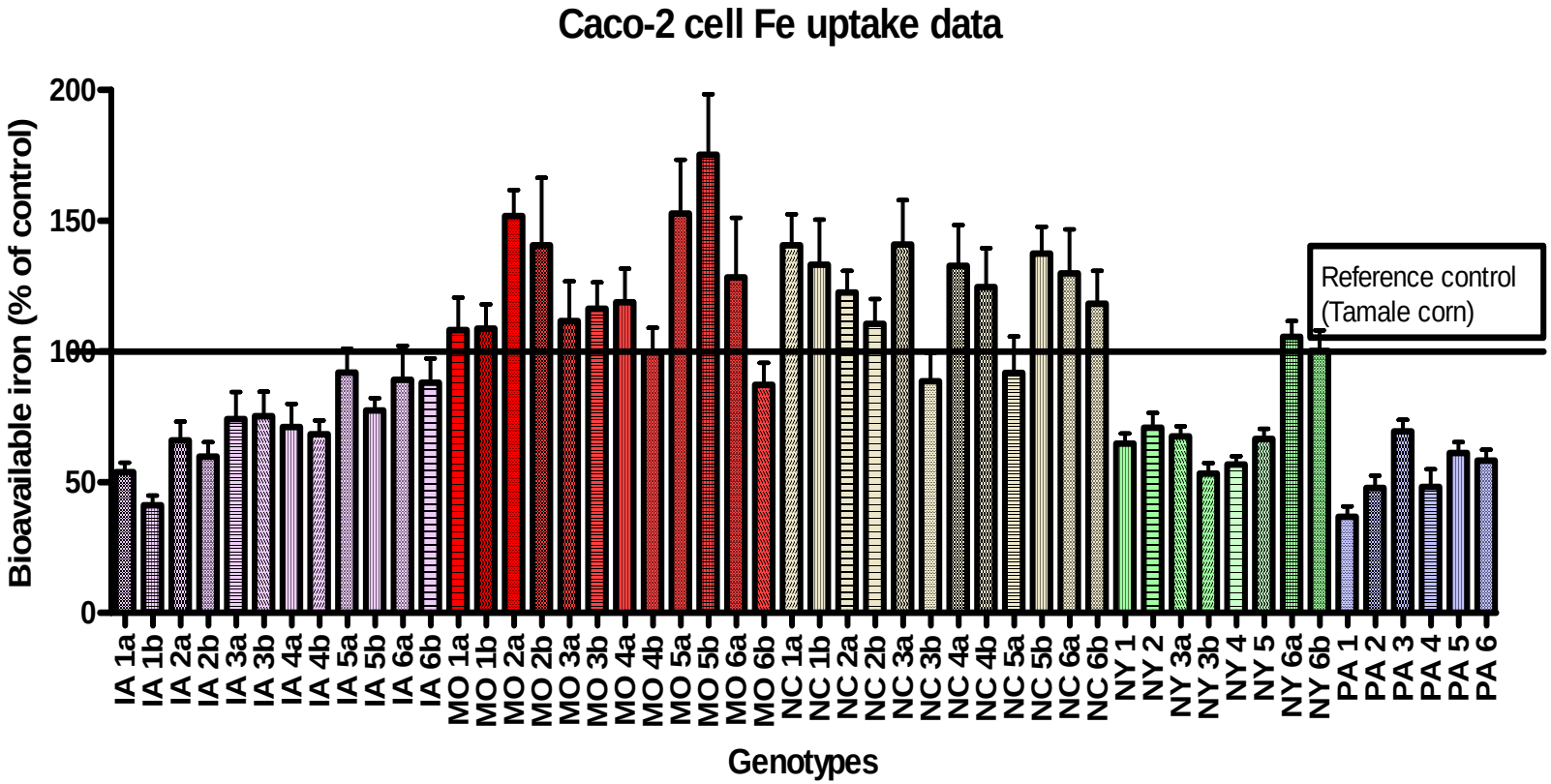

Figure 3.1: Caco-2 cell ferritin formation. The amount of bioavailable iron in G×E maize lines was assessed with the use of the Caco-2 cell model. Ferritin formation in the cells is an index of iron bioavailability. One gram (dry weight) of each sample was analyzed in the model. Bar values (mean ± SEM, *n* = 6). G×E = Genotype by Environment.

Table 3.7: Fixed-effects tests for grain iron bioavailability (*JMP* v7)

Source of variation	DF	F Ratio	Prob > F
Environment	4	72.6915	0.0025
Genotype	5	7.5441	<.0001
Genotype*Environment	20	5.0377	<.0001

Further analysis of the G×E interaction based on environment (Table 3.8) revealed significant differences in grain iron bioavailability for all six genotypes across all five locations.

Table 3.8: Comparison of grain iron bioavailability across environments (*JMP* v7)

Source of variation: ***Environment***	DF	F Ratio	Prob > F
Genotype 1	4	39.5151	<.0001
Genotype 2	4	26.4247	<.0001
Genotype 3	4	12.4183	<.0001
Genotype 4	4	20.8360	<.0001
Genotype 5	4	18.6711	<.0001
Genotype 6	4	8.1758	<.0001

Additionally, the assessment of the G×E interaction for grain iron bioavailability based on genotype (Table 3.9) showed a strong significant difference in grain iron bioavailability among the six genotypes in the Iowa, Missouri, New York, and Pennsylvania locations. There was no significant difference in grain iron bioavailability among genotypes grown in the North Carolina location.

Table 3.9: Comparison of grain iron bioavailability among genotypes (*JMP* v7)

Source of variation: ***Genotype***	DF	F Ratio	Prob > F
IOWA	5	9.090	<.0001
MISSOURI	5	5.7933	<.0001
NORTH CAROLINA	5	1.1087	0.3561
NEW YORK	5	6.8874	<.0001
PENNSYLVANIA	5	4.9439	0.0002

(2) Individual data analysis: As earlier mentioned, six maize genotypes were grown in five diverse environments to evaluate the G×E interaction. The objective of the individual data analysis was to further analyze the six maize genotypes in more detail, so as to evaluate location-specific changes in the magnitude of differences between genotypes or changes in the relative ranking of the genotypes. It was expected, based on genotypic data, that samples 1, 1a, 1b, 2, 2a, 2b, 3, 3a, and 3b would exhibit low bioavailability potential, while samples 4, 4a, 4b, 5, 5a, 5b, 6, 6a, and 6b were expected to exhibit high bioavailability potential, with significant differences between the two groups. The data analysis also compared the B73, Mo17, and hybrid pair lines- LB vs. LM, HB vs. HM, and low vs. high respectively to determine which of the two lines showed the expected responses in the individual locations.

(i) Grain iron bioavailability – **Iowa**: There was a strong significant difference in grain iron bioavailability among the six genotypes in the Iowa location ($P < .0001$). Table 3.10 shows least square means differences in grain iron bioavailability determined using Tukey HSD. Iron bioavailability of low bioavailability potential inbreds and hybrids - 1a, 2a, 2b, 3a and 3b - was to expectation, with no significant differences between these samples. Only sample 1b was significantly different from the hybrids. Iron bioavailability of high bioavailability potential inbreds and hybrids – 4a, 4b, 5a, 5b, 6a and 6b - was to expectation, with no significant differences between these samples. Comparing inbred and hybrid pair lines - samples 1 vs. 4, 2 vs. 5 and 3 vs. 6 - that were expected to have different iron bioavailability potentials, the data showed significant differences in iron bioavailability in some of the inbreds but not the hybrids.

Table 3.10: Least square means differences in grain iron bioavailability (Iowa)

Sample	Iron bioavailability potential					Least Sq Mean
IA 5a	High Mo17	A				61.214394
IA 6b	High hybrid	A	B			58.113211
IA 6a	High hybrid	A	B			57.775122
IA 5b	High Mo17	A	B	C		51.418968
IA 3b	**Low hybrid**	A	B	C		48.682535
IA 3a	**Low hybrid**	A	B	C		47.804331
IA 4a	High B73	A	B	C		46.648502
IA 4b	High B73	A	B	C		45.234433
IA 2a	**Low Mo17**		B	C	D	43.494930
IA 2b	**Low Mo17**			C	D	39.508113
IA 1a	**Low B73**			C	D	36.003422
IA 1b	**Low B73**				D	27.242046

Levels not connected by same letter are significantly different.

(ii) Grain iron bioavailability – **Missouri**: There was a strong significant difference in grain iron bioavailability among the six genotypes in the Missouri location ($P < .0001$). Table 3.11 shows least square means differences in grain iron bioavailability determined using Tukey HSD. Iron bioavailability of low bioavailability potential inbreds and hybrids - 1a, 1b, 2b, 3a, and 3b was similar but significantly differently from sample 2a. Iron bioavailability of high bioavailability potential inbreds and hybrids – 4a, 4b, 5a, 5b, 6a and 6b - was erratic, with significant differences between sample 5b and samples 4a, 4b, 6a and 6b; and sample 5a and samples 4b and 6b. Comparing inbred and hybrid pair lines - samples 1 vs. 4, 2 vs. 5 and 3 vs. 6 - that were expected to have different iron bioavailability potentials, the data showed no significant differences in iron bioavailability of the inbreds or hybrids.

Table 3.11: Least square means differences in grain iron bioavailability (Missouri)

Level	Iron bioavailability potential						Least Sq Mean
MO 5b	High Mo17	A					61.826652
MO 2a	**Low Mo17**	A	B				55.229579
MO 5a	High Mo17	A	B	C			53.613752
MO 2b	Low Mo17	A	B	C	D		48.195062
MO 6a	**High hybrid**		B	C	D	E	43.961985
MO 4a	**High B73**		B	C	D	E	42.068521
MO 3b	Low hybrid		B	C	D	E	41.608298
MO 3a	Low hybrid			C	D	E	39.640411
MO 1b	**Low B73**				D	E	39.019928
MO 1a	**Low B73**				D	E	38.229385
MO 4b	**High B73**				D	E	35.876057
MO 6b	**High hybrid**					E	31.276452

Levels not connected by same letter are significantly different.

(iii) Grain iron bioavailability – **North Carolina**: There was a strong significant difference in grain iron bioavailability among the six genotypes in the North Carolina location ($P < .0001$). Table 3.12 shows least square means differences in grain iron bioavailability determined using Tukey HSD. Iron bioavailability was significantly different in some of the low bioavailability potential inbreds and hybrids, and some of the high bioavailability potential inbreds and hybrids. Comparing inbred and hybrid pair lines - samples 1 vs. 4, 2 vs. 5 and 3 vs. 6 - that were expected to have different iron bioavailability potentials, the data showed no significant differences in iron bioavailability between the pair groups but showed block differences between samples 3a and 3b, and samples 5a and 5b.

Table 3.12: Least square means differences in grain iron bioavailability (North Carolina)

Level	Iron bioavailability potential			Least Sq Mean
NC 1a	Low B73	A		60.436106
NC 3a	Low hybrid	A		59.734394
NC 5b	High Mo17	A		59.261932
NC 4a	High B73	A		56.705525
NC 1b	Low B73	A		56.222450
NC 6a	High hybrid	A		55.375447
NC 2a	Low Mo17	A	B	53.093886
NC 4b	High B73	A	B	52.981395
NC 6b	High hybrid	A	B	50.779334
NC 2b	Low Mo17	A	B	47.491052
NC 5a	High Mo17		B	38.570019
NC 3b	Low hybrid		B	37.541426

Levels not connected by same letter are significantly different.

(iv) Grain iron bioavailability – **New York**: There was a strong significant difference in grain iron bioavailability among the six genotypes in the New York location ($P < .0001$). Table 3.13 shows least square means differences in grain iron bioavailability determined using Tukey HSD. Iron bioavailability of low bioavailability potential inbreds and hybrids - 1, 2, 3a and 3b was similar. Iron bioavailability of the high bioavailability potential hybrids (sample 6a and 6b) was significantly different from that of the inbreds (samples 4 and 5). Comparing inbred and hybrid pair lines - samples 1 vs. 4, 2 vs. 5 and 3 vs. 6 - that were expected to have different iron bioavailability potentials, the data showed significant differences in iron bioavailability only in the hybrid pairs – samples 3 vs. 6.

Table 3.13: Least square means differences in grain iron bioavailability (New York)

Level	Iron bioavailability potential			Least Sq Mean
NY 6b	High hybrid	A		59.430278
NY 6a	High hybrid	A		55.778394
NY 2	Low Mo17		B	39.701743
NY 3b	Low Hybrid		B	37.855076
NY 5	High Mo17		B	37.714671
NY 1	Low B73		B	36.388594
NY 4	High B73		B	31.960686
NY 3a	Low Hybrid		B	29.945763

Levels not connected by same letter are significantly different.

(v) Grain iron bioavailability – **Pennsylvania**: There was a significant difference in grain iron bioavailability among the six genotypes in the Pennsylvania location ($P < .0003$). Table 3.14 shows least square means differences in grain iron bioavailability determined using Tukey HSD. Iron bioavailability of low bioavailability potential inbreds (samples 1 and 2) were similar but significantly different from the hybrid (sample 3). Iron bioavailability of the high bioavailability potential inbreds and hybrids (samples 4, 5 and 6) was not significantly different. Comparing inbred and hybrid pair lines - samples 1 vs. 4, 2 vs. 5 and 3 vs. 6 - that were expected to have different iron bioavailability potentials, the data showed no significant differences between samples.

Table 3.14: Least square means differences in grain iron bioavailability (Pennsylvania)

Level	Iron bioavailability potential				Least Sq Mean
PA 3	Low hybrid	A			57.838923
PA 5	High Mo17	A	B		51.332505
PA 6	High hybrid	A	B		48.445494
PA 4	High B73		B	C	41.599891
PA 2	Low Mo17		B	C	39.595536
PA 1	Low B73			C	30.189753

Levels not connected by same letter are significantly different.

Overall, analysis of the individual locations showed the expected trend more in superior allele lines (samples 4, 5 and 6) compared to inferior allele lines (samples 1, 2 and 3). Also, iron bioavailability in Mo17 lines was more to expectation compared to B73 lines, which may suggest that B73 lines are more subject to G×E interaction.

(c) Soil Analysis: An important goal in this study was to determine the association between soil iron status and both grain iron concentration and amount of bioavailable iron from grain samples grown in our selected locations. Table 3.15 highlights some of the soil data collected in this study.

Table 3.15: Highlights of soil data collected from locations where trials were planted

Location	*Soil depth (cm)*	*Row Number*	*Extracted Fe (mg/kg)*	*Total Fe (mg/g)*	*pH*
NEW YORK	**10**	**40**	**19.4**	**8.0**	**7.5**
NEW YORK	**30**	**40**	**18.0**	**8.9**	**7.9**
NEW YORK	**10**	**80**	**29.5**	**8.2**	**7.7**
NEW YORK	**30**	**80**	**36.8**	**7.4**	**7.8**
NEW YORK	**10**	**120**	**26.5**	**7.8**	**7.5**
NEW YORK	**30**	**120**	**26.2**	**7.7**	**7.8**
NEW YORK	**10**	**180**	**19.2**	**7.8**	**7.6**
NEW YORK	**30**	**180**	**20.4**	**7.3**	**7.7**
NEW YORK	**10**	**221**	**23.6**	**7.7**	**7.6**
NEW YORK	**30**	**221**	**26.3**	**7.9**	**7.8**
NEW YORK	**10**	**261**	**19.8**	**7.5**	**7.6**
NEW YORK	**30**	**261**	**20.5**	**7.7**	**7.8**
IOWA	**10**	**-**	**47.3**	**5.2**	**6.1**
IOWA	**30**	**-**	**41.5**	**5.8**	**6.1**
MISSOURI	**10**	**-**	**66.1**	**4.9**	**6.2**
MISSOURI	**30**	**-**	**59.0**	**4.9**	**6.4**
NCAROLINA	**10**	**-**	**13.7**	**1.7**	**6.3**
NCAROLINA	**30**	**-**	**18.0**	**2.4**	**6.4**
PENNSYLVANIA	**10**	**-**	**22.6**	**7.3**	**6.1**
PENNSYLVANIA	**30**	**-**	**28.9**	**7.3**	**5.7**

Figure 3.2 shows a one-way analysis of variance (ANOVA) of extracted soil iron by location. As shown, there was a significant difference in the amount of extracted iron from the five locations ($P < .0001$). The mean extracted soil iron was 29.17 mg/kg, and the mean range was 13.7 – 66.1 mg/kg.

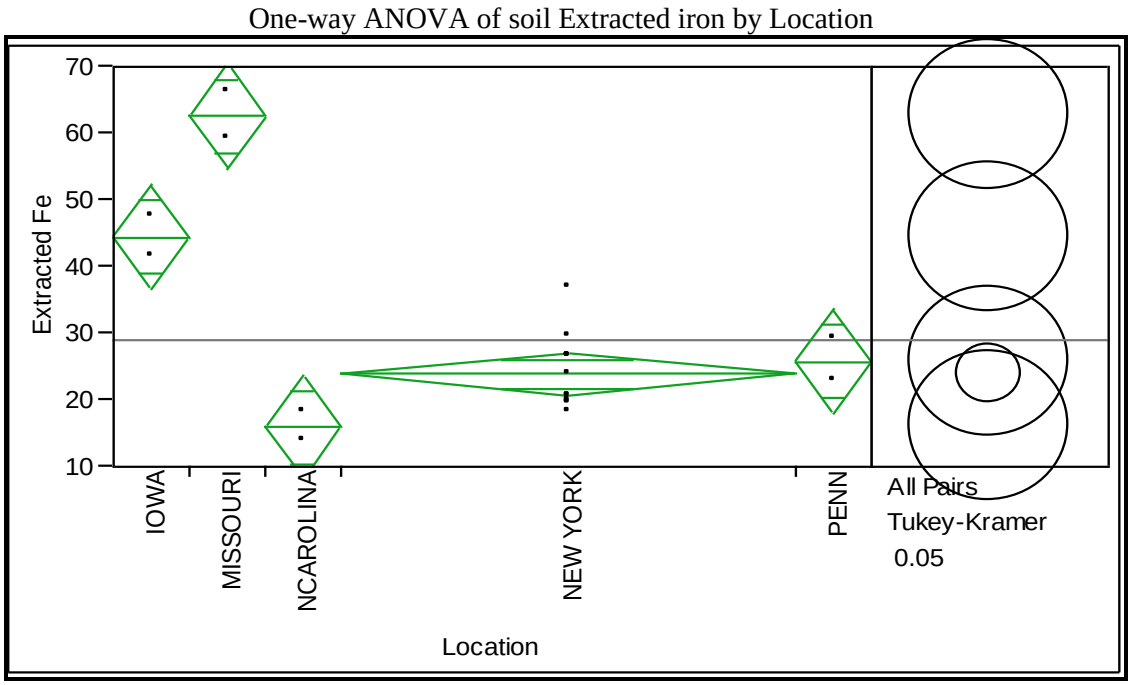

Figure 3.2: One-way analysis of variance (ANOVA) for iron extracted soil by location. Analysis of all pairs was done using Tukey-Kramer (*JMP* v7). NCAROLINA = North Carolina. PENN = Pennsylvania.

Figure 3.3 shows a one-way ANOVA of total soil iron by location. There was a significant difference in the amount of total soil iron from the five locations ($P < .0001$). The mean total soil iron was 6.67 mg/g, and the range was 1.7 – 8.9 mg/g.

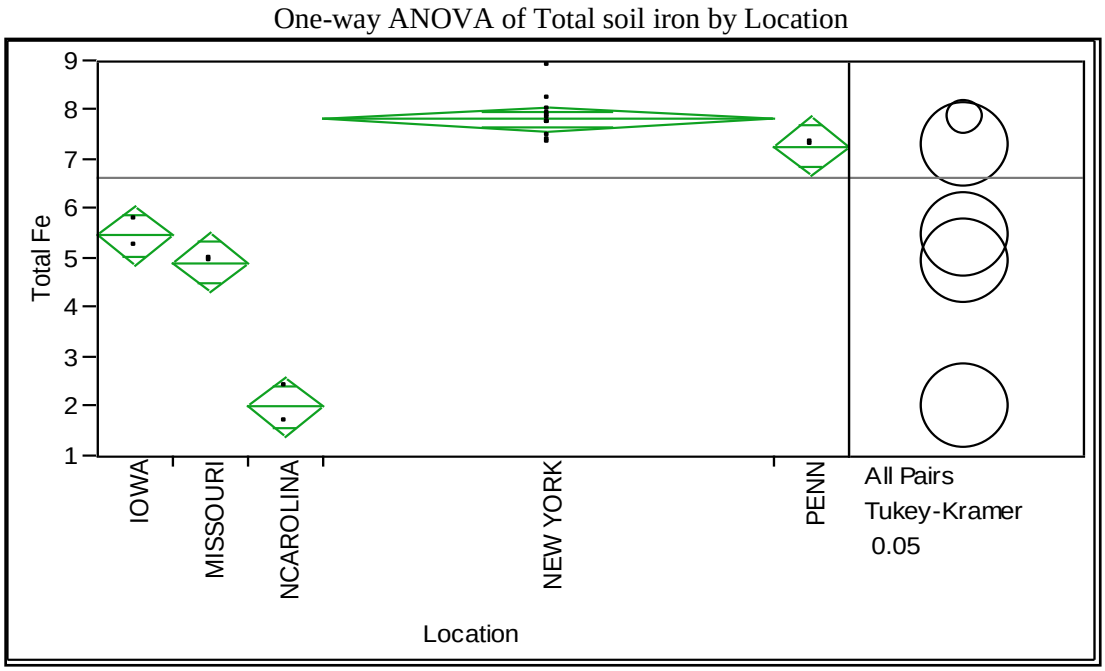

Figure 3.3: One-way ANOVA for total soil iron by location. Analysis of all pairs was done using Tukey-Kramer (*JMP* v7). NCAROLINA = North Carolina. PENN = Pennsylvania.

Table 3.16 shows pairwise correlations for grain iron concentration, grain iron bioavailability, soil extracted iron, total soil iron, and soil pH correlations. The aim of the analysis was to determine the association between soil Fe status and both grain iron concentration and amount of bioavailable iron from grain samples grown in our selected location. The correlation data show a significant but negative correlation between total soil iron and grain iron bioavailability. The correlation between soil pH and soil extractable iron was also negative and significant, while the correlation between soil pH and total soil iron was positive and significant. It is also important to note that the correlation between grain iron concentration and grain iron bioavailability was small and not significant, while that between total soil iron and grain iron concentration was negative though not significant.

Table 3.16: Pairwise correlations for grain iron concentration, grain iron bioavailability, soil extracted iron, total soil iron and soil pH.

Variable	by Variable	Correlation	Prob > F
Grain Fe Bioavailability	Grain Fe concentration	0.2187	0.1270
Total soil Fe	Grain Fe concentration	-0.0264	0.8555
Total soil Fe	***Grain Fe Bioavailability***	***-0.6517***	***<.0001***
Soil extractable Fe	Grain Fe concentration	0.0052	0.9712
Soil extractable Fe	Grain Fe Bioavailability	0.1714	0.2339
Soil extractable Fe	Total soil Fe	0.1811	0.2081
Soil pH	Grain Fe concentration	0.2437	0.0881
Soil pH	Grain Fe Bioavailability	-0.0548	0.7053
Soil pH	***Total soil Fe***	***0.3531***	***0.0119***
Soil pH	***Soil extractable Fe***	***-0.3127***	***0.0270***

The results presented clearly indicate a significant G×E interaction for both grain iron concentration and grain iron bioavailability.

Discussion

As mentioned earlier, the G×E interaction is a result of inconsistent genotype performance across environments. In this study we analyzed the effects of G×E interaction on grain iron concentration and grain iron bioavailability for six genotypes grown across five diverse locations. If the iron traits were relatively stable across these locations, we expected that the expression of iron traits for individual genotypes would be consistent across the locations, and the magnitude of difference between genotypes or relative ranking of the genotypes within individual locations would similarly be consistent. Overall there was a significant effect of G×E interaction for both grain iron concentration and grain iron bioavailability. However, a further analysis of G×E interaction for grain iron concentration showed that grain iron concentration for

genotypes 2 and 5 was consistently expressed across the five locations. In addition, the North Carolina location stood out, as there was no significant difference in grain iron concentration between the six genotypes grown there. A similar analysis for grain iron bioavailability showed no significant difference in grain iron bioavailability among the six genotypes grown in the North Carolina location. It is not clear if these results can be attributed to soil properties or altitude, but it is worth noting that the North Carolina location had the lowest altitude, mean total soil iron, and mean soil extractable iron.

Another goal of this study was to determine the association between soil iron status and both grain iron concentration and amount of bioavailable iron from grain samples grown in our selected locations. The data show no significant association between soil extractable iron and either grain iron concentration or grain iron bioavailability. There was however a highly significant ($P<.0001$) negative correlation between total soil iron and grain iron bioavailability. This may suggest that our maize genotypes would express more bioavailable iron if grown in soils that had low or poor iron status. This is significant because land owned by poor farmers in target locations such as Sub-Saharan Africa is likely to have mineral-deficient soils.

Conclusion

Micronutrient trait expressions and the extent of G×E interaction across diverse environments can influence both screening and breeding methods used in an iron biofortification program (Pfeiffer & McClafferty, 2007). The impact of biofortified maize on the iron status of those suffering from iron deficiency will depend to a large extent on the iron bioavailability of the maize genotypes. Our studies have consistently shown no significant association between grain iron concentration and grain iron bioavailability (Hoekenga et al, unpublished data). Our screening and breeding methodologies have thus been based on grain iron bioavailability. Analysis

of grain iron bioavailability shows high G×E interaction. Since the major target areas for biofortified crops are located in developing countries, additional progress in breeding for iron biofortified maize requires that further breeding and G×E testing be carried out in these target locations.

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CHAPTER 4

ASSESSMENT OF IRON BIOAVAILABILITY FROM IRON BIOFORTIFIED MAIZE

Abstract

The aim of our study was to use the Caco-2 cell *in vitro* model and a poultry model to test iron bioavailability in two select maize hybrid lines. The Caco-2 cell model was used as an initial screening tool to estimate the amount of bioavailable iron in the maize hybrid lines. The poultry iron model was used to confirm the *in vitro* results and validate the breeding method employed in iron biofortification of the hybrids. The Caco-2 cell bioassay data showed that the amount of bioavailable iron differed significantly (P =.0014) in the two hybrid lines. The maize lines were then used to formulate nutritionally balanced chicken diets (except for iron) that were fed ad libitum to day-old Cornish Cross broiler chicks for 4 weeks. Feed intakes were measured daily while weight and hemoglobin (Hb) were monitored weekly. Hb values were used to estimate iron bioavailability from the diets. Although iron concentrations in the two diets were similar (high bioavailability maize diet = 24.79±0.70 ppm and low bioavailability maize diet = 24.39±0.32 ppm), significant differences in blood Hb concentrations were observed between chicks consuming the two diets (P = .0004). We conclude that conventional breeding can improve iron nutritional quality in maize grain, thus providing significantly more bioavailable iron to growing chicks. Human feeding trials should be conducted to determine the efficacy of consuming the high bioavailable iron maize.

Introduction

Iron deficiency is the most widespread micronutrient deficiency in the world, affecting approximately two billion people (Zimmermann & Hurrell, 2007). The causal factors responsible for iron deficiency across the globe are complex and multi-factorial, but its root cause is thought to be the prevalence of dysfunctional food systems that fall short of delivering sufficient micronutrients to meet human requirements (Welch & Graham, 2005). The consequences of iron deficiency anemia (IDA) include impaired growth, retarded psychomotor and cognitive development in children, damaged immune mechanisms with increased morbidity and mortality rates in all age groups, and reduced work capacity in adults (WHO, 2001; Neumann, Gewa, & Bwibo, 2004).

The traditional strategies that have been employed to address IDA include dietary diversification, iron supplementation, and food fortification. Dietary diversification is an ideal approach that aims to improve iron intake and bioavailability but requires an educational component to achieve the desired changes in dietary behaviors. Iron supplementation is a therapeutic strategy aimed at groups that are highly susceptible to IDA. However, logistical constraints such as poor infrastructure and poor compliance at the individual level can reduce the effectiveness of supplementation (Zimmermann & Hurrell, 2007). In addition, recent studies show that untargeted iron supplementation in children living in regions to which malaria is endemic may result in increased risk of morbidity and mortality (Sazawal et al., 2006). On the other hand, iron fortification of foods is a practical and cost-effective prophylactic approach to iron deficiency and anemia. Nevertheless, in populations with limited access to fortified foods it is less effective (Zimmermann & Hurrell, 2007). These traditional strategies have had some success in reducing the burden of IDA in some populations.

In 1995, the Consultative Group on International Agriculture Research (CGIAR) initiated a micronutrients project that focused on linking agriculture and nutrition through "biofortification" of staple food crops (Graham, Welch & Bouis, 2001). Biofortification is defined as the process of breeding improved food crops that are rich in bioavailable iron, beta carotene and/or zinc. Biofortification of staple foods is thought to be a potential complementary strategy for alleviating IDA in at-risk populations, and it presents a number of advantages, especially the potential for providing more micronutrients at an affordable cost via the agricultural system. Thus, by targeting resource-poor populations that grow and consume their own food, the biofortification strategy would more effectively reach populations with limited access to diet diversification, iron fortification, or supplementation (Nestle et al., 2006; Mayer, Pfeiffer & Beyer, 2008).

Successful micronutrient biofortification requires, of course, that nutritionally improved crop lines be efficacious (Welch & Graham, 2004; Nestle et al., 2006). Simply increasing iron concentration in a staple food like maize does not necessarily reduce IDA incidence because this does not necessarily enhance bioavailability (Borg et al., 2009). Because the importance of bioavailability in the uptake of micronutrients such as iron is widely recognized, bioavailability studies will play a key role in assessing the biofortification process (Welch et al, 2000). The ideal approach would be to conduct iron bioavailability studies in humans. Such studies are, however, extremely expensive and often require refinement of objectives via appropriate *in vitro* and *in vivo* animal iron models in order to ensure success.

A number of *in vitro* and *in vivo* animal models are available for determining iron bioavailability (Wienk, Marx & Beynen, 1999). *In vitro* methods provide an attractive, rapid, and low-cost option for initial screening of iron bioavailability. Solubility and dialyzability are *in vitro* techniques that have previously been used to

predict iron bioavailability. They do not, however, measure iron bioavailability completely, greatly diminishing their usefulness (Fairweather-Tait et al, 2007; Miller & Berner 1989). The development of an *in vitro* iron bioavailability model that mimics the gastric and intestinal digestion of humans, coupled with cultures of human Caco-2 cells, has shown great promise in addressing iron bioavailability issues (Yun et al., 2004; Au & Reddy, 2000; Glahn et al., 1998). The *in vitro* digestion/Caco-2 cell iron model has been used in previous studies to predict iron bioavailability, and was used in this study as a screening tool (Glahn et al., 2002; Pynaert et al., 2006).

In vivo animal models are very useful in studying the mechanism of iron absorption and understanding iron bioavailability. Rodents and more recently piglets have been the models of choice for iron bioavailability studies. Although similarities in gastrointestinal anatomy and physiology between pigs and humans attract researchers to that model, the large size of pigs makes it an expensive model to employ. The rat model is relatively less expensive but may not be ideal given that rats are much more efficient at absorbing iron than are humans (Welch et al., 2000). The poultry model has been used effectively in some iron bioavailability studies (Wienk, Marx & Beynen, 1999), and has been proposed as a suitable *in vivo* model for iron bioavailability (Tako, Rutzke & Glahn, 2010). The poultry model was thus adopted for initial screening of biofortified maize in this study because it has been shown to respond appropriately to differences in iron bioavailability in foods. In addition, a feeding trial using the poultry model can accommodate modest amounts of sample material produced by breeders with restricted plot sizes or in the initial stages of a biofortification project.

The focus of our project has been iron biofortification in maize. This chapter evaluates two hybrid maize lines that were identified during the genotype screening process using the Caco-2 cell model, with the expectation that they would have

significant differences in iron nutritional quality while being largely similar in other respects. We present results from the Caco-2 cell assay and a 4-week poultry feeding trial. The goal of this chapter is to validate the quantitative trait loci (QTL) approach for iron bioavailability while addressing the following question: Are significant differences in iron bioavailability between the hybrids as seen in the *in vitro* digestion/Caco-2 cell iron model reflected in the *in vivo* poultry model?

Materials and Methods

Chemicals, enzymes, and hormones: Unless otherwise stated, all chemicals, enzymes, and hormones were purchased from Sigma Chemicals Co. To prepare reagents for cell culture, 18 MΩ water was used. Glassware and utensils used in the experiments were soaked in 1.2 M HCl for at least 4 hours and rinsed in deionized water prior to use.

Maize samples: The two maize varieties tested in the poultry feeding trial are derived from the *I*ntermated *B*73 x *M*o17 (*IBM*) recombinant inbred (RI) maize population. These lines were selected for the poultry feeding trial based on their parents' Caco-2 cell assay data. The parents are near isogenic line sister pairs (more that 90% genetically identical to each other) that were isolated from a Caco-2 cell assay screening of 54 maize lines planted in 2007 on a Cornell University research farm in Aurora, NY (see Figure 4.1). The parental genotypes that exhibited high bioavailability potential (high bioavailability B73; C-025 and high bioavailability Mo17; C-052) were crossed to produce a daughter genotype referred to in the data as the High×High hybrid. The parental genotypes that exhibited low bioavailability potential (low bioavailability B73; C-028 and low bioavailability Mo17; C-030) were crossed to produce a daughter genotype is referred to in the data as the Low×Low hybrid. For the poultry feeding trial, the High×High hybrid forms the Group H diet—

the high bioavailability maize diet— and the Low×Low hybrid forms the Group L diet—the low bioavailability maize diet. The maize lines were bred via conventional breeding guided by the Caco-2 cell/ quantitative trait loci (QTL) model.

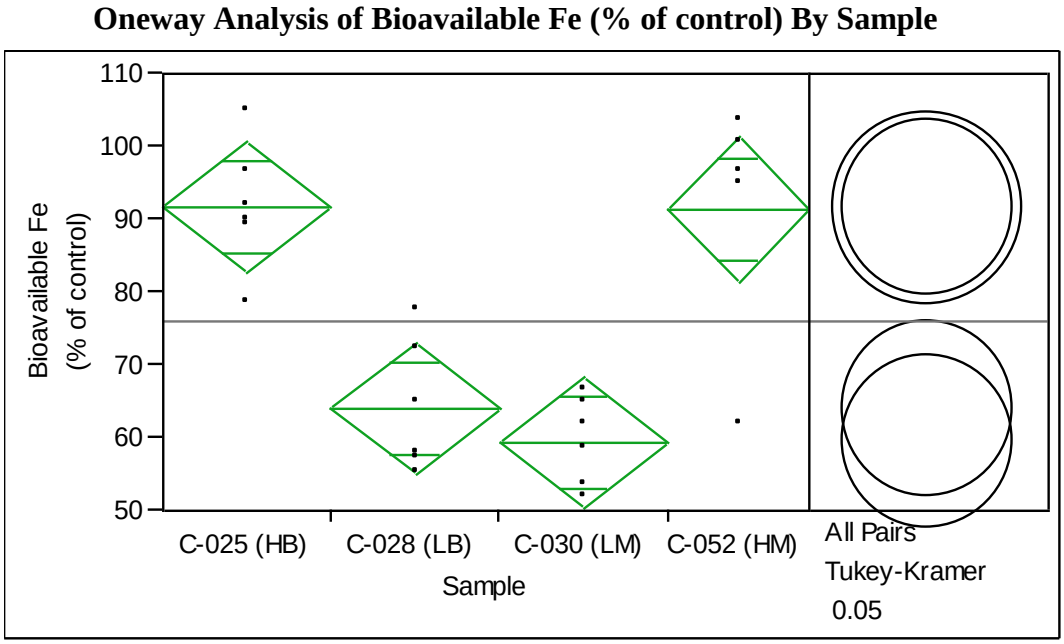

Figure 4.1: One way analysis of bioavailable iron in inbred parents of hybrids tested in the poultry feeding trial. The parental genotypes that exhibited high bioavailability potential were high bioavailability B73 (HB) C-025 and high bioavailability Mo17 (HM) C-052. The parental genotypes that exhibited low bioavailability potential were low bioavailability B73 (LB) C-028 and low bioavailability Mo17 (LM) C-030. The high bioavailability inbreds had significantly more bioavailable iron than the low bioavailable inbreds ($P < .0001$). HB and HM were crossed to produce a High×High hybrid. LB and LM were crossed to produce a Low×Low hybrid.

Maize sample preparation: Maize kernels (20g) were sorted to remove any debris or damaged seeds and then placed in a 50 mL centrifuge tube and covered with 25 mL of 18 MΩ water. Samples were then autoclaved at 121 °C and at a pressure of 115 kPa for 40 min, allowed to cool at room temperature, and then frozen overnight at –20 °C. Samples were then freeze-dried at 100 millTorr and a temperature of –50 °C for 7 days, ground to a fine powder with a coffee mill (90 sec), and stored in 50 mL

centrifuge tubes at 25°C. A tamale maize sample, used as a control in the Caco-2 cell bioassay, was prepared in an identical manner.

Mineral analysis for maize and liver samples: A 0.3g dry ground maize sample or a 0.4 g chicken liver tissue sample was weighed into borosilicate glass test tubes and chemically digested using 4 mL of concentrated nitric acid at 120°C until the residue was light brown to yellow in color. Exactly 1.0 ml of a 50/50 mixture of concentrated nitric acid and perchloric acid was then added, and the temperature was increased to 180°C. After 2 hours, the temperature was further increased to 240°C until the digested samples were dry. After cooling, 0.25 mL of concentrated hydrochloric acid was added to dissolve the ash. One hour later, the sample was diluted with 10 mL of 5% nitric acid. The ashed sample was then mixed and transferred into 15 mL auto sampler tubes and analyzed on an axially viewed inductively coupled plasma (ICP) trace analyzer emission spectrometer (model ICAP 61E trace analyzer, Thermo Electron, Waltham, MA; see Rutzke, 2002).

Caco-2 cell screening*:* The *in vitro* Caco-2 cell iron model was used as a screening tool to estimate the amount of bioavailable iron in the maize samples.

Cell Culture: Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) at passage 17 and used in experiments at passages 25–33. Cells were seeded at a density of 50,000 cells/cm^2 in collagen-treated six-well plates (Costar Corp., Cambridge, MA). The cells were grown in Dulbecco's Modified Eagle Medium (GIBCO, Grand Island, NY) with 10% v/v fetal calf serum (GIBCO), 25 mmol/L N-(2-Hydroxyethyl) piperazine-N'-2-ethane sulfonic acid (HEPES), and 1% antibiotic antimycotic solution (GIBCO). The cells were maintained at 37°C in an incubator with a 5% CO_2/95% air atmosphere at constant humidity, and the medium was changed every 2 days. The cells were used in the iron uptake experiments at 13 days post seeding.

In Vitro Digestion: The preparation of the digestion solutions—pepsin, pancreatin, and bile extract—and the *in vitro* digestion was performed as previously published (Glahn et al., 1998). Briefly, 1.0 g of dry samples was used for each sample digestion. Ascorbic acid (AA) was added to enhance iron bioavailability using a 20:1 AA: Fe molar ratio. Then 10 ml of pH 2 140 mM NaCl, 5 mM KCl buffer was added to the samples, and the mixture was pH re-adjusted to pH 2. Then 0.5 mL of pepsin solution was added to each of the samples prior to incubation. After a 1-hour incubation, the sample pH was adjusted to pH 5.5 to 6.0 with 1.0 M $NaHCO_3$, and after the addition of 2.5 mL of pancreatin-bile solution, the sample pH was further adjusted to pH 6.9-7.0 with 1.0 M $NaHCO_3$. Sample volumes were then adjusted (by weight) to tube weight plus 15 g using the 140 mM NaCl, 5 mM KCl pH 6.7. Finally, 1.5 mL of the samples was transferred to appropriate inserts on the Caco-2 cell plates.

Harvesting: The harvesting of the Caco-2 cell monolayers was performed as previously published (Glahn et al., 1998). The cells were harvested after a 24-hour incubation period. First, growth media were carefully aspirated off the cells. The cells were then rinsed twice with 2 mL of 130mM NaCl, 5 mM PIPES, pH 6.7 buffer. To harvest the cells, 2 mL of 18.2 MΩ water was added to the cells, and the cells were placed in a sonicator (Lab-line Instruments, Melrose Park, IL) at 4°C for 15 minutes. The cells were then scraped off the plates, suspended in the 2 mL of 18.2 MΩ water, and transferred to pre-labeled 5 ml tubes in anticipation of protein and ferritin assays.

Cell Protein Analysis: Caco-2 cell protein was measured on samples that had been solubilized in 0.5 mol/L NaOH, using a semimicro adaptation of the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). A 25 μL sample of the sonicated Caco-2 cell monolayer, harvested in 2 mL of water, was used for each protein measurement expressed in mg.

Ferritin Analysis: A one-stage, two-site immunoradiometric assay was used to measure Caco-2 cell ferritin content (FER-Iron II Ferritin Assay, RAMCO Laboratories, Houston, TX). A 10 µL sample of the sonicated Caco-2 cell monolayer, harvested in 2 mL of water, was used for each ferritin measurement expressed per unit cell protein (ng ferritin/mg cell protein).

Poultry Feeding Trial*:* The *in vivo* poultry model was used to estimate iron bioavailability in the two maize samples. The experiment was conducted at the Cornell University Poultry farm in Ithaca, NY. All animal protocols were approved by the Cornell University Institutional Animal Care and Use Committee. Figure 4.2 shows an overview of the study design.

Subjects: Fertile chicken eggs were incubated for 21 days before hatching. Seventeen day-old Cornish Cross broiler chicks were then randomized based on weight, into three groups—6 chicks in each of the 2 study groups, and 5 chicks in the positive-control group. Each group was housed in a temperature-controlled metabolic cage on a 12-hour light/dark cycle.

Diet and diet administration: The chicks were introduced to the experimental diet immediately after hatching and allowed ad libitum feed and water intake. Maize iron and phytate concentration, and diet composition and iron concentration are shown in Table 4.1.

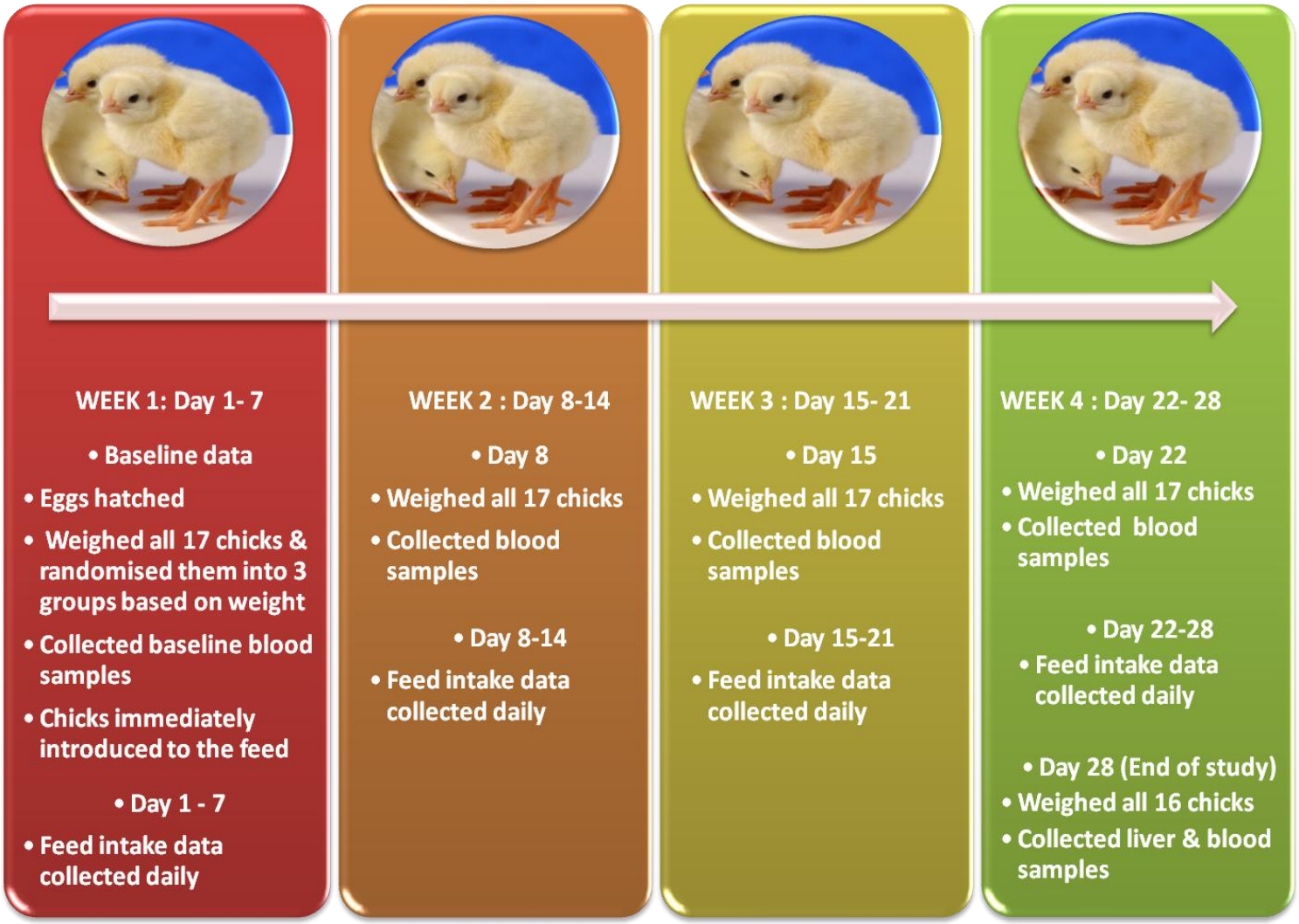

Figure 4.2: Overview of the study design

Data and tissue collection: As highlighted in Figure 4.1, feed intake data were collected daily. Body weight and blood samples were taken at baseline, then weekly for 4 weeks. At the end of the study, liver tissue was also sampled.

Hemoglobin (Hb) measurements: Blood Hb concentrations were determined using the cyanmethemoglobin method following the manufacturer's kit instructions. Hb values were used in this study to calculate total body hemoglobin iron and hemoglobin maintenance efficiency values for the chickens in the feeding trial.

Table 4.1: Diet composition and iron concentration data for poultry trial

Ingredient	Group H	Group L	Group C
		g/kg diet	
Ground maize[1]	750	750	750
Choline Chloride	0.75	0.75	0.75
Maize oil	30 mL	30 mL	30 mL
Maize starch	46.25	46.25	46.25
DL-Methionine	2.5	2.5	2.5
Dry skim milk	100	100	100
Ferric citrate	-	-	0.5
Vitamin/Mineral premix (no iron)[2]	70	70	70
	Iron & Phytate concentrations		
Iron in maize (ppm) *Mean ± SD, n= 3*	21.23±0.23	18.79±0.41	18.07±1.04
Iron in diet (ppm) *Mean ± SD, n= 3*	24.79±0.70	24.39±0.32	116.54±2.49
Phytate in maize (µmoles/g) *Mean ± SD, n= 2*	10.88±0.08	10.28±0.08	-

[1]Ground maize—group H: high bioavailability hybrid maize (cooked); group L: low bioavailability hybrid maize (cooked); group C (Positive-control group): commercially purchased maize (uncooked).

[2]A vitamin/mineral premix was purchased from Dyets, Inc (Bethlehem, PA). The premix provided (per kg diet): retinyl palmitate, 1208 μg; ergocalciferol, 5.5 μg; dl-α-tocopheryl acetate, 10.72 mg; menadione, 0.5 mg; d-biotin, 0.05 mg; choline chloride, 0.5 g; folic acid, 0.3 mg; niacin, 15 mg; Ca-D panthothenate, 10 mg; riboflavin, 3.5 mg; thiamin, 1 mg; pyridoxine, 1.5 mg; cyanocobalamin, 17.5 μg; $CuSO_4 \cdot 5H_2O$, 6 mg; $C_2H_8N_2 \cdot 2HI$ (ethylene diamine dihydroiodine), 0.14 mg; MnO, 4 mg; Na_2SeO_3, 0.3 mg; ZnO, 100 mg.

Total body hemoglobin iron (Hb Fe): Total body hemoglobin iron for each chick was calculated from body weight (BW), Hb concentration and estimated blood volume using the following formula:

Hb Fe = BW (kg) × [0.06 L blood/kg BW] × Hb (g/L blood) × [3.35 mg Fe/g Hb]

Hemoglobin maintenance efficiency (HME): Hemoglobin maintenance efficiency values were calculated as follows:

$$\text{HME} = \frac{\text{Hb Fe, mg (final)} - \text{Hb Fe, mg (initial)}}{\text{Total Fe intake, mg}} \times 100\%$$

Statistical Analyses: Statistical analyses of the data were performed using the GraphPad Prism v4 (GraphPad Software, San Diego, CA) and *JMP* v7.0 (SAS Institute Inc., Cary, NC) software packages. Repeated measures analysis was done for all repeated measurements. In some cases, the data were log-transformed prior to analysis. Means were considered to be significantly different for P values ≤ 0.05.

Results

The initial screening of the maize hybrid lines using the Caco-2 cell bioassay showed significant differences between varieties. The goal of our study was to establish if the differences seen in the *in vivo* iron model would be evident in the avian model. In this study we analyzed two near isogenic hybrid lines derived from the IBM RI maize population. The hybrids were identical essentially everywhere except for the iron grain bioavailability QTL where they had either all superior or inferior alleles. Here we present results from the Caco-2 cell bioassay and the poultry feeding trial.

(a) Caco-2 cell experiment

Figure 4.3 shows *in vitro* estimates of the amount of bioavailable iron (ng/mg) in the daughter hybrid lines. As in the parental lines, the amount of bioavailable iron *in vitro* was significantly different between the daughter hybrid lines (P =.0014).

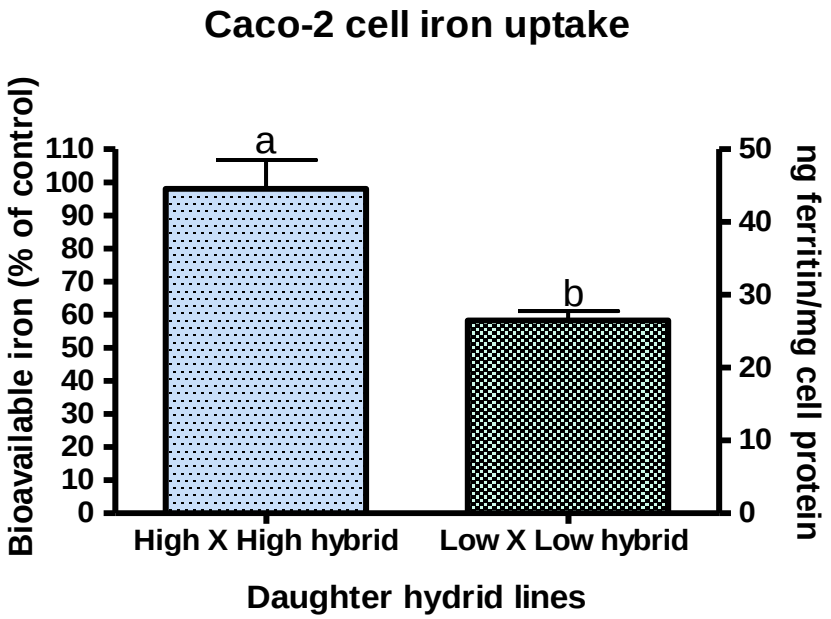

Figure 4.3: Caco-2 cell ferritin formation. The amount of bioavailable iron in maize hybrids (daughter lines) was assessed with the use of the Caco-2 cell model. Ferritin formation in the cells is an index of iron bioavailability. One gram (dry weight) of each sample was analyzed in the model. Statistical analysis was performed by one-way ANOVA and student's t test. Column values (mean ± SEM, n = 6) with no letters in common are significantly different ($P < 0.05$).

(b) Poultry Feeding Trial

***(i)* Feed intake, Daily iron intake, and Body weight:** The cumulative feed intake, mean daily iron intake, and body weight development of the chicks are summarized in Table 4.2.

Feed intake: Statistical analysis revealed that there was a week-and-diet effect on feed intake but no interaction (diet × week) effect. Throughout the study, feed intake was lowest in Group C and this differed significantly from that measured in

Groups H and L (P <.0001). However, feed intake increased consistently in all three groups during the study, and feed intake for each week was significantly different from that of every other week (P <.0001).

Table 4.2: Cumulative feed intake, daily iron intake, and body weight of the chicks.

	Study week			
	Week 1	*Week 2*	*Week 3*	*Week 4*
	Feed intake (g)			
Group H[3]	$378.3\pm24.84^{d\alpha}$	$773.6\pm16.59^{c\alpha}$	$1235.8\pm27.12^{b\alpha}$	$1732.9\pm23.40^{*a\alpha}$
Group L	$402.0\pm28.28^{d\alpha}$	$817.4\pm 11.57^{c\alpha}$	$1125.9\pm 20.05^{b\alpha}$	$1569.5\pm 24.71^{a\alpha}$
Group C	$314.1\pm 9.82^{d\beta}$	$550.2\pm 8.30^{c\beta}$	$858.2\pm 20.86^{b\beta}$	$1436.2\pm34.21^{a\beta}$
	Week 1	*Week 2*	*Week 3*	*Week 4*
	Daily iron intake (mg)[1]			
Group H	$1.56 \pm 0.62^{d\beta}$	$2.74 \pm 0.4^{c\beta}$	$4.38 \pm 0.67^{b\beta}$	$6.14 \pm 0.58^{a\beta}$
Group L	$1.63 \pm 0.69^{d\beta}$	$2.85 \pm 0.28^{c\beta}$	$3.92 \pm 0.49^{b\beta}$	$5.47 \pm 0.60^{a\beta}$
Group C	$6.10 \pm 1.14^{d\alpha}$	$9.16 \pm 0.97^{c\alpha}$	$14.29 \pm 2.43^{b\alpha}$	$23.91\pm 3.99^{a\alpha}$
	Week 1	*Week 2*	*Week 3*	*Week 4*
	Body weight (g)[1]			
Group H	$89.65\pm24.08^{d\alpha}$	$150.90\pm55.86^{c\alpha}$	$256.55\pm106.81^{b\alpha}$	$382.24\pm102.68^{a\alpha}$
Group L	$91.03\pm 8.68^{d\alpha}$	$142.42\pm44.95^{c\alpha}$	$223.07\pm 102.65^{b\alpha}$	$303.07\pm166.39^{a\alpha}$
Group C	$77.02\pm 5.38^{d\alpha}$	$119.30\pm28.13^{c\alpha}$	$180.66\pm 72.00^{b\alpha}$	$237.08\pm112.17^{a\alpha}$

[1]Mean±SD.

[2] Values in same row (English alphabet)/column (Greek alphabet) with different superscript letters are significantly different; (P < 0.05).

[3]Group H (n = 6): High bioavailability maize diet, Group L (n = 6): low bioavailability maize diet, Group C (n = 5): Positive-control maize diet, [*]1 mortality.

Daily iron intake: Daily iron intake values were computed from daily feed intake and diet iron concentration data. Group C had the highest daily iron intake, which was significantly different from that of Groups H and L (P <.0001). Daily iron intake did increase consistently in all three groups during the study and each week's result was significantly different from that of every other week (P <.0001).

Body weight: The chicks grew well over the course of the study. Body weight was not significantly different by treatment or diet (P = .3250) but was significantly different by time (P <.0001).

(ii) **Hemoglobin, Liver iron concentration, Hemoglobin iron and Hemoglobin maintenance efficiency:** Hemoglobin, liver iron concentration, total body hemoglobin iron and hemoglobin maintenance efficiency are shown in Figure 4.4, Figure 4.5, Table 4.3 and Figure 4.6 respectively.

Hemoglobin: After an initial dip in hemoglobin (Hb) concentration, from day 8 on, chicks in Groups H and C showed an increase in Hb concentration, which leveled off by the end of the study. However, chicks in Group L showed a steady decline in Hb concentration (Figure 4.4). There was a strong diet and week effect on Hb concentration. Hb concentration in Groups H and C was significantly different from Group L (P = .0004) and Hb concentration differed significantly across weeks 2, 3 and 4 (P <.0001).

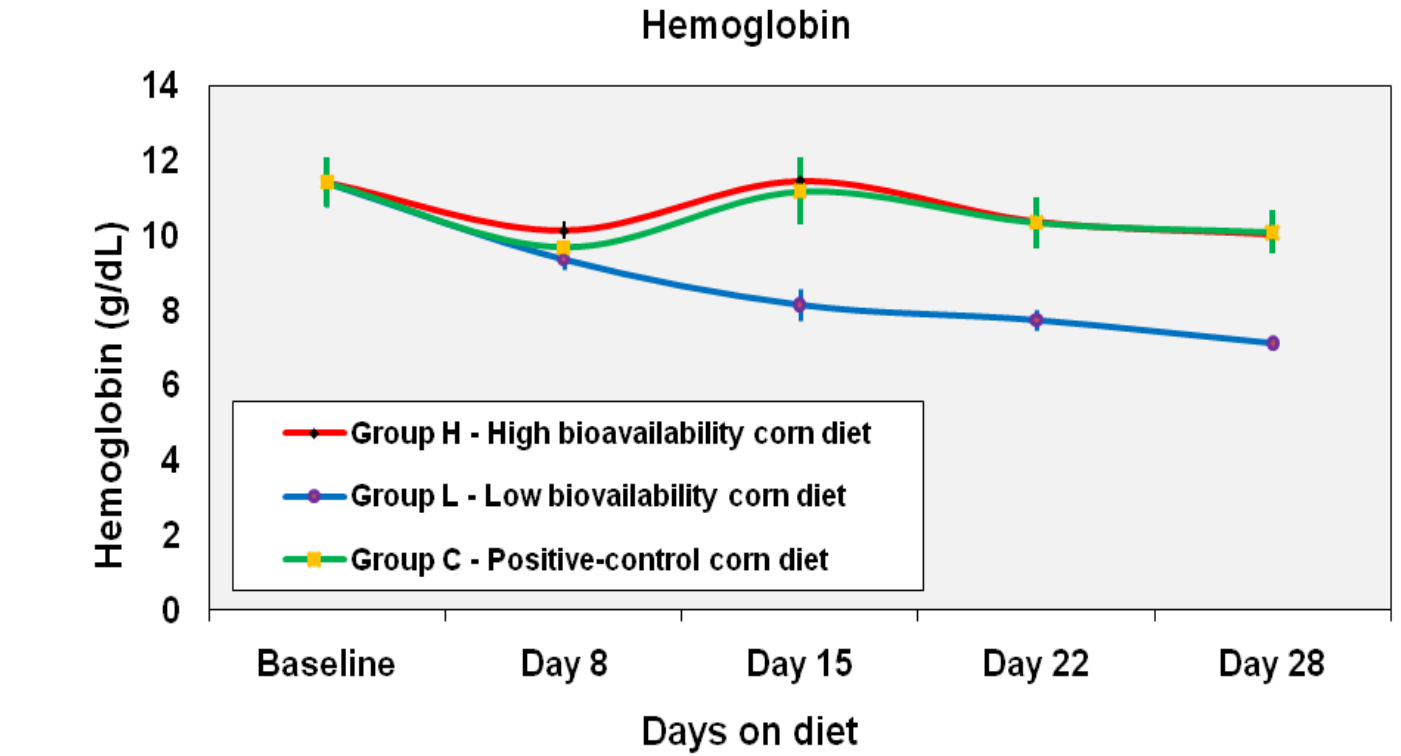

Figure 4.4: Hemoglobin trend during the poultry feeding trial. Blood samples were collected at baseline, day 8, day 15, day 22 and day 28, and hemoglobin concentration (Hb) analyzed using the cyanmethemoglobin method. Statistical analysis was performed by repeated measures ANOVA. Hb concentration in Groups H and C was significantly different from Group L (P = .0004). Hb concentration for weeks 2, 3 and 4 were significantly different from each other (P <.0001). Hb values (mean ± *SEM*). Group H [n = 6 *(*n = 5 on day 28)]: High bioavailability maize diet, Group L (n = 6): low bioavailability maize diet, Group C (n = 5): Positive-control maize diet.

Liver iron concentration: Liver iron concentration data are shown in Figure 4.5. Chicken livers were harvested at the end of the study and analyzed for iron concentration by ICP-ES. Liver iron was highest in Group C, and differed significantly (P <.0001) across all groups.

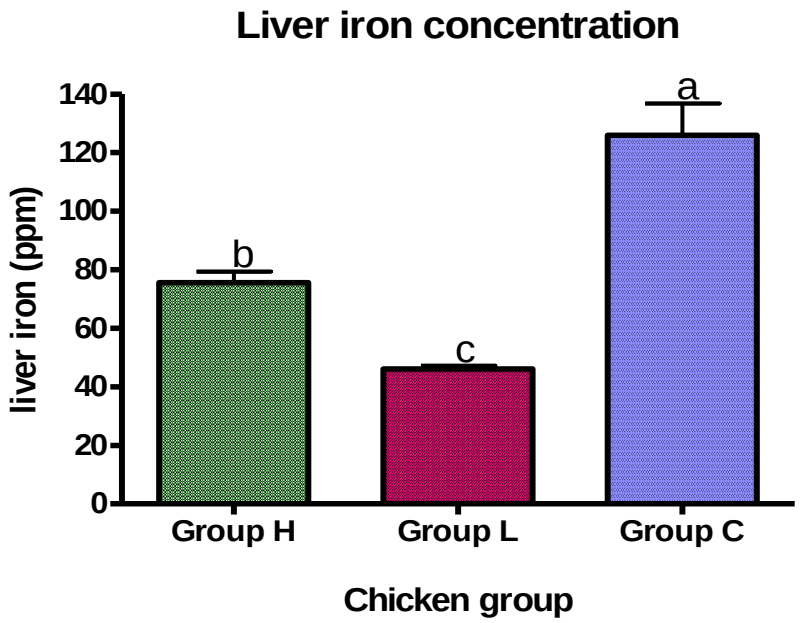

Figure 4.5: Liver iron concentration. Chicken livers were harvested on day 28 of study and iron concentration determined using ICP-ES. 0.4 grams (wet weight) of each sample (n=3) was analyzed. Statistical analysis was performed by Tukeys-HSD. Bar values (mean ± SEM) with no letters in common are significantly different (p < 0.05). Group H (n=5): High bioavailability maize diet, Group L (n=6): low bioavailability maize diet, Group C (n=5): Positive-control maize diet.

Total body hemoglobin iron (Hb Fe): As seen in Table 4.3, Group H had relatively higher body Hb Fe during the study, which was significantly different from Groups L and C (P =.0023). Body Hb Fe was also significantly different between weeks (P <.0001) with weeks 3 and 4 being significantly different from week 1 and 2, and week 1 being significantly different from week 2.

Table 4.3: Total body hemoglobin iron

	Study day				
	Baseline	*Week 1*	*Week 2*	*Week 3*	*Week 4*
			Hb Fe (mg)		
Group H[3]	-	$1.83\pm0.49^{c\alpha}$	$3.53\pm 1.53^{b\alpha}$	$5.33\pm2.09^{a\alpha}$	$7.65 \pm1.82^{a\alpha*}$
Group L	-	$1.71\pm 0.16^{c\beta}$	$2.30\pm0.61^{b\beta}$	$3.43\pm1.47^{a\beta}$	$4.30\pm2.26^{a\beta}$
Group C	-	$1.50\pm 0.10^{c\beta}$	$2.71\pm0.90^{b\beta}$	$3.81\pm1.76^{a\beta}$	$4.76\pm2.25^{a\beta}$

[1]Mean±SD.
[2] Values in same row (English alphabet) /column (Greek alphabet) with different superscript letters are significantly different; ($p < 0.05$).
[3]Group H (n=6): High bioavailability maize diet, Group L (n=6): low bioavailability maize diet, Group C (n=5): Positive-control maize diet.
[*]1 mortality

Hemoglobin maintenance efficiency (HME): HME provides an estimate of the percentage of ingested iron that is absorbed and utilized to maintain hemoglobin synthesis and the values were calculated using data collected during week 1, 2, 3 and 4. It is a slight underestimate because some of absorbed iron is incorporated into myoglobin and other iron containing proteins and some is excreted. HME values for Group H were significantly different from Group L ($P < .0001$), and differed across weeks ($P = 0.0147$, see Figure 4.6). Because the goal of this study was to compare the high bioavailable iron maize with the low bioavailable iron maize, only data from these two groups are presented in Figure 4.6.

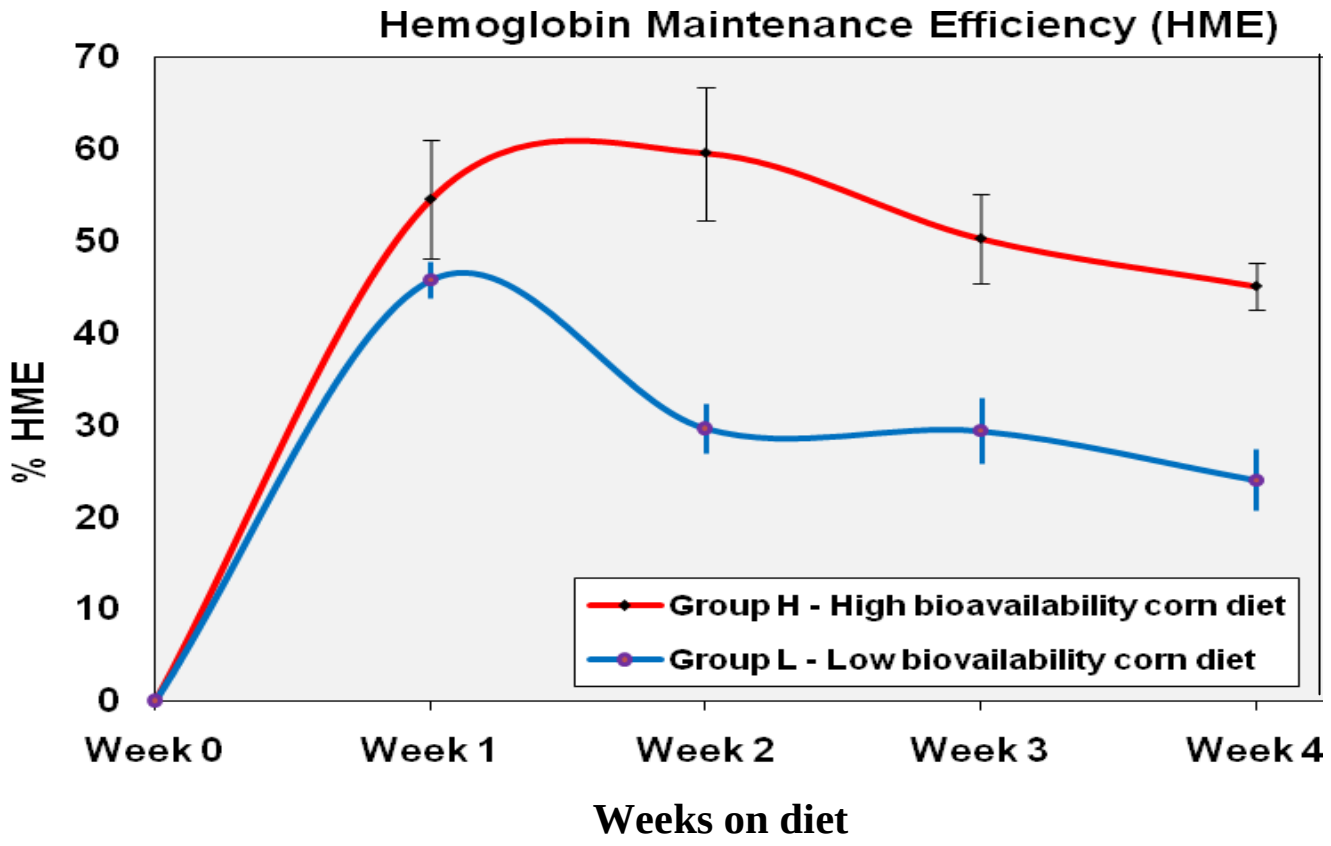

Figure 4.6: Hemoglobin maintenance efficiency values. HME values for Group H were significantly different from those of Group L ($P < .0001$), and differed across weeks ($P = 0.0147$). Values (mean ± *SEM*). Group H [$n = 6$ *(*$n = 5$ on day 28)]: High bioavailability maize diet, Group L ($n = 6$): low bioavailability maize diet.

Discussion

This chapter presents results collected on the bioavailability of iron in two maize hybrids grown under field conditions in Poplar Ridge, New York. The maize samples were selected for the poultry feeding trial based on the bioavailability potential of their parents—high bioavailability maize vs. low bioavailability maize. The daughter lines showed the same pattern in the Caco-2 cell model (Figure 4.3) and were therefore formulated into nutritionally balanced diets (except for iron) for a poultry feeding trial.

Our goal with the poultry feeding trial was to confirm the *in vitro* results and validate the utility of the Caco-2 cell bioassay as a screening tool for use in maize improvement programs. The chicken diets were formulated so that for the two experimental diets—the high bioavailability maize diet and the low bioavailability maize diet—the maize component would provide most of the iron to the growing chicks. The control group was a positive control group, which served as an experimental reference. The control diet was formulated according to National Research Council (NRC) standards and a 0.5 g ferric citrate/kg diet was added to the formula to provide additional iron to the chicks.

Although the two experimental diets exhibited similar levels of iron concentration, as shown in Table 4.1, Hb concentration in blood collected from chicks in the high bioavailability maize diet (Group H) differed significantly from that of the chicks in the low bioavailability maize diet (Group L; $P = .0004$) during the course of the study (weeks 2, 3, and 4; $P < .0001$). No significant differences were observed between the high bioavailability maize diet (Group H) and the positive control group (Group C) for blood Hb despite the differences in diet iron concentration. Group C diet had a higher iron concentration (116.54 ppm) compared to the Group H diet

(24.79 ppm). This would suggest that absorption of iron citrate is different from the absorption of native iron.

In addition, liver iron concentration was used to expand the bioavailability data. As shown in Figure 4.5, liver iron values for the groups were significantly different (P <.0001). The positive control group (Group C) had the highest liver iron concentration, which indicates more storage iron compared to the other two groups. Iron intake was similar between the high bioavailability maize diet (Group H) and the low bioavailability maize diet (Group L), yet liver iron was significantly different between the two groups. This would suggest that the high bioavailability maize diet provided more bioavailable iron to the chicks than the low bioavailability maize diet.

The hemoglobin maintenance efficiency (HME) values were another piece of data that demonstrated that the iron in the high bioavailability maize diet is more bioavailable than that in the low bioavailability maize diet. As seen in Figure 4.6, HME values for Group H were significantly different from Group L (P < .0001). The two diets - the high bioavailability maize diet (Group H) and the low bioavailability maize diet (Group L) - had the same iron concentrations, but different amounts of absorbed iron as shown by the hemoglobin data. The two maize lines were identical in every respect except for their iron grain bioavailability alleles. The high bioavailability maize line had superior alleles while the low bioavailability maize line had inferior alleles. It is plausible that the difference in the amount of bioavailable iron in the two hybrids lines can be attributed to either the presence of iron promoter compounds in the high bioavailability maize hybrid line or the presence iron inhibitory compounds in the low bioavailability maize hybrid line or both. Further research is needed to identify and isolate the compounds.

As already defined, biofortification is the process of enriching the nutrient quality of staple food crops via plant breeding or biotechnology. Biofortification as an

agricultural intervention has the potential to provide a sustainable solution to populations who are at high risk of micronutrient malnutrition. The success of such a strategy requires, however, that the nutrients in food crops be bioavailable. Iron is a critical nutrient in the diet as it plays a fundamental role in oxygen transport and energy metabolism. However, the bioavailability of non-heme iron from plant foods is influenced and hindered by many factors. Thus it is vital that the potential enhancement of iron nutritional quality in foods include bioavailability testing.

Crop improvement activities in conventional breeding can produce a vast amount of varieties. The same is true of iron biofortification in maize. Therefore in order to rank samples, or determine which plant lines have potential or show promise for enhanced iron content or quality, a screening method is required. The Caco-2 cell iron model has been used as a screening bioassay for *in vitro* assessment of the amount of bioavailable iron in numerous studies and was likewise used in our study to conduct the initial screening of the maize lines. One objective for the poultry feeding trial was to confirm the *in vitro* results. The agreement between the *in vivo* and *in vitro* data therefore validates the Caco-2 cell bioassay as a screening method for iron biofortified maize.

Another objective of the study was to validate the breeding method employed in iron biofortification of the hybrids. Genotype data generated from the QTL model indicated that the two hybrid lines were dissimilar, exhibiting different bioavailability potentials. This was reflected in both the Caco-2 cell data and the poultry feeding trial data. All three data sets are in agreement, which validates the QTL model. These results are a clear indication that iron biofortification of maize is feasible.

Previous iron bioavailability studies on iron biofortified staple foods have been very successful and have paved the way for subsequent research. A case in point is the study of iron bioavailability from iron biofortified rice. The initial screening of the

enriched lines was done using a rat model (Welch et al., 2000). In their study, Welch et al. identified a rice genotype—IR68144—developed at the International Rice Research Institute (IRRI) to be of special interest. Based on these results, Hass et al. (2005) conducted a human efficacy trial designed to test the biological effects of consuming the biofortified rice. The results from a 9-month human feeding trial provided the first indication that breeding for enhanced micronutrient content can result in a measurable improvement in nutritional status. Our initial efforts toward breeding for improved iron quality in maize have been encouraging. However, a human feeding trial similar to the one conducted by Haas et al. is required to further validate the QTL breeding strategy and to determine whether iron biofortification in maize is a practical and sustainable strategy for mitigating iron malnutrition in at-risk populations.

Conclusion

Iron deficiency is a worldwide, endemic public health problem. Food-system-based interventions such as biofortification may provide a practical and sustainable solution for at-risk populations. If biofortification is to succeed, however, enriched nutrients in foods must be bioavailable. Bioavailability testing is thus an essential aspect of the biofortification process. The *in vivo* results presented in this chapter using two selected maize hybrid genotypes show that conventional breeding can improve the iron quality in maize, thus providing significantly more bioavailable iron to growing chicks. These findings support the biofortification strategy for iron in maize but a human feeding trial is required to confirm the efficacy of the iron biofortified lines and should thus be prioritized.

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CHAPTER 5

A CONCEPTUAL FRAMEWORK FOR BIOFORTIFICATION

Despite advances in science and medicine, micronutrient deficiencies, including iron deficiency (ID), still affect over two billion people globally (Zimmermann & Hurrell, 2007; WHO, 2001). From a purely food policy perspective, there are three questions that must be answered if we are to understand better why nutritional iron deficiency persists, especially in developing nations: 1) Who suffers from iron deficiency? 2) Where are they located? 3) Why are they still suffering? In many developing countries, populations at risk of developing iron deficiency are usually economically poor. They comprise predominantly women of child-bearing age, infants, and children. A majority reside in rural areas, where they grow their own food and consume mainly what they produce. These populations are forced to rely on dysfunctional food systems that fail to provide adequate quantities of the nutrients they need. Under such conditions, nutritional deficiencies are inevitable.

In their quest to address micronutrient deficiencies, including ID, nutritionists have focused on interventions such as diet diversification, supplementation as well as food fortification. And although agriculture is the principal source of nutrients that sustain human life, its role in addressing human nutrition issues, including nutrient deficiencies, has not been fully exploited until recent years (Pinstrup-Andersen, 2000). The emergence of biofortification as an agricultural intervention to address micronutrient deficiencies among the rural poor who grow and consume their own staple foods has provided the opportunity for a new paradigm for world agriculture (Welch & Graham, 2000). The main advantage of biofortification is that it is a sustainable approach that can complement traditional food-based approaches for addressing micronutrient malnutrition.

Traditional interventions—diet diversification, supplementation, and food fortification—have helped reduced the burden of iron deficiency, especially in developed countries. The advantage of these interventions is that we have reliable information about their implementation, efficacy, sustainability, and impact in different parts of the world. Therefore they are safe and easy options in programs designed to address micronutrient deficiencies. These interventions have, however, produced relatively better results in developed countries than in developing countries. Factors such as poor infrastructure, the need for centralized processing, limited human resources, lack of financial resources, and lack of political will have resulted in limited success in developing countries. In developing countries in which these interventions have had some success, the urban population is usually better placed to benefit from such intervention programs, leaving the rural poor sidelined with little benefit from traditional interventions. Biofortification of staple foods can help bridge this gap by directly targeting the rural poor, especially those who grow and consume their own foods.

As shown in the conceptual framework (Figure 5.1), biofortification has the potential to more effectively address iron deficiency among at-risk populations because it addresses the root cause of the problem. If properly implemented, iron biofortification of staple foods can result in increased iron intake or bioavailability that can help increase body iron and maintain iron status for non-anemic individuals (Haas et al, 2005). On the other hand, when coupled with other interventions such as supplementation or post-harvest fortification, it is plausible that biofortification can help improve the iron status of previously anemic individuals and help maintain normal iron status (Bouis &Welch, 2010).

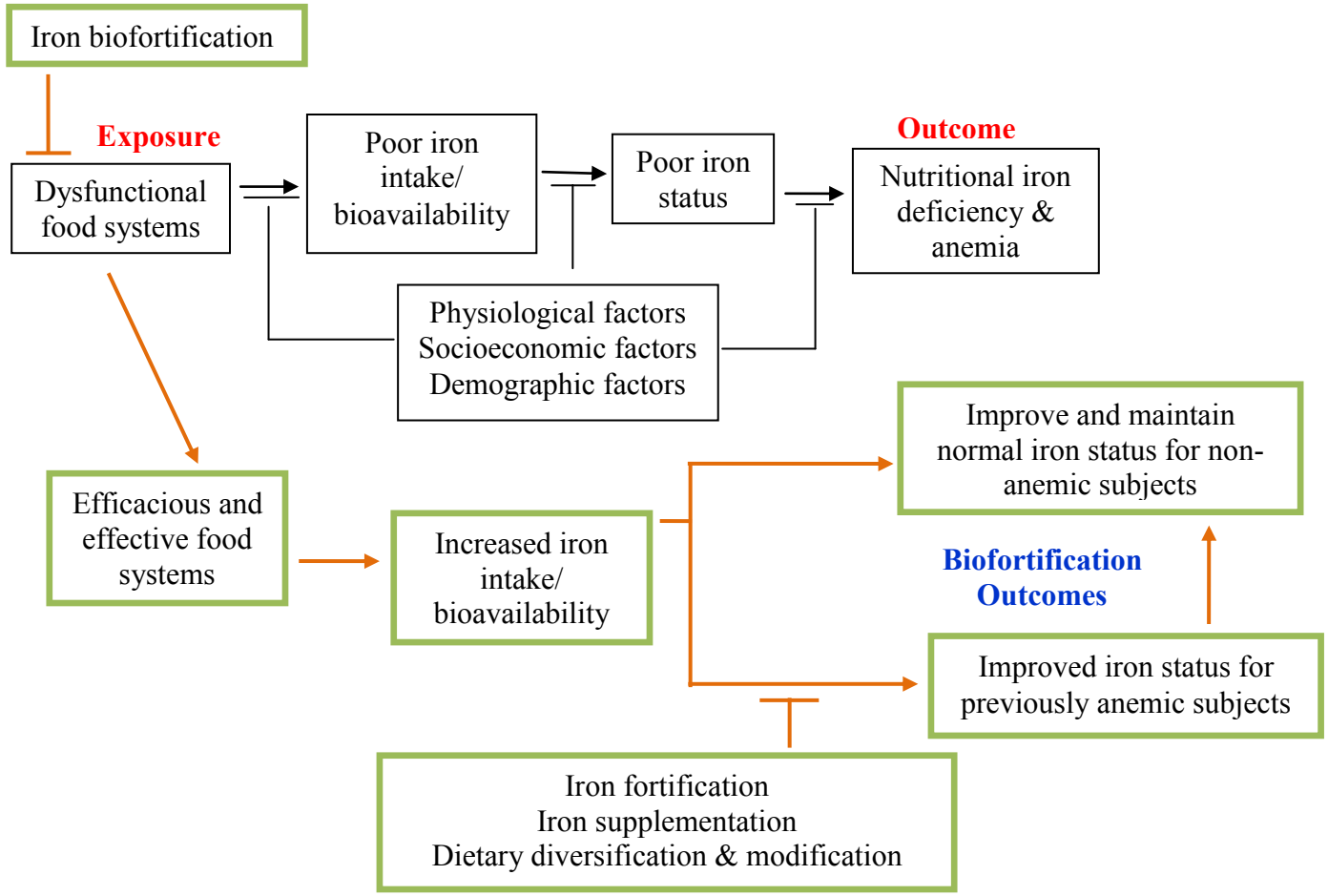

Figure 5.1: Conceptual framework for biofortification.

Farmer adoption and consumer acceptance are critical to the success of biofortification (Welch & Graham, 2004). However, upstream research is equally important. This research work has focused on three important goals: 1) to determine if both iron concentration and iron bioavailability in maize are genetically tractable traits, 2) to evaluate the magnitude of Genotype x Environment interaction in biofortified maize, and 3) to assess iron bioavailability from iron biofortified maize. Table 5.1 provides a summary of the research conducted and lessons learned.

The ultimate goal of our biofortification project should be to create elite maize lines that can be adopted by farmers and accepted by consumers in target regions such as Sub-Saharan Africa, where iron deficiency and anemia are significant public health problems. The maize lines produced must therefore have excellent agronomical qualities and be effective in reducing iron deficiency. Our research work, presented in three studies (chapters 2–4), has highlighted a conventional breeding method—the Caco-2 cell/QTL approach—that can be used to breed for enhanced iron bioavailability in maize. We have also stressed the need to assess Genotype x Environment interaction and shown proof of concept that conventionally bred iron biofortified maize provides significantly more bioavailable iron to growing chicks. The next step in the research work can be similarly study-specific, as shown:

Study 1: *Enhancing iron bioavailability in maize using a Caco-2 cell/ QTL model*. As earlier mentioned, the alleles present in the maize population used in this research work may not represent the best alleles available for iron biofortification. Thus additional libraries should be screened for enhanced or diminished iron traits. In addition, studies on the effects of various postharvest treatments, cooking, and storage on grain iron nutrition retention should also be conducted.

Table 5.1: Summary of the research conducted and lessons learned.

Study	Research Question(s)	Lessons Learned & Recommendations
Study 1: Enhancing iron bioavailability in maize using a Caco-2 cell/QTL model.	♣ Is iron concentration in maize a genetically tractable trait? ♣ Is iron bioavailability in maize a genetically tractable trait?	Grain iron content and grain iron bioavailability can be genetically manipulated and enhanced through breeding. The better approach to iron biofortification in maize requires breeding for enhanced grain iron bioavailability relative to grain iron content.
Study 2: Evaluating Genotype x Environment interaction in biofortified maize.	♣ Does growing biofortified maize in diverse locations affect iron concentration and/or iron bioavailability in the maize lines?	Genotype x Environment interaction has an effect on iron trait expression. Soil properties may also influence iron trait expression. Interdisciplinary research that includes soil scientists and agronomists is needed to better understand the soil effect.

Table 5.1 (Continued).

Study	Research Question(s)	Lessons Learned & Recommendations
Study 3: Assessment of iron bioavailability from iron biofortified maize.	♣ Are significant differences in iron bioavailability as measured by the Caco-2 cell *in vitro* iron model reflected in an *in vivo* iron model?	The Caco-2 cell bioassay and the poultry model showed similar results on iron bioavailability from the maize samples. Caco-2 cell bioassay is an effective tool for screening iron biofortified maize. Conventional breeding can improve iron nutritional quality in maize grain, thus providing significantly more bioavailable iron to growing chicks. Human feeding trials should be conducted to determine the efficacy of consuming the high bioavailable iron maize.

Study 2: *Evaluating Genotype x Environment interaction in biofortified maize*. This study called attention to the soil effect on iron bioavailability in the grain. Figure 5.2 presents data from a previous study by Hoekenga et al. that presented a similar point.

As seen in Figure 5.2, maize lines from the IBM RI population were grown three times in New York (NY; 2001, 2003, 2005) and once in North Carolina (NC; 2005) in replicated trials. What is striking about the data is that the grain iron concentrations for maize grown in NC was double that grown in NY, as shown by the median grain iron concentration numbers. The soil type in Aurora, NY was Lima silt series Loam (alfisol) with pH 6.7, while the soil type in Clayton, NC was Norfolk loamy series sand (ultisol) with pH 4.8. It is reasonable to assume that the low pH of the NC soils allowed the plants to assimilate more iron from the rhizosphere, which was then partly compartmentalized in the grain. The data thus suggest the need to include soil scientists in iron biofortification projects, with the goal of identifying and mapping soils in target regions that would be ideal for growing iron biofortified maize.

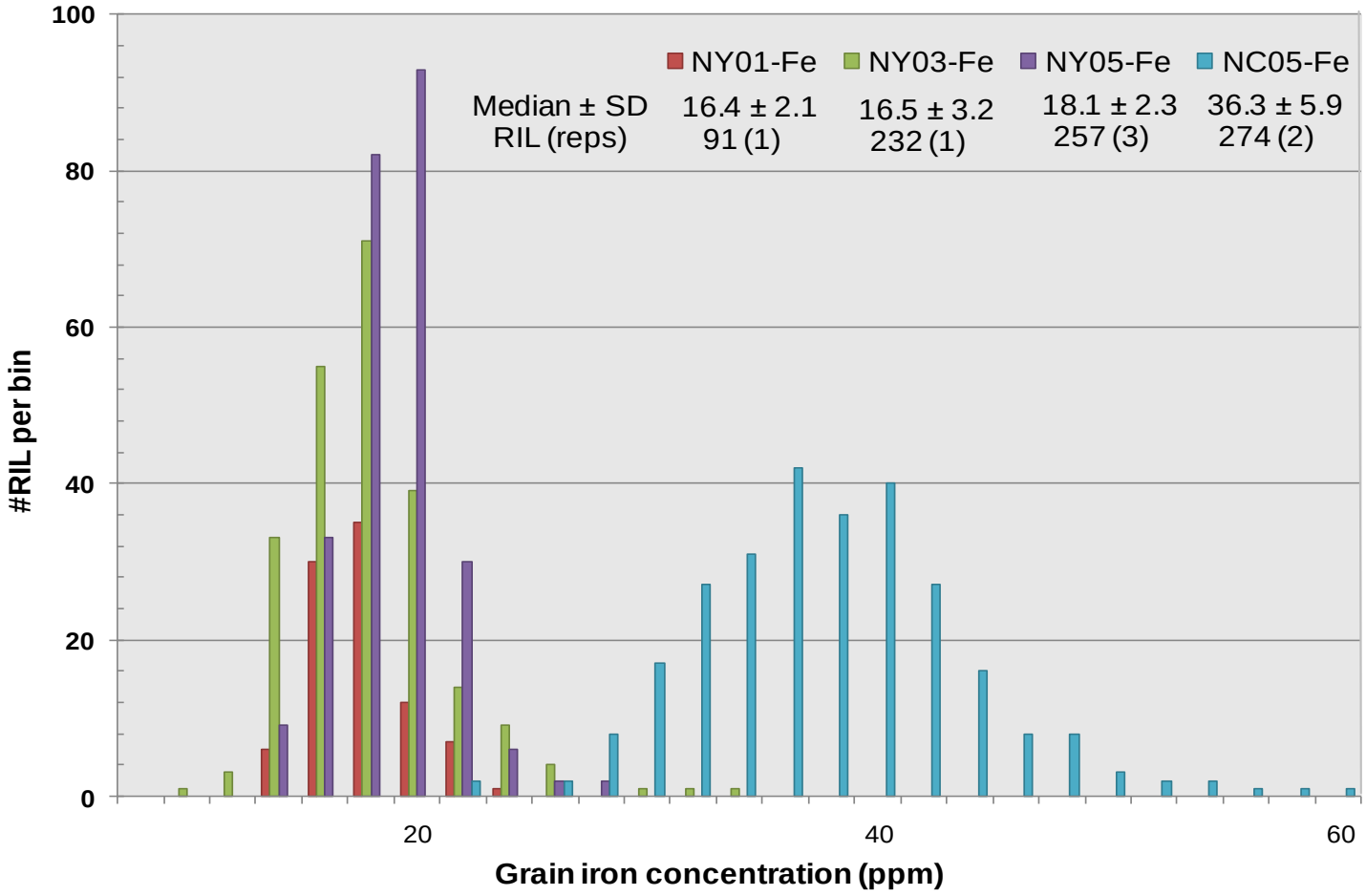

Figure 5.2: Grain iron concentration of maize grown in New York and North Carolina

Study 3: *Assessment of iron bioavailability from iron biofortified maize.* This study raises positive prospects for iron biofortified maize. It is clear that human feeding trials should be conducted to determine the efficacy of consuming high bioavailable iron maize. Based on the amount of resources available and the research question raised, single-meal or long-term studies can be used to assess iron bioavailability in human subjects. This next step however raises two important study questions:

Question 1. Long-term feeding trials are the definitive test for iron biofortified foods. These ideally work with mildly anemic or non-anemic subjects. However, if a long-term feeding trial is carried out in a malaria-endemic region, the 'iron trap" is brought into question. How can this be best addressed?

The iron trap is defined as the interaction between increased intake of non-native iron (via iron supplementation or food fortification) and susceptibility to infectious diseases such as malaria (Friedman et al, 2009, Sazawal et al., 2006). The best approach if working in a malaria-endemic area would be to have procedures in place with which to closely monitor malaria, morbidity, and mortality and to have intermittent preventive treatment built into the efficacy trial. If the research goal is simply to show proof of concept, then an alternative approach would be to conduct the study in an area that is not classified by the Centers for Disease Control or the World Health Organization as a malaria-risk region.

Question 2. If on the other hand a single-meal study is selected, does it matter whether extrinsic or intrinsic labeling is used? To better approximate iron bioavailability from foods, many assessment techniques have incorporated either radioisotopes or stable isotopes of iron into a meal. Intrinsic labeling of plants requires growing plants in nutrient media labeled with an iron isotope. In contrast, extrinsic labeling would involve adding iron isotope labels during preparation of test meals. The

extrinsic labeling technique is based on the assumption that complete isotopic exchange takes place between an extrinsically added iron isotope label and the native iron in the food so that the fractional absorption of the extrinsic label by human subjects predicts total iron absorption. Data presented in a study by Cook et al (1972) suggested that iron absorption from an intrinsic tag was very similar to that from an extrinsic tag. Welch et al. (2000) argue, however, that although extrinsic labeling of foods is easy and relatively inexpensive, poor equilibration of the extrinsic label would cast the results from such studies into question. Some studies that have compared intrinsic and extrinsic labeling in foods have questioned the use of extrinsic tags (Jin et al, 2008; Heaney et al, 2000). Research on the subject in the context of biofortified maize may be required to conclusively address this research issue.

Last but not least, additional research is required to further refine model bioavailability systems used for screening iron biofortified crops. The Caco-2 cell *in vitro* iron model has been invaluable in screening maize lines in this study. However, to compare results from one experiment with those of another requires a control sample to be used in the all experiments so that the sample: control ratios can be compared. After careful thought, we decided to use Tamale maize grown and consumed in Mexico as a control sample in our research work. If not wisely selected, the control sample can be a limiting factor in the study, and this fact raises the issue of how best to select a control sample. Is it necessary to have a synthetic control sample in addition to a researcher's control sample? Further research should be dedicated to addressing these questions.

The poultry model has been previously used to understand iron bioavailability, and shows promise as an initial *in vivo* screening tool for iron biofortified foods (Tako, Rutzke & Glahn, 2010). However, data and information about how to optimize the model for an iron bioavailability trial is limited to a certain extent. This is especially

true when we consider the extensive information available on the pig and rat models. It would thus be useful to devote further research to developing an iron bioavailability study protocol for the poultry model and establishing hematological indices to guide researchers as they conduct these studies.

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