

Improving nutritional qualities of maize endosperm: Introduction

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Malnutrition

The problem of malnutrition

Malnutrition is the result of a diet lacking in one or more nutrients. While undernutrition due to a lack of overall calories is a problem that certainly needs attention, malnutrition has gone virtually unnoticed. The hidden hunger of malnutrition affects an astonishing one in three people worldwide, according to the Micronutrient Initiative [1]. Lack of key nutrients, especially in the first 1000 days of life (from conception to the second birthday), results in adverse effects to cognitive and physical development as well as a reduction in immune function [2]. In this dissertation, methods that contribute to the reduction of malnutrition due to inadequate iron or protein are considered.

Potential solutions

The most desirable method of improving nutrition is to improve accessibility to nutritious foods, but this solution requires long-term efforts to reduce poverty. A diverse diet including fruits, vegetables, and animal products is simply not accessible to many people in the developing world. Many different strategies have been used to improve nutrition, including supplementation, fortification, changes in diet, and biofortification. Each strategy has its own benefits and challenges.

Nutritional supplements deliver a concentrated and often highly bioavailable form of a nutrient. They are used successfully in the developed world to treat nutritional deficiencies under care of medical professionals, but problems associated with supplementation in developing countries include irregular delivery, low acceptance rates, quality control, potential side effects such as nausea, and potential over-supplementation [3]. Food-based approaches may be more sustainable compared to supplementation. Fortification can be a cost effective means of delivering additional nutrients when foods are processed in industrial settings before distribution to consumers [3]. This type of fortification can potentially improve the nutrient intake of a large proportion of the

population. Successes of fortification on a large scale include iodine in salt [4] and iron in fish sauce and soy sauce [5]. However, for fortification to be effective, persons with nutritional deficiencies must have regular access to industrially processed foods, which is not the case in many developing countries that lack consistent supply chains to rural areas. On smaller scales, multi-nutrient fortification of foods for school age children can improve micronutrient status and reduce incidence of anemia [6]. A combination of large scale and smaller scale fortification efforts with targeted supplementation for those at greatest risk is likely needed to ensure that the largest possible number of persons are receiving needed nutrients [7].

A more immediate way to improve nutrition is to increase the bioavailability of existing nutrients in the diet. For example, different processing methods of cassava affect bioavailability of beta-carotene [8] and addition of lactic acid during tortilla preparation can increase bioavailability of iron in maize flour [9]. Enhancement of nutrient bioavailability through changes in preparation methods of staple foods is particularly important as prices of staple foods are increasing. When the price of staples and other foods goes up, consumption of nutrient rich foods like vegetables and meat goes down (Figure 1) [10]. Due to higher grain prices, families in Africa are finding themselves unable to afford vegetables and dairy to supplement their maize-based diet. [11]. Improving the nutrients in staple foods with biofortification could help decrease the negative nutritional effects of a monotonous diet.

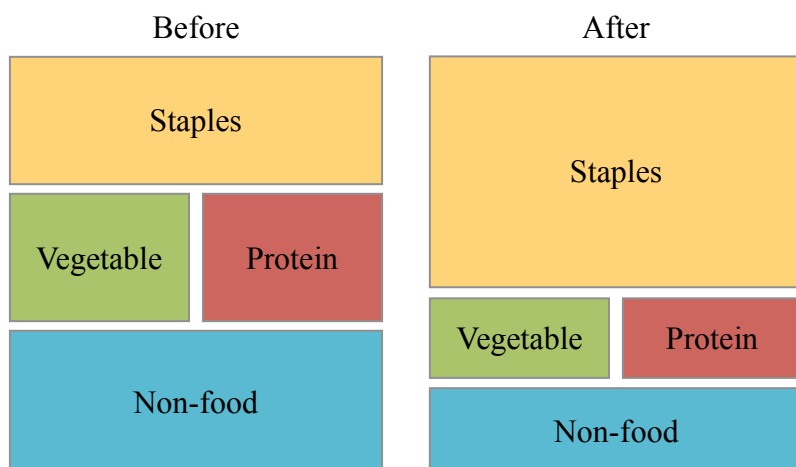


Figure 1. Percentage of funds spent by families on different items before and after a 50% increase in food prices, with income held constant. Image adapted from Bouis et al. [10].

Biofortification is a potential solution for malnutrition

Biofortification is the improvement of intrinsic nutritional qualities of crops. Some nutrients can be successfully increased through fertilization, such as zinc [12]. In the case of iron, soil abundance is rarely a problem [13], but the availability of iron in the soil is negatively affected by multiple factors. These include: temperatures that are too low or too high, high organic content, poorly aerated soils, nematode infection of roots, and high levels of some nutrients, including phosphorus, zinc, copper, and magnesium [14]. Soil pH is a particularly important factor; at higher pH, iron is converted to insoluble forms [14]. With such a wide variety of factors involved in iron absorption, soil improvement is not a viable way to quickly improve iron content of crops. Fertilization with nitrogen results in increased total protein for many crops [15] but does not change the quality of the protein.

Biofortification with selective breeding or biotechnology is a way to improve the nutritional qualities of crops that eliminates the need for a central distribution system or soil improvement, needing no outside intervention once seeds are distributed. These qualities make biofortification an attractive interim solution for malnutrition in developing countries [16], at least until broader strategies to reduce poverty are successful. Biofortified seed must be made available at low or no cost in order to be effective, so there is little incentive for corporations to provide private funding for research. Public funding has been the primary source for projects using breeding and biotechnology to improve nutritional qualities of crops [17]. However, there is evidence that corporations see value in public-private partnerships.

Monsanto is developing drought tolerant maize in the Water Efficient Maize for Africa (WEMA) project in partnership with the Gates Foundation and other non-profit organizations. Monsanto plans to distribute improved seed with the water efficient trait to low income farmers at no cost, while relatively wealthy farmers may be required to pay royalties for using the trait [18]. WEMA may prove to be a model for new public-private partnerships focusing on biofortification.

Depending on the trait, it may be possible to sell biofortified seed in developed countries as a specialty variety. For example, maize with improved protein quality might be useful as animal feed in developed countries but would help reduce human protein-energy malnutrition in developing countries.

There has been success in improving single nutritional traits of various crops with breeding, including improvement of iron, zinc, and manganese in wheat, and of iron in rice and in beans [19]. The most well known example of biofortification is Golden Rice, engineered to express high levels of beta-carotene, the precursor of vitamin A [20]. Biotechnology has also been used to increase not only minerals but also such diverse nutrients as folate, resistant starch, and fiber [19]. Previous biofortification research specific to improvement of bioavailable iron and protein quality in maize will be discussed in subsequent sections.

Many of these previous biofortification efforts using biotechnology made use of genes from species other than the target crop. Biotechnology that makes use of genes from different species is controversial, and may not be well accepted by the target consumers in developing countries or by activists in developed countries. A cisgenic approach, in which genes and genetic elements from the species of interest are used to produce a desired phenotype, may prove more acceptable [21]. Maize and relatives of maize contain many genes that have the potential to improve nutritional quality of maize grain, including maize globin for bioavailable iron and seed storage proteins for protein quality.

Iron deficiency and improvements in dietary iron

Iron deficiency and absorption

Approximately 1.62 billion people are affected by anemia, many due to poor iron status [22]. The regions most affected are Africa, South East Asia, and parts of South America [23]. Globally, anemia is most prevalent among pre-school age children (47.4%), followed by pregnant women (41.8%), and school-age children (25.4%) [22]. The prevalence of iron deficiency may be even greater than that of anemia, because tests for anemia do not identify iron deficiency [24]. Even mild iron deficiency has a wide range of negative health effects [24]. Two problems are of particular concern: the cognitive and physical development of infants is compromised [25], and the immune system of all age groups is inhibited [26,27].

Iron status affects multiple health outcomes even when a direct link is not present. For example, iron deficiency increases mortality of pregnant women because they are less likely to survive blood loss in childbirth [28]. There is evidence that iron deficiency increases absorption capacity for all divalent heavy metals, increasing susceptibility to heavy metal poisoning [29]. There are

also direct economic consequences of iron deficiency. For example, iron deficient adults have decreased work productivity than adults with adequate iron [24,28].

Absorption of iron in the diet is affected by the state of iron deficiency. Most studies suggest that individuals that are iron deficient absorb about 20% of dietary iron, while individuals that have healthy levels of iron absorb about 10% of dietary iron [30]. Because of this variation in absorption, it is difficult to set a recommended daily value (RDV) for iron. The amount of dietary iron that must be absorbed per day to maintain a healthy iron status depends on age and menstruation status. Adult men require about 1mg while menstruating women require 1.5mg to as much as 3.4mg, pregnant women require 4-5mg toward the end of pregnancy, and children have high iron requirements during periods of rapid growth [31]. The iron RDV is 8mg for men and post menopausal women, 18mg for menstruating women, and 27mg for pregnant women [32]. Upper limits for daily dietary intake of iron are 40mg for infants and children, and 45mg for adolescents and adults [32]. The World Health Organization recommends rates of iron fortification that are higher than the upper limits listed here, but those rates are based on the bioavailability of the particular fortificant [33] (Table 1).

Inhibitors and enhancers of iron bioavailability

In addition to iron status, bioavailability of iron depends on compounds in a meal that enhance or inhibit iron bioavailability. While heme iron is absorbed intact [34], absorption of non-heme iron in the diet can be greatly affected by preservation, processing, and preparation of food. Other foods eaten with the iron source can also affect bioavailability of non-heme iron. Changes in dietary habits can be effective in increasing bioavailability of existing iron in the diet, but they require large-scale educational efforts and changes in cultural practices as well as increasing access to food that enhance iron absorption.

Phytates are the primary storage form of phosphorous in plants. As such, they are common compounds in plant-based diets [35]. Phytate binds iron as well as other minerals such as calcium, copper, and zinc [13] in a form that is not readily bioavailable. Enzymatic deactivation of phytate, also known as phytic acid, with fermentation or germination, increases iron bioavailability of grains [9,36]. Consumption of polyphenols [37] such as tannins, found in coffee, tea [38], and some herbs, with meals can also decrease iron absorption [35].

Ascorbic acid can overcome inhibitors of iron absorption, including calcium, phytic acid, and polyphenols [39]. Ascorbic acid enhances non-heme iron absorption [35], so preparation methods that include the addition of foods rich in vitamin C, such as citrus juice or tomatoes, will increase iron bioavailability of the meal. Unknown factors in meat increase the bioavailability of iron from plant sources [40]. Recently, nicotianamine was found to be an greater enhancer of iron bioavailability than ascorbic acid [41], and both EDTA and folic acid have been found to be effective co-forticants of iron as well [42].

Iron forticants

Finding the right iron forticant is a challenge. It must be inexpensive, easily absorbed, able to overcome dietary inhibitors, and cause minimal changes in taste or color [39]. Ferrous sulfate (FeSO_4) is a highly bioavailable iron forticant, and is considered the standard by which other iron forticants are measured. Iron bioavailability is often expressed as relative biological availability (RBA, also known as relative biological value, RBV), or the bioavailability of the iron compound compared to the bioavailability of ferrous sulfate within the same experiment, regardless of how bioavailability is measured. Despite its high bioavailability and low cost, ferrous sulfate is far from being an ideal forticant. It has a dark red color, a metallic taste, and can cause fats in flour to go rancid [39]. In addition, cooking can cause bioavailability of ferrous sulfate to decrease [43].

Iron forticants can be divided into four categories based on their solubility [44]. Forticants in category 1 are water-soluble, have high bioavailability, but are likely to have unpleasant sensory changes. Forticants in category 2 are poorly water-soluble but soluble in weak acid, with high bioavailability and few sensory changes. Forticants in category 3 are insoluble in water and poorly soluble in weak acid, with variable bioavailability and few sensory changes. Forticants in category 4 are chelates that tightly bind iron, have high bioavailability in the presence of the inhibitor phytic acid, and have some sensory changes. Table 1 shows common forticants with their percentage of iron by weight and bioavailability, as collected by Hurrell [44]. Each of these potential iron forticants has advantages and disadvantages. For example, NaFeEDTA has been shown to be more bioavailable than ferrous sulfate when added to wheat flour [44], but is expensive compared to ferrous sulfate [39]. Electrolytic iron causes the least color change when added to corn flour tortillas, while ferrous sulfate causes the most color change [16,45].

Common iron fortificants categorized by solubility

Category	Compound	% Iron	Bioavailability
1	Ferrous sulfate 7 H ₂ O	20	100
	Ferrous sulfate, dried	33	100
	Ferrous gluconate	12	89
	Ferrous lactate	19	67 to 106 ^a
	Ferric ammonium citrate	17	50 to 70
2	Ferrous fumarate	33	30 to 100 ^b
	Ferrous succinate	33	92
3	Ferric pyrophosphate	25	21 to 75
	Micronized dispersible ferric pyrophosphate	1.2	15 to 93
	Ferric orthophosphate	29	25 to 32
	Elemental iron: hydrogen reduced	96	13 to 148
	Elemental iron: electrolytic	97	75
	Elemental iron: carbonyl	99	5 to 20
4	Ferrous bisglycinate	20	90 to 350
	NaFeEDTA	13	200 to 400

Table 1. Percent iron by weight and relative bioavailability of common iron fortificants categorized by solubility. RBAs reported here are from human isotope absorption studies. ^a RBA of 106 for ferrous lactate was with added ascorbic acid. ^b For ferrous fumarate, RBA of 100 is for adults, RBA of 30 is for infants. Adapted from Hurrell [44].

Recommended amounts of fortificant by daily flour consumption

Compound	< 75 g/day	75 to 149 g/day	150 to 300 g/day	> 300 g/day
NaFeEDTA	40 ppm	40 ppm	20 ppm	15 ppm
Ferrous sulfate	60 ppm	60 ppm	30 ppm	20 ppm
Ferrous fumarate	60 ppm	60 ppm	30 ppm	20 ppm
Electrolytic iron	not recommended	not recommended	60 ppm	40 ppm

Table 2. Average levels of fortificant recommend for addition to wheat flour based on fortificant type and estimated per capita flour consumption. Adapted from WHO [33].

The Department of Nutrition for Health and Development (NHD) of the World Health Organization has prepared guidelines for flour fortification that take into account the relative bioavailability of fortificants and per capita consumption [33], as shown in Table 2. Fortification of flour alone is not sufficient for menstruating women when per capita flour consumption is less than 75g per day. Electrolytic iron is not recommended when per capita flour consumption is low; due to the large amount required, sensory qualities may be negatively affected [33].

Biofortification of iron

Some studies have shown that there is sufficient genotypic variation in major cereal crops to breed for higher total iron [46], but an evaluation of more than 1000 improved maize genotypes and 400 maize landraces found little variation of total iron in the grain [47]. Recent research on breeding for bioavailable iron rather than total iron has had some success [48], but the Caco-2 cell culture model and animal models for iron bioavailability make screening germplasm for improved bioavailability costly. Key to improving iron in the grain is mobilizing and redistribution of iron from the leaves to the grain [46]. Also important is ensuring that iron accumulates in the endosperm, because the embryo is often removed prior to consumption, although this practice is not as prevalent in maize as in wheat and rice.

A variety of transgenic approaches have been used to improve total iron or bioavailable iron. Attempts to increase total iron by overexpressing iron transporters have had mixed success [13]. Expression of human lactoferrin with an endosperm specific promoter in rice caused a two-fold increase in total iron [46]. Expression of soybean ferritin in rice caused a two to three fold increase of total iron which was as bioavailable as ferrous sulfate [46]. Another approach is to reduce the antinutrient phytate. This can be accomplished with the gene for the enzyme phytase from a microorganism such as the fungus *Aspergillus niger*. Reduction in phytic acid may not be an optimal solution, as low phytic acid mutants have reduced germination rates, increased oxidative stress, reduced tocopherols, and DNA damage, indicating that phytate acts as an antioxidant [49]. These problems were not observed in maize with low phytic acid due to expression of fungal phytase [49]. Co-expression of ferritin and phytase increased iron bioavailability significantly [50]. There has been little research into increasing iron bioavailability by expressing absorption enhancers, but this method shows promise. For example, over-expression of rice nicotianamine, a metal chelator present in all higher plants, in rice grain resulted in increased total iron and total zinc as well as an increase in iron bioavailability compared to wild-type rice [41].

Hemoglobin

Iron in hemoglobin is not susceptible to inhibition of bioavailability caused by compounds like phytate. This is because heme is taken up by the digestive system intact, with heme oxygenase

releasing the iron within mucosal cells [34] of the small intestine. Hemoglobin was originally isolated in mammals, but the gene for globin has been found in a variety of organisms, including bacteria and plants [51]. Hemoglobin consists of the protein globin and the iron binding heme. In this dissertation, globin protein that has been determined to have heme will be referred to as hemoglobin. Globin protein that does not contain heme or that has not yet been determined to contain heme will be referred to simply as globin.

Bovine hemoglobin was shown to have bioavailability similar to that of ferrous sulfate [52]. Despite its high bioavailability, heme iron from animal sources is not currently used as a fortificant. Problems include source, stability, and color. Hemoglobin may be obtained from waste blood from slaughterhouses, but this source is not available to many iron deficient populations, and may not be feasible for cultural or religious reasons. Whole dried blood, purified hemoglobin, and heme alone may be used as fortificants. Using heme alone decreases the amount of fortificant that must be added because 94% of hemoglobin is the globin protein, however this decreases iron bioavailability due to aggregation of free heme in the intestine [53]. When refrigerated, pure dry animal hemoglobin has a shelf life of about two years, but when reconstituted the shelf life is reduced to 10 days refrigerated and 21 days frozen [54]. The dark color of heme limits it as a fortificant to dark colored foods such as chocolate and black beans [53].

Plant hemoglobin

At least one globin gene has been found in each plant that has been investigated for such genes [51]. The three dimensional structures of multiple plant globins have been determined to have a heme binding pocket [51,55], indicating that plant globin could contain heme in vivo. Plant heme is the result of a complex biosynthetic pathway [56], as shown in Figure 2. Heme has a ring of four nitrogens that interact with four of the six binding sites of an iron atom. The terminal histidine, an amino acid from the globin protein, binds to one of the remaining sites of an iron atom. In pentacoordinate hemoglobins, the sixth site binds strongly with oxygen and other gases, including nitric oxide and carbon monoxide. In hexacoordinate hemoglobins, the sixth site is bound by a second histidine [51].

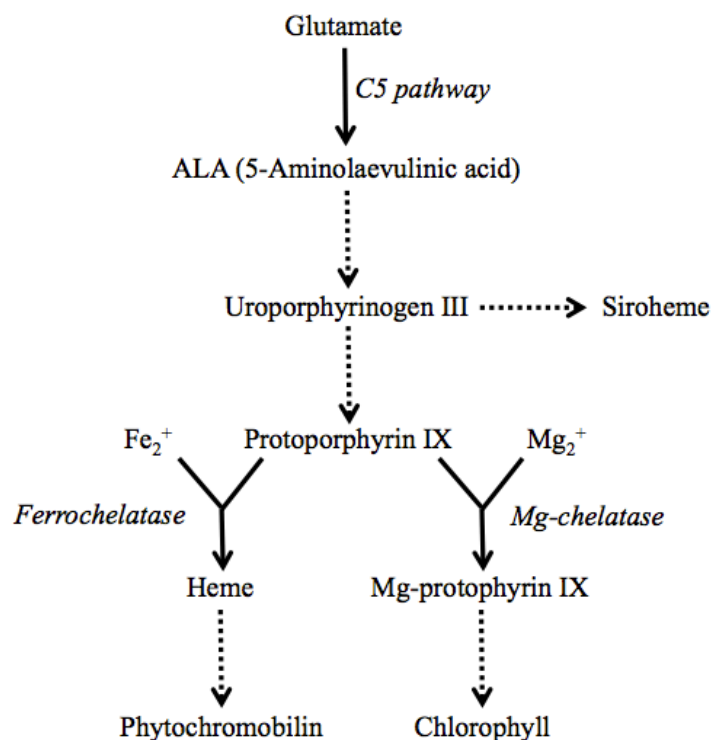


Figure 2. Simplified plant tetrapyrrole synthesis pathway. Dashed lines represent multiple steps. Adapted from Cornah [56].

Three groups of globins have been found in plants: symbiotic, non-symbiotic, and truncated. There is evidence that the three groups evolved from a common ancestral hemoglobin [55]. Symbiotic hemoglobins are found in the root nodules of legumes and some other plants in association with nitrogen fixing bacteria. These pentacoordinate hemoglobins use iron to bind oxygen to create an anoxic, or low oxygen, environment for the bacteria. Non-symbiotic globins are present in low concentrations in plant tissue, so are unlikely to function as oxygen transporters like symbiotic hemoglobins [51]. They are hexacoordinate, but still show strong affinity for oxygen [51]. Truncated hemoglobins are so-named because one of their domains is shorter than that of other globins [51]. The presence of heme in non-symbiotic and truncated globins *in vivo* has not yet been determined.

The function of non-symbiotic globins remains unclear, but seems to be involved with stress response [51]. Non-symbiotic plant globins are involved in stress tolerance, allowing mitochondria to function and allowing plants to grow under conditions of low oxygen [57]. In maize seedlings, maize globin mRNA levels increased in root tips under stress due to high salt and low oxygen, and expression of maize globin in tobacco allowed greater tolerance to

submergence and salt [58]. When barley globin was overexpressed in maize tissue culture cells under low oxygen, nitrous oxide levels were reduced [59].

Only one study to date has tested the bioavailability of a plant hemoglobin. Proulx et al. compared the bioavailability of symbiotic leghemoglobin from soybean root nodules to that of bovine hemoglobin and ferrous sulfate [43]. They found that, when tested without a food matrix in a Caco-2 cell model, leghemoglobin was slightly more bioavailable than ferrous sulfate and bovine hemoglobin was twice as bioavailable as ferrous sulfate [43]. Notably, when added to a food matrix and cooked, bioavailability of ferrous sulfate was significantly decreased while the hemoglobins were unaffected [43].

Using biofortification to increase levels of heme iron in crops may bypass some of the problems presented by fortification with mammalian heme or hemoglobin, but more testing is needed to determine the bioavailability of non-symbiotic plant hemoglobins and to determine whether non-symbiotic globin in plants is associated with heme.

Protein deficiency and seed storage proteins in maize

Protein deficiency

Protein-energy malnutrition (PEM), occurs when protein and nutrient intake do not meet requirements for growth and bodily maintenance [60]. Protein deficiency is associated with increased susceptibility to disease and subsequent mortality [61]. PEM can be defined by multiple measures, but underweight is the measure that can be found in most global data. Underweight is defined as a weight that is 80% of what is expected for the age. This anthropometric indicator is primarily used for pre-adolescent children because height varies widely in adults [62]. The World Health Organization estimated the prevalence of underweight preschool children in 2010 was 16.2% percent (103.7 million). The prevalence was 4.3% in Central America and 19.3% in Africa (1.8 and 30.3 million respectively) [63,64], both including areas where maize is a staple crop.

When inadequate protein intake is combined with adequate carbohydrate consumption over a long period of time, there is decreased synthesis of visceral proteins, including albumin. This results in a decrease in hydrostatic pressure in the vascular system and extravascular fluid

accumulation under the skin and in body cavities [60]. Other measures of malnutrition are stunting (low height for age) and wasting (low weight for height). A low height for age is caused by chronic malnutrition that occurs when there is a protein and energy imbalance for an extended period of time [62]. A low weight for height is caused by acute malnutrition, or malnutrition of a high severity over a shorter period of time. A person can be affected by both chronic and acute malnutrition, being both stunted and wasting at the same time. In sub-Saharan Africa, it is estimated that 10% of children are wasting and stunting can be as high as 40% in some areas [65].

Dietary requirements for protein

Dietary needs for protein vary, with 0.66g/kg of protein being the average daily requirement for adults, and 0.83g/kg meeting or exceeding the requirements for 97.5% of the healthy adult population [66]. Pregnant and lactating women require additional protein, up to 31g per day above pre-pregnancy requirements, depending on trimester or lactation status. There are no differences in protein requirements for age or sex in adults, based on available evidence. In addition to the 0.66g/kg daily protein requirement for body maintenance, children require additional protein for growth based on age. Infants require much more protein on a body weight basis, with an average daily requirement of 1.12g/kg, and 1.31mg/kg meeting or exceeding the requirements for 97.5% of healthy infants. The daily protein requirement falls rapidly up to age two, then gradually decreases until meeting the adult value at or shortly after age 18. Adolescent males require a little more protein for growth than adolescent females [66].

The bioavailability of protein depends on relative amounts of non-essential and essential amino acids and on amounts of other nitrogen sources in the diet. Essential amino acids are those that cannot be made by the human body, thus must be acquired in the diet. A protein source that contains amino acids in approximately the same ratios as human dietary requirements is a complete or balanced protein and will be digested more efficiently than an incomplete protein [67]. Larger quantities of unbalanced proteins are needed to meet dietary needs. Small amounts of some essential amino acids are synthesized by bacteria in the gut, but not in quantities large enough to meet daily requirements [66]. For humans, the essential amino acids are: leucine, lysine, valine, phenylalanine, tyrosine, isoleucine, threonine, methionine, histidine, cysteine, tryptophan [66]. Of these, lysine is of the greatest concern because of the relatively low levels of

lysine present in staple foods, including maize. Amino acid requirements for adults and amino acid content of maize are shown in Figure 3.

Dietary protein needs of children and adults who are suffering from disease have higher protein and/or specific amino acid requirements, depending on the particular disease. For example, Kurpad et al. found that undernourished men with intestinal parasites required about 50% more lysine than uninfected men [68]. Previously undernourished children require additional protein and other nutrients above average required daily amounts [66]. These additional requirements must be considered in any efforts to increase total protein or protein quality of maize.

Maize protein content and quality

The total protein content of maize grain is highly elastic. The most well known experiment that altered total protein content in maize is the Illinois Long Term Selection Experiment. Starting with a single open pollinated variety, selection for high and low protein has been conducted since 1896 [69]. Typical maize lines consist of 8-12% protein, but selection has resulted in a high protein line with over 32% protein and a low protein line with 4% protein [70]. Even though high protein is possible in maize, selection for yield has increased starch and decreased total protein [71]. The decrease in total protein is not associated with a change in ratios of the essential amino acids lysine, methionine, and tryptophan [71], but the low content of these amino acids in maize has contributed to nutritional deficiency in regions where maize is the staple food, such as in Africa and Central America, particularly in cases where maize makes up more than 50% of the diet [65]. These amino acids are also important when maize is used as an animal feed, such that maize must be combined with other grains or with synthetic amino acids to provide a complete protein source.

While there is little variation in amino acid content in modern inbred lines, some mutations such as *opaque2* significantly alter maize amino acid composition. Zarkadas et al. examined amino acid content of a quality protein maize (QPM) line, which contains the *opaque2* mutation, alongside flint and dent inbreds. They showed that the QPM line had approximately twice as much lysine as the flint and dent inbred lines (Figure 3). Using estimates of lysine content in QPM and non-QPM maize, Nuss and Tanumihardjo showed that 40% less QPM maize was needed to meet daily requirements for lysine compared to non-QPM maize, at 500g per day for

adults and 100g per day for children [65]. Lysine, along with methionine and tryptophan, are important targets for improvement of maize protein quality.

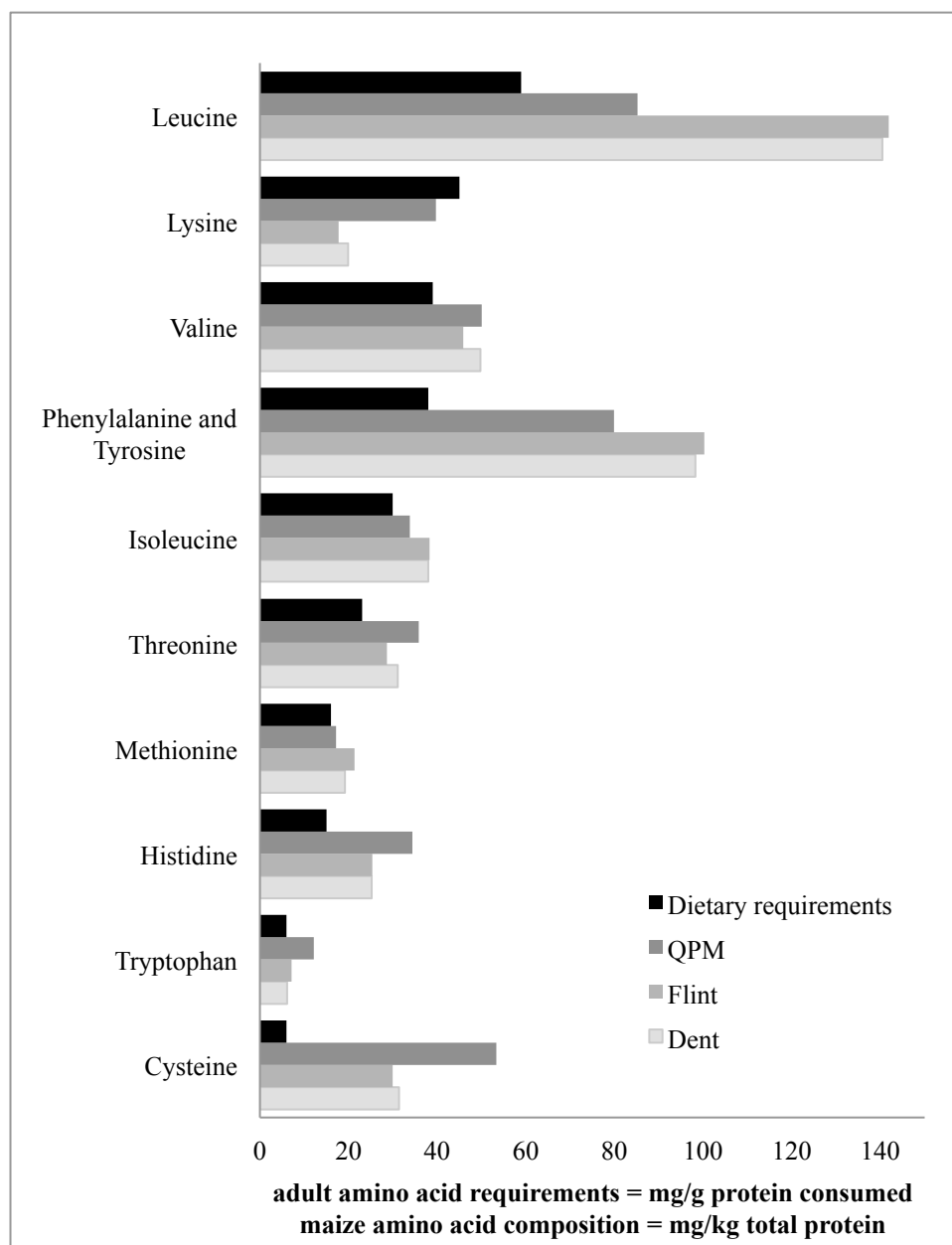


Figure 3. Adult amino acid requirements and amino acid requirements of three maize genotypes. Data from WHO [66] and Zarkadas [72]. The required mg/g protein is based on a mean nitrogen requirement of 105mg nitrogen/kg of body weight per day (0.66g protein/kg per day).

Zein description and function

Zeins make up about 50% of total protein in the endosperm [73] and are not expressed elsewhere in the plant [74]. These seed storage proteins serve as a source of nitrogen for the seedling during

germination and have no enzymatic activity [75]. Due to their localized expression at high levels, zein promoters are useful for many biotechnology applications [76], including expression of proteins for extraction and for biofortification. Zeins play an important role in total protein and amino acid content in maize. For example, in the Illinois Long Term Selection experiment, the high protein line had increased zeins while the low protein line had decreased zeins [77].

Genetic similarity, solubility, and amino acid content are used to classify zeins into groups: alpha, beta, gamma, and delta [78]. Zeins aggregate into protein bodies in the rough endoplasmic reticulum of the lumen in endosperm cells. The alpha zeins accumulate in the center of protein bodies, after a shell consisting of beta and gamma zeins forms [79]. The gamma and delta zeins are also deposited on the surface of starch granules in the endosperm [80]. The alpha zeins (19 and 22kDa) are encoded by large group of genes, producing proteins that are abundant and variable across maize lines [81]. They contain primarily leucine and alanine, so are not desirable from a nutrition standpoint. The beta (14kDa), gamma (16 and 27kDa), and delta (10 and 18kDa) zeins are less abundant but high in methionine and cysteine compared to alpha zeins. As the endosperm matures, zeins encapsulate starch granules into a hydrophobic starch-protein matrix [80,82]. This matrix has been shown to reduce the digestibility of starch when corn grain is fed to lactating dairy cows [83,84], which results in lower feed efficiency.

None of the zeins are high in lysine, so mutations that decrease zein expression are of interest in improving amino acid balance of maize. In the homozygous state, the *opaque2* mutation results in an increase in lysine greater than 50% [85]. *opaque2* is a transcription factor that controls expression of zeins and of an enzyme that degrades free lysine. Maize with the *opaque2* mutation has decreased zeins and higher free lysine [85]. However, the endosperm of *opaque2* maize is soft so is more susceptible to storage pests and fungus than non-opaque maize. Breeding efforts to reduce these negative characteristics has resulted in quality protein maize (QPM).

Upregulation of 27kDa gamma zein is associated with increased kernel hardness in QPM, possibly due to its role in protein body formation [86,87]. The 27kDa gamma zein is also notable because its promoter has been shown to drive high expression of transgenes in maize endosperm [88], making up 5% of endosperm transcripts [86]. Better understanding of the 27kDa gamma zein and other seed storage proteins may allow for improvement of protein quality in maize without reduction of yield or agronomic qualities.

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