Investigation of factors impacting provitamin A bioavailability and indicators of micronutrient status

By

Kara A. Bresnahan

A dissertation submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

(Nutritional Sciences)

at the

UNIVERSITY OF WISCONSIN-MADISON

2014

Date of final oral examination: May 8th, 2014

This dissertation is approved by the following members of the Final Oral Committee:
Sherry Tanumihardjo, Associate Professor, Nutritional Sciences
David Eide, Professor, Nutritional Sciences
HuiChaun Lai, Associate Professor, Nutritional Sciences
Susan Smith, Professor, Nutritional Sciences
Philipp Simon, Professor, Horticulture

Acknowledgements

I thank Dr. Sherry Tanumihardjo for her exceptional mentorship over my six-year tenure as an undergraduate and graduate student in her laboratory. Her guidance has expanded my understanding on the role of the scientist to someone who seeks to manifest their work to the greatest benefit of humanity. The unique opportunities she has encouraged me to pursue have helped me develop a diverse scientific skill set, a broad understanding of global health issues, and a deep appreciation of multi-disciplinary collaboration. I thank my committee for their shared knowledge and reassurance throughout my studies. I thank my colleagues for their friendship and assistance. Finally, I thank my family for their unwavering love and support.

Table of Contents

Index of Tables

Index of Figures

Index of Abbreviations

9*c*, 9-*cis*

13*c*, 13-*cis*

βC, β-carotene

βCE, β-carotene equivalent

ACT, antichymotrypsin

ACTH, adrenocorticotropic hormone

AGP, α_1-acid glycoprotein

APP, acute phase protein

APR, acute phase response

a*t*, all-*trans*

BC, Butobe cooked

BR, Butobe raw

C-A-, neither CRP nor AGP raised

C+A-, CRP raised only

C-A+, AGP raised only

C+A+, both CRP and AGP raised

CB, control banana

CG, Cavendish green

CR, Cavendish ripe

CRP, C-reactive protein

IOM, Institute of Medicine

KG, Kluai Khai Bong green

LG, Lady Finger green

LR, Lady Finger ripe

MC, M9 cooked

MR, M9 raw

MRDR, modified relative dose response

NC, Nakinyika cooked

NR, Nakinyika raw

PEM, protein energy malnutrition

SAA, serum amyloid A

SN, Sukali Ndizi

TDRC, Tropical Disease Research Center

VA, vitamin A

VA+, vitamin A positive control

VA-, vitamin A negative control

VAD, vitamin A deficiency

ABSTRACT

Vitamin A is an essential nutrient that is important for visual, reproductive, developmental, and immunological health. Vitamin A deficiency is a major global health concern, impacting 190 million children under 5 years of age. The introduction of provitamin A carotenoid biofortified staple crops may provide a sustainable and cost effective solution for alleviating vitamin A deficiency, particularly in the rural poor who are disproportionately missed by supplementation interventions.

In order to develop biofortified crops that serve as effective vehicles for the delivery of vitamin A to target populations, the investigation of factors impacting the bioavailability of provitamin A carotenoids from plant foods is essential. *Cis*- isomers of β-carotene are increased by up to 50% percent during thermal processing of plant foods and are currently assigned a vitamin A value equivalent to 50% that of all-*trans* β-carotene. However, the unique bioefficacies of 9-*cis*- and 13-*cis*-β-carotene have not been evaluated using physiological doses in an animal model that metabolizes vitamin A and carotenoids similarly to humans. Bananas are a staple crop, which contain varying levels of provitamin A carotenoids, in many tropical and subtropical regions where vitamin A deficiency persists. It is important to elucidate the impact of matrix effects on provitamin A carotenoid bioavailability in different cultivars and preparations of banana so as to assist plant breeders in biofortification efforts.

The burden of infectious disease is high in many regions where vitamin A deficiency is prevalent. Infection suppresses appetite and alters blood-based indicators of micronutrient status. Few studies have quantified the effect of common infections, such

as malaria, on dietary intake. As provitamin A carotenoid biofortification targets are currently set to meet 50% of the EAR in children and based on food intake, infection-related appetite suppression is important to consider. Alterations to micronutrient status indicators can lead to inaccurate estimations of deficiency in populations with high or unknown prevalence of infectious disease. Quantitative investigations into the impact of infection by time stage on blood-based micronutrient status indicators will support the development of a standardized method for adjustment.

Preface

Explanation of dissertation format:

Evidence supporting the following dissertation is derived from studies implementing the gerbil as a model of human vitamin A and carotenoid metabolism and from a randomized, controlled feeding trial in rural Zambian children with high provitamin A carotenoid biofortified maize. Chapter 1 reviews the synergistic relation between infection and malnutrition and considers the impact of infection on indicators of micronutrient status. Chapter 2 examines the unique bioefficacies of 9-*cis*- and 13-*cis*-β-carotene as compared to the all-*trans* isomer when dosed at physiological levels in Mongolian gerbils. Chapter 3 investigates differences in provitamin A carotenoid bioavailability from green versus ripe and raw versus cooked banana diets in Mongolian gerbils. Chapters 4 and 5 consider the effect of infection on dietary intake and indicators of micronutrient status in preschool-aged, rural Zambian children. Chapter 6 summarizes the main findings from these investigations. The appendix contains a published paper on work I participated in as a research intern and graduate student in the Tanumihardjo laboratory.

CHAPTER 1

REVIEW: Undernutrition, the Acute Phase Response to Infection, and its Effects on Micronutrient Status Indicators

Kara Bresnahan

Interdepartmental Graduate Program in Nutritional Sciences

University of Wisconsin, Madison, WI 53706, USA

To be submitted for peer review

ABSTRACT

Infection and undernutrition are prevalent in developing regions of the world and demonstrate an synergistic relationship. The acute phase response (APR) to infection is a systematic inflammatory reaction to disruptions in a host's homeostasis. Released from immune cells in response to deleterious stimuli, proinflammatory cytokines act on distant tissues to induce the behavioral and systemic effects of the APR. Cytokines act to increase energy and protein requirements in order to manifest fever and support hepatic acute phase protein (APP) production. Blood concentrations of glucose and lipid are augmented to provide energy to immune cells in response to cytokines. Additionally, infection suppresses appetite, decreases intestinal absorption of nutrients, and can cause greater direct loss of micronutrients. Traditional indicators of iron, zinc, and vitamin A status are altered during the APR, leading to inaccurate estimations of deficiency in populations with high or unknown prevalence of infection. Blood concentrations of APPs can be employed in nutrition interventions to assess the time stage and severity of infection. A standardized method for adjusting micronutrient status indicators that are sensitive to infection is needed and should include universalized elevation cutoffs for APPs. Malnutrition increases infection-related morbidity and mortality. Protein energy malnutrition leads to increased gut permeability to pathogens, abnormal immune cell populations, and impaired APP response. Micronutrient deficiencies cause specific immune impairments that impact both innate and adaptive responses. The antagonistic interaction between the APR and nutritional status epitomizes the need for integrated interventions to address undernutrition and reduce disease burden in developing countries.

INTRODUCTION

Undernutrition persists as a major global health concern, particularly in Sub-Saharan Africa, South Asia, and regions of Latin America (1-3). Dietary intakes of populations in these areas are often chronically deficient in macronutrients [leading to protein-energy malnutrition (PEM)], micronutrients (leading to specific micronutrient deficiencies), or both (2,4-6). Poverty is the foundational cause of undernutrition and associated health determinants, such as infectious disease risk (7-9).

Over half of all deaths from infection are associated with malnutrition in children under 5 years of age (10), and a synergistic relationship exists among nutritional status, immunity, and infectious disease. Undernutrition increases vulnerability to infection through impaired immunity, and infection exacerbates the condition, further weakening the immune response (5). Even mild febrile infections negatively impact nutritional status; however, prior nutritional status of the host, severity of illness, and dietary intake during recovery determine the severity of the consequences (11-13). It is not surprising to find high prevalence of microbial and parasitic diseases occurring in developing countries where undernutrition is common (6,14).

PEM is associated with abnormal immune cell populations and impaired intestinal barrier function, which increases risk for microbial infection (5,15-17). Micronutrient deficiencies can lead to specific immune impairments that negatively impact both innate and adaptive immune responses (16,18). Innate immunity includes the acute phase response (APR), a systematic inflammatory reaction to imbalances in an individual's homeostasis (19). During the APR, metabolic processes and nutrient requirements are altered (19,20). Important in public health assessments, blood concentrations of micronutrients are also impacted, leading to inaccurate

assessments of micronutrient status and misestimates of deficiency prevalence during infection (21-23).

We review here the relation between infection and nutritional status. We will first consider the effects of the APR to infection on nutrient metabolism and then the impact of nutrient deficiency on immunity. We will also consider the impact of the APR on micronutrient status indicators and outcomes of nutrition interventions.

THE ACUTE PHASE RESPONSE TO INFECTION

The APR is a systemic inflammatory reaction to disruptions in the body's homeostasis due to infection, tissue damage, tumor development, certain chronic disease states, and immunological disorders. Inflammation is believed to be protective to the host as it removes injurious stimuli and promotes the healing of damaged tissue. The magnitude of the APR varies with the severity of injury and by time stage.

Generally, the APR initiates when a pathogen is detected by pattern recognition receptors, including Toll-like receptors, on a variety of immune cells. The activation of these receptors causes cells to secrete prostaglandins and cytokines that promote proinflammatory mechanisms, such as increases in vascular permeability and the recruitment of more cytokine secreting neutrophils and macrophages. Cytokines serve as short-lived, long-range mediators that act on various tissues to mount the systemic response, which is commonly characterized by fever, leukocytosis, increased secretion of adrenocorticotropic hormone (ACTH) and glucocorticoids, and alterations in plasma protein concentrations. Protein levels that are increased or decreased in the plasma during the APR are respectively called positive or negative acute phase proteins (APPs) and are primarily regulated by the liver. The APR is limited by

counter-regulatory mechanisms, including diffuse cell-mediated and targeted neural anti-inflammatory pathways. These mechanisms also promote tissue repair once the deleterious stimuli have been removed. The pathophysiological changes that occur during the APR have been previously reviewed in detail (19,24-36). Aspects of the APR that relate to nutrient metabolism and nutritional status will be examined further herein.

Cytokine Biology and Nutrient Metabolism

Most cytokines are multi-functional and exhibit effects on diverse cell types. Acute phase cytokines can be divided into three general categories: (i) proinflammatory cytokines, which include TNFα and interleukin (IL)-1, (ii) IL-6-type cytokines that promote the systemic features of the APR, and (iii) anti-inflammatory cytokines (26). These cytokines modulate the immune response and associated metabolic effects through complex interactions that can be additive, synergistic, or antagonistic. Generally, cytokines regulate gene expression through intracellular signal transduction pathways that activate transcription factors (24,39,40,41).

TNFα and IL-1 are generated shortly after the initial deleterious stimulus, promoting the production of a variety of other cytokines, such as IL-6, and inducing sickness behavior by acting on distant tissue sites through cell-mediated and neural communication pathways (24,42,43). TNFα, IL-1, and IL-6 are responsible for cytokine-induced sickness behavior, which is characterized by symptoms of fever, anorexia, weakness, malaise, somnolence, and an inability to concentrate (19,43-46). The metabolic cost of fever is high, increasing energy expenditure by about 7-11% per unit rise in fever (°C) in children (**Table 1**) (47,48). This, coupled with anorexia, produces a state of negative energy balance during infection. The fatigue

and discomfort associated with illness is believed to have the adaptive advantage of energy-conservation (43,44).

Proinflammatory cytokines shift metabolic processes to a catabolic state during infection. TNFα and IL-1 inhibit muscle protein synthesis and promote wasting (49-53). Through the stimulation of glucocorticoid release, IL-1 increases the whole body flux of amino acids by promoting muscle catabolism and liver anabolism (54). During infection, mean losses are 0.6 g protein/kg body weight/day, matching the estimated protein requirement of adults, and can be as high as 1.14 g protein/kg body weight/d during peak febrile response (11). Considering differences in the amino acid composition of muscle and APPs, Reeds et al. (55) concluded that the high demand for aromatic amino acids in APP production is responsible for a significant portion of the net loss in body nitrogen during the APR.

In order to ensure an adequate supply to obligate glucose-consuming cells, hyperglycemia is directly induced by cytokines during infection and via the stimulation of glucoregulatory hormones. Proinflammatory cytokines act on the hypothalamic-pituitary-adrenal axis to increase production of ACTH and glucocorticoids, which promote glycogenolysis and insulin resistance (56). Additionally, TNFα stimulates glucagon production, and both TNFα and IL-1 promote gluconeogenesis (56,57). The duration of hyperglycemia is dependent upon severity of infection and typically normalizes within 10 days (56); however, when infection is severe or septic, hypoglycemia can pose potentially lethal health risks (11).

Proinflammatory cytokines and IL-6 quickly increase serum triglyceride concentrations, which are maintained for at least 17 hours (57,58). This response is accomplished by: (i) promoting peripheral lipolysis, (ii) increasing intracellular citrate concentrations to up-regulate *de novo* fatty acid synthesis and hepatic triglyceride production, and (iii) enhancing VLDL

secretion (57,58). Hepatic fatty acid oxidation and ketogenesis are suppressed to ensure sufficient fatty acid substrate for these processes. Serum cholesterol concentrations are decreased by TNFα through suppression of LDL and HDL. Alterations in lipid profile are believed to be protective to the host during infection because lipoproteins have the ability to bind bacterial endotoxin and viruses, resulting in rapid clearance by the liver and reduced activation of cytokine-induced inflammatory mechanisms. Additionally, the increase in serum triglycerides may provide fuel to macrophages. During inflammatory chronic disease states and sustained APR, the prolonged alteration of lipid profile is associated with an increased risk of atherosclerosis (58).

IL-6 is the major regulator of the APR in hepatocytes and promotes the systemic effects of the APR by stimulating the growth and differentiation of many cell types. While TNFα and IL-1 can promote the production of certain APPs in the liver, only IL-6 induces the production of the full spectrum of APPs known in humans (19,24). APP production is regulated by IL-6 in a dose-dependent manner, thus modulating the response to the severity of infection (24,39). Additional APR functions facilitated by IL-6 include the maturation of B- and T-cells, and hematopoiesis (24,39,59). IL-6 also displays anti-inflammatory attributes, by suppressing TNFα and IL-1 production in mononuclear cells and promoting ACTH and glucocorticoid production (24, 60,61).

Infection and Nutrient Requirements

Infection can lead to a reduction in food intake, impairment of nutrient absorption, and increased nutrient requirements (**Figure 1**). Although breast milk intake appears to be unaffected, food intake is suppressed in children during the APR according to the magnitude of infection. Caloric

intake decreases between 8 to 22% in children with diarrheal diseases, malaria, and acute respiratory infection (62-64). During more severe infections, such as cholera and measles, decreases in caloric intake have been reported as 44% and 75%, respectively. In Peruvian infants with diarrhea or fever, breast milk intake was unaffected; however, caloric intakes of non-breast-milk food decreased by 20-30%, resulting in an overall decrease in caloric intake of 5-6% (65). Infection-related caloric suppression results in both decreased macro- and micronutrient intake (63,64).

Nutrient absorption can be decreased during enteric and nonenteric infection. Gut helminthes and diarrheal diseases can impair nutrient absorption by damaging epithelial cells (20,63,66). The extent of malabsorption corresponds to the severity of enteric infection and diarrheal output (20). Nutrient absorption appears to also be reduced during nonenteric infection. Carbohydrate malabsorption has been observed in hospitalized American children with HIV infection (67). Using a labeled tracer dose of vitamin A, Sivakumar and Reddy (68,69) found uninfected children absorbed 99% of the dose, children with diarrhea and *Ascaris* infection absorbed 70% and 80%, respectively, and children with nonenteric infection absorbed 74%.

In addition to increased energy requirements due to the metabolic cost of fever, macro- and micronutrient demands may be augmented through greater direct loss. Leakage of low molecular weight proteins in the urine is common during febrile infection (20). This phenomenon leads to protein loss in the urine, as well as loss of vitamin A bound to retinol-binding protein that can exceed the daily requirement for children (70,71). Tissue damage from parasitic infection can lead to blood loss and iron-deficiency anemia (20).

Acute Phase Proteins as Markers of Infection for Nutrition Interventions

The liver plays an important role in modulating the immune response and removing pathogens (72,73). In mice, approximately 7% of hepatic genes respond to APR cytokines, inducing a wide array of functional changes, including those in macronutrient metabolism and APP production (74,75). Positive APPs broadly work to mitigate the consequences of infection and modify the host's immune response (19,28,30,33). The role of negative APPs remains unclear, but the down-regulation is speculated to increase the availability of amino acids for positive APP production (28,30). Many negative APPs are transporters; the hepatic suppression of these proteins allows for a temporarily increased free availability of their associated nutrients and hormones in the plasma followed by decreases in plasma concentration due to sequestration (19,28,33).

The manner in which the liver alters APP concentrations in the plasma is dependent on the time stage of infection, such that positive APPs become elevated in the plasma at different timepoints post-stimulus. In nutrition interventions, C-reactive protein (CRP) and serum amyloid A (SAA) are employed as early markers of infection because they become elevated within 24-hours post-stimulus and normalize rapidly as infection resolves (23,24,33,76,77). Antichymotrypsin (ACT) and α_1-acid glycoprotein (AGP) are implemented as late-stage markers of infection because they increase after 48 hours and can remain elevated after the infection convalesces (23,33,77,78). Using these proteins, it is possible to identify different stages of infection including incubation (early stage APP only raised), early convalescence (both early and late stage APPs raised), convalescence (late stage APP only raised), and return to a healthy state (neither early nor late stage APP raised) (23,77). Positive APPs provide a superior assessment of infection when compared with cytokines, which possess short half-lives.

The magnitude of the APP response is dependent upon the severity of injury to the host. Contreras-Manzano et al. (79) reported positive APPs to be more elevated in children with respiratory infections than diarrhea. Furthermore, direct correlations between plasma concentrations of positive APPs and indirect correlations between positive APP concentration and transporter-requiring micronutrients have been found in children (23,81-85). Duncan et al. (86) also found that alteration for several micronutrients assessed in the blood corresponded to the extent of the APR when assessed by CRP concentration in adults.

APP elevation cutoff points used to determine infection are not standardized and may be age, gender, or nutrient specific. For example, elevation cutoffs for CRP of 5 or 10 mg/L are commonly reported in the literature to describe the APR (21,23,77,87-90). Bresnahan et al. (23), found that a CRP concentration of 5 mg/L was an effective elevation cutoff point to show impact on traditional blood-based indicators of vitamin A and iron status in Zambian children, whereas Abraham et al. (92) found significant changes occurring at only 0.6 mg/L using high-sensitivity assays. In adults, CRP cutoffs between 5-20 mg/L have been necessary to detect changes in blood concentration for various vitamins and minerals (86). CRP concentrations increase with age and tend to be higher in women than men (93). This increase, in part, is related to chronic inflammatory conditions, suggesting a higher CRP cutoff may be necessary to assess acute infection in adults (87,94). In order to develop standardized methods for adjustment of nutritional status indicators that are reactive to the APR, more research is imperative to establish universal APP cutoff points that are nutrient and population specific.

Micronutrient Status Indicators during the Acute Phase Response

The impact of the APR on micronutrient status indicators is well-documented in the literature, yet no standardized method for adjustment during infection has been developed. In

public health assessments, micronutrient status is commonly determined using concentrations in blood as a surrogate for total body status. Blood concentrations of retinol, iron, and zinc can be altered by the APR to infection (**Table 2**) (21-23,77,80-87,90,92,95-110). Alterations in blood micronutrient concentration result from hepatic suppression of transport proteins (retinol-binding protein, transthyretin, albumin, and transferrin), and increases in serum ferritin and hepatic metallothionein, positive APPs that assist in iron and zinc sequestration (28,111-115). Although micronutrient demands can be increased during infection, significant changes in micronutrient status do not likely occur as early as decreases in blood concentrations suggest during the APR. Furthermore, blood concentrations are typically restored to pre-infection values as the APR resolves, supporting measurements taken during febrile infection as inaccurate reflections of micronutrient status (80,95,96,104).

The APR rapidly impacts common biomarkers for iron status including serum iron, transferrin, and ferritin (115). Of the iron biomarkers, serum ferritin concentrations are the most significantly affected by infection with reported increases of 30 to 1,400% depending on the time stage and severity of infection in children (21,23,84,85,97,102,109). Serum iron and transferrin are suppressed by 50 and 30%, respectively, and percent transferrin saturation is lowered to about 20% (104,115). The impacts of infection on soluble transferrin receptors and hemoglobin remain unclear. While iron status appears to be the main factor impacting soluble transferrin receptor concentration, it has been reported to be decreased in surgical patients and in individuals with malarial and HIV infection (104,116-118). Hemoglobin was suppressed in British, Zambian, and Zanzibari children during the APR and found inversely related to serum ferritin concentration, supporting its role as a reactive iron status indicator (23,84,119). However,

Wieringa et al. (21) reported no effect of the APR on hemoglobin, and Das et al. (102) found no association between hemoglobin and serum ferritin.

Serum zinc concentration is suppressed by 12% in children during infection (21,97). Similarly, in HIV positive adults, a 12% decrease in serum zinc has been observed (22). In children with malaria, CRP concentration and parasite density predicted low serum zinc concentration (108). It is important to also consider the definition of zinc deficiency when assessing the impact of infection on estimates of deficiency prevalence as cutoffs vary by gender, age, and time of day (120).

Serum retinol concentration is a widely used indicator for vitamin A status and is recommended for population assessment by the WHO (121,122). The current deficiency cutoff is set at 0.7 μmol retinol/L serum (or plasma) regardless of inflammation (122). However, in children, serum retinol concentrations are suppressed by approximately 25% during common infections (23,77,98) and up to 69% in children with *Shigellosis* (80). This decrease in serum retinol concentration is independent of vitamin A liver reserves (123). Therefore, individuals who are experiencing infection but have an adequate vitamin A status may be misclassified as deficient, leading to overestimation of deficiency prevalence in a population. The modified relative dose response (MRDR) test for vitamin A status is not impacted by the APR and is more sensitive than serum retinol to changes in liver retinol reserves, supporting it as a superior tool for assessment, particularly in populations with high or unknown prevalence of infection (21,23,121). Plasma concentrations of provitamin A carotenoids are also suppressed during inflammation (100,124). This may suggest an increased demand for vitamin A during infection, a reflection of alterations in blood lipid profile, or a decrease in dietary intake (124).

Developing a standardized method for adjusting blood-based micronutrient status indicators that are reactive to the APR is challenging because: (i) discerning the difference between infection suppressed values and deficiency is difficult in regions where dietary intake is insufficient, (ii) the relationship between blood concentrations of micronutrients and APPs is not linear, and (iii) correction factors based on a healthy reference population may misestimate deficiency prevalence to the opposite extreme (22,110,125). The measurement of APP concentrations when assessing micronutrient status should become standard practice as APPs are elevated during subclinical inflammation (22,126). Moreover, both early and late stage markers of infection should be evaluated because it is common to find more individuals in a population with elevated AGP or ACT than CRP (21-23). It is important to also consider sex differences when adjusting micronutrient status indicators for the APR.

UNDERNUTRITION AND IMMUNE FUNCTION

PEM and micronutrient deficiencies lead to underweight and stunting, which are defined by the World Health Organization as weight-for-height and height-for-age Z-scores below -2 standard deviations, respectively (127). As of 2011, 16% of children below 5 years of age were underweight and 26% of children were stunted, which marks great achievement in combating global undernutrition (127). However, half of all infectious deaths in developing countries are associated with a low weight-for-age (10). While many interventions and research studies focus on severe PEM because of the associated sharp increase in mortality, Pelletier et al. (128) demonstrated that mild-to-moderate PEM accounts for 80% of childhood mortality in developing countries. Stunting also appears to compromise immune function, but the effect has not been studied comprehensively (15,20).

PEM leads to increased susceptibility to and mortality from infection (129-131). Gut barrier function is decreased, increasing the risk of microbial infection (16). Undernourished individuals also experience a reduction in leukotrienes, which promote leukocyte accumulation and enhance macrophage phagocytosis, negatively impacting the host's ability to kill microbial, fungal, and parasitic agents (132). During PEM, the structure and function of the thymus gland is damaged, leading to abnormal T-cell populations and reduced antigen response (133,134). Compared with healthy children, the B-cell response is lowered during malnutrition (135). However, the humoral response appears to be well-preserved during the short-term seroconversion to whole inactivated viral and polysaccharide vaccines (136). Additionally, the development of antibody titers to common pathogens appeared to be normal in Kenyan children (137).

Conflicting evidence exists regarding the ability of children with severe PEM to mount a complete immune response. While Doherty et al. (138) reported decreased TNFα and IL-6 in an *in vitro* study using whole blood from malnourished children, Sauerwein et al. (139) found that children with PEM mount a greater immune response than healthy children. A study in Kenyan children supports PEM as a proinflammatory state with a generalized increase in IL-6 (140). The APP response is impaired in children with severe PEM, with a greater deficit occurring in kwashiorkor as compared with marasmus (140-142). In children with marasmus, rates of protein catabolism are similar to healthy children during infection and higher than in kwashiorkor (143). These comparative differences in immunity may reflect an inappropriate response during kwashiorkor and are consistent with observations of a lower fatality rate in marasmic children with infection (144).

A high burden of infectious disease is associated with delayed linear growth and stunting (145-149). Similar to underweight, stunted children display an abnormal lymphocyte profile (15). Rural children tend to experience more severe stunting and have higher plasma levels of APPs than children living in urban settings (128).

Severe deficiencies frequently occur for multiple micronutrients simultaneously and can complicate PEM (17,18). Certain micronutrients, including vitamin A, iron, and zinc, are important immunomodulators and, thereby, are crucial to the host in mounting an effective response to infection. Iron deficiency is a major global health concern, impacting 3.5 billion people (150). Iron is essential for redox reactions, gene regulation, oxygen delivery to tissues, and cell growth (114,151,152). Iron supports immunity through its involvement with peroxide and nitric oxide producing enzymes, cytokine production and function, and lymphocyte proliferation (114,151,152). Iron homeostasis is paramount as deficiency and overload negatively impact immune function (151). Many pathogens also require iron to thrive, and therefore sequestration during infection is protective to the host (151,152). Iron supplementation is controversial because excess and timing of repletion may promote pathogenic infection (152). In deficient children, iron supplementation improves status and promotes related growth and development (153). However, iron supplementation in children has not been found to affect the incidence of infection or associated mortality and may increase the risk of diarrheal diseases and malaria (153,154).

Zinc is essential for highly proliferating cells in the immune system and influences both innate and acquired immune functions (155-157). Zinc is a coenzyme in many important reactions during the immune response and is essential for thymic hormone function (156,158). Phagocyte and lymphocyte activity are impaired or completely suppressed in zinc deficiency,

resulting in weakened cytokine and antibody responses (155-157). Zinc deficiency impacts approximately one-third of the global population and often coexists with PEM (18,159). Zinc supplementation in children promotes gains in weight and height and protects against respiratory infections (160-162). While zinc supplementation protected against diarrheal disease in children under 5 years of age (161), Negi et al. (163) recently reported supplements to be ineffective in children between 5 to 12 years of age.

Vitamin A deficiency compromises innate immunity through the degradation of mucosal epithelial barriers and impaired development of neutrophils, macrophages, and natural killer cells (164). Pathogen-specific immunity is also lessened during vitamin A deficiency via reduction of antibody production in T-cells (164). Vitamin A deficiency impacts 190 million children and is the leading cause of preventable blindness (165). Xerophthalmia is associated with respiratory tract infection, measles, and diarrhea in children (166). This is not surprising considering that infection can worsen deficiency through anorexia and increase direct loss of vitamin A in the urine. The semi-annual vitamin A supplementation program recommended by the World Health Organization (165,167) reduced malnutrition and was protective against infection in Nepalese children (168). Additionally, vitamin A supplementation reduced the frequency of malarial episodes by 30% in children in Papua New Guinea and promoted ponderal growth and protection against malaria-related death in Tanzanian children (169,170). Provitamin A carotenoid biofortified staple crops provide a potentially effective method for alleviating vitamin A deficiency in the rural poor, who are disproportionally missed by supplementation interventions (171,172). However, biofortification target concentrations of provitamin A carotenoids should be increased in areas with high prevalence of infection to account for reduced food intake and increased requirements (23).

SUMMARY

The synergism between nutritional status and the APR is apparent where malnutrition and infectious diseases are prevalent. The APR to infection increases energy expenditure to generate fever and accelerates catabolism of macronutrients. Further, infection suppresses food intake, decreases nutrient absorption, and can increase requirements by augmenting direct loss of micronutrients. Malnutrition, in turn, increases susceptibility to infection by impairing both innate and adaptive immune responses. Integrated interventions are paramount to improve nutritional status and reduce disease burden in the developing world.

Blood-based micronutrient status indicators can be altered during the APR leading to misestimates of deficiency prevalence in populations with high or unknown prevalence of infection. Currently, a universally accepted, standardized method for adjusting micronutrient status indicators that are reactive to the APR is lacking. In order to develop a system for adjustment, elevation cutoffs for early and late stage APPs must be universally established and may need to be nutrient and population specific. Additionally, the relation between APP and micronutrient indicator concentration requires further elucidation because both vary according to the magnitude of infection and correspond in a non-linear fashion.

REFERENCES

1. Shofield C, Ashworth A. Why have mortality rates for severe malnutrition remained so high? Bull World Health Organ. 1996;74:223-9.

2. World Health Organization. The world health report 2002. Geneva: World Health Organization; 2002.

3. Food and Agriculture Organization. The state of food insecurity in the world 2010. Rome: Food and Agriculture Organization; 2010.

4. Millward DJ, Jackson AA. Protein/energy ratios of current diets in developing countries compared with a safe protein/energy ratio: implications for recommended protein and amino acid intakes. Public Health Nutr. 2004;7:387-405.

5. Neumann CG, Gewa C, Bwibo NO. Child nutrition in developing countries. Pediatr Ann. 2004;33:658-74.

6. Jamison DT, Breman JG, Measham AR, Alleyne G, Claeson M, Evans DB, Jha P, Mills A, Musgrove P, editors. Disease control priorities in developing countries. 2nd ed. New York: Oxford University Press; 2006.

7. Fernandez ID, Himes JH, de Onis M. Prevalence of nutritional wasting in populations: building explanatory models using secondary data. Bull World Health Organ. 2002;80:282-91.

8. Sachs JD, McArthur JW. The Millenium Project: a plan for meeting the Millenium Development Goals. Lancet. 2005;365:347-53.

9. Tanumihardjo SA, Anderson C, Kaufer-Horwitz M, Bode L, Emenaker NJ, Haqq AM, Satia JA, Silver HJ, Stadler DD. Poverty, obesity, and malnutrition: an international perspective recognizing the paradox. J Am Diet Assoc. 2007;107:1966-72.

10. Pelletier DL, Frongillo EA. Changes in child survival are strongly associated with changes in malnutrition in developing countries. J Nutr. 2003;133:107-19.

11. Scrimshaw NS, SanGiovanni JP. Synergism of nutrition, infection, and immunity: an overview. Am J Clin Nutr. 1997;66:464S-77S.

12. Woodward B. Protein, calories, and immune defenses. Nutr Rev. 1998;56:S84-92.

13. Solomons NW. Malnutrition and infection: an update. Br J Nutr. 2007;98:S5-10.

14. World Health Organization. World malaria report 2013. Geneva: World Health Organization; 2013.

15. Chandra RK, Sarchielli P. Body size and immune responses. Nutr Res. 1996;16:1813-9.

16. Welsh FKS, Farmery SM, MacLennan K, Sheridan MB, Barclay GR, Guillou PJ, Reynolds JV. Gut barrier function in malnourished patients. Gut. 1998;42:396-401.

17. Cunningham-Rundles S, McNeely DF, Moon A. Mechanisms of nutrient modulation of the immune response. J Allergy Clin Immunol. 2005;115:1119-28.

18. Bhaskaram P. Immunobiology of mild micronutrient deficiencies. Br J Nutr. 2001;85:S75-80.

19. Gruys E, Toussaint MJM, Niewold TA, Koopmans SJ. Acute phase reaction and acute phase proteins. J Zhejiang Univ Sci B. 2005;6B:1045-56.

20. Stephensen CB. Burden of infection on growth failure. J Nutr. 1999;129:534S-8S.

21. Wieringa FT, Dijkhuizen MA, West CE, Northrop-Clewes CA, Muhilal. Estimation of the effect of the acute phase response on indicators of micronutrient status in Indonesian infants. J Nutr. 2002;132:3061-6.

22. Thurnham DI, Mburu ASW, Mwaniki DL, Wagt AD. Micronutrients in childhood and the influence of subclinical inflammation. Proc Nutr Soc. 2005;64:502-9.

23. Bresnahan KA, Chileshe J, Arscott SA, Nuss E, Surles R, Masi C, Kafwembe E, Tanumihardjo SA. The acute phase response affects traditional measures of micronutrient status in rural Zambian children during a randomized controlled feeding trial. J Nutr. 2014; *In press.*

24. Heinrich PC, Castell JV, Andus T. Interleukin-6 and the acute phase response. Biochem J. 1990;265:621-36.

25. Baumann H, Gauldie J. The acute phase response. Immunol Today. 1994;15:74-80.

26. Koj A. Initiation of the acute phase response and synthesis of cytokines. Biochim Biophys Acta. 1996;1317:84-94.

27. Suffredini AF, Fantuzzi G, Badolato R, Oppenheim JJ, O'Grady NP. New insights into the biology of the acute phase response. J Clin Immunol. 1999;19:203-14.

28. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. New Eng J Med. 1999;340:448-54.

29. Medzhitov R. Toll-like receptors and innate immunity. Nat Rev Immunol. 2001;1:135-45.

30. Ceciliani F, Giordano A, Spangnolo V. The systemic reaction during inflammation: the acute-phase proteins. Protein Pept Lett. 2002;9:211-23.

31. Janeway, Jr. CA, Medzhitov R. Innate immune recognition. Ann Rev Immunol. 2002;20:197-216.

32. Tracy KJ. The inflammatory reflex. Nature. 2002;420:853-9.

33. Raynes JG. The acute phase response. In: Kaufmann SHK, Steward MW, editors. Tropley and Wilson's microbiology and microbial infection, 10th ed. London, England: Hodder Arnold Ltd, 2014. p. 1-22.

34. Akira A, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell. 2006;124:783-801.

35. Medzhitov R. Origin and physiological roles of inflammation. Nature. 2008;454:428-35.

36. Tracey KJ. Reflex control of immunity. Nature. 2009;9:418-28.

37. Rosas-Ballina M, Tracey KJ. The neurology of the immune system: neural reflexes regulate immunity. Neuron. 2009;64:28-32.

38. Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell. 2010;140:805-20.

39. Heinrich PC, Behrmann I, Müller-Newen G, Schaper F, Graeve L. Interleukin-6-type cytokine signaling through the gp130/Jak/STAT pathway. Biochem J. 1998;334:297-314.

40. Hanada T, Yoshimura A. Regulation of cytokine signaling and inflammation. Cytokine Growth Factor Rev. 2002;13:413-21.

41. O'Shea JJ, Murray PJ. Cytokine signaling modules in inflammatory responses. Immunity. 2008;28:477-87.

42. Kelley KW, Bluthé RM, Dantzer R, Zhou JH, Shen WH, Johnson RW, Broussard SR. Cytokine-induced sickness behavior. Brain Behav Immun. 2003;17:S112-8.

43. Danzer R. Cytokine-induced sickness behavior: a neuroimmune response to activation of innate immunity. Eur J Pharmacol. 2004;500:399-411.

44. Exton MS. Infection-induced anorexia: active host defense strategy. Appetite. 1997;29:369-83.

45. Bluthé RM, Michaud B, Poli V, Dantzer R. Role of IL-6 in cytokine-induced sickness behavior: a study with IL-6 deficient mice. Physiol Behav. 2000;70:367-73.

46. Dantzer R, Kelley KW. Twenty years of research on cytokine-induced sickness behavior. Brain Behav Immun. 2007;21:153-60.

47. Stettler N, Shutz Y, Whitehead R, Jequier E. Effect of malaria and fever on energy metabolism in Gambian children. Pediatr Res. 1992;31:102-6.

48. Benhariz M, Goulet O, Salas J, Colomb V, Ricour C. Energy cost of fever in children on total parenteral nutrition. Clin Nutr. 1997;16:251-5.

49. Goodman MN. Tumor necrosis factor induces skeletal muscle protein breakdown in rats. Am J Physiol. 1991;260:E727-30.

50. Lang CH, Frost RA, Nairn AC, MacLean DA, Vary TC. TNF-α impairs heart and skeletal muscle protein synthesis by altering translation initiation. Am J Physiol Endocrinol Metab. 2002;282:E336-47.

51. Conney R, Owens E, Jurasinski C, Gray K, Vannice J, Vary T. Interleukin-1 receptor antagonist prevents sepsis-induced inhibition of protein synthesis. Am J Physiol. 1994;267:E636-41.

52. Zamir O, Hasselgren PO, Kunkel SL, Frederick J, Higashiguchi PO. Evidence that tumor necrosis factor participates in the regulation of muscle proteolysis during sepsis. Arch Surg. 1992;127:170-4.

53. Zamir O, I'Brien W, Thompson R, Bloedow DC, Fisher JE, Hasselgren PO. Reduced muscle protein breakdown in septic rats following treatment with interleukin-1 receptor antagonist. Int J Biochem. 1994;26:943-50.

54. Le Floc'h N, Melchior D, Obled C. Modifications of protein and amino acid metabolism during inflammation and immune system activation. Livest Prod Sci. 2004;87:37-45.

55. Reeds PJ, Fjeld CR, Jahoor F. Do the differences between the amino acid compositions of the acute-phase and muscle proteins have a bearing on nitrogen loss in traumatic states? J Nutr. 1994;124:906-10.

56. Mizock BA. Alterations in fuel metabolism in critical illness: hyperglycaemia. Best Pract Res Clin Endocrinol Metab. 2001;15:533-51.

57. Van Der Poll T, Romijn JA, Endert E, Borm JJJ, Büller HR, Sauerwein HP. Tumor necrosis factor mimics the metabolic response to acute infection in healthy humans. Am J Physiol. 1991; 261:E457-65.

58. Khovidhunkit W, Kim MS, Memon RA, Shigenaga JK, Moser AH, Feingold KR, Grunfeld C. Effects of infection and inflammation on lipid and lipoprotein metabolism: mechanisms and consequences to the host. J Lipid Res. 2004;45:1169-96.

59. Scheller J, Garbers C, Rose-John S. Interleukin-6: from basic biology to selective blockade of pro-inflammatory activities. Sem Immunol. 2014;26:2-12.

60. Schindler R, Mancilla J, Endres S, Ghorbani R, Clark SC, Dinarella CA. Correlations and interactions in the production of interleukin-6 (IL-6), IL-1, and tumor necrosis factor (TNF) in human blood mononuclear cells: IL-6 suppresses IL-1 and TNF. Blood. 1990;1:40-7.

61. Xing Z, Gauldie J, Cox G, Baumann H, Jordana M, Lei XF, Achong MK. IL-6 is an anti-inflammatory cytokine required for controlling local or systemic acute inflammatory responses. J Clin Invest. 1998;101:311-20.

62. Martorell R, Yarbrough C, Yarbrough S, Klein RE. The impact of ordinary illnesses on the dietary intakes of malnourished children. Am J Clin Nutr. 1980;33:345-50.

63. Molla A, Malla AM, Rahim A, Sarker SA, Mozaffar Z, Rahaman M. Intake and absorption of nutrients in children with cholera and rotavirus infection during acute diarrhea and after recovery. Nutr Res. 1982;2:233-42.

64. Bresnahan KA, Chileshe J, Tanumihardjo SA. Quantification of food and nutrient intakes in Zambian children with and without malaria under controlled feeding conditions. Exp Biol Med. 2014;239:45-51.

65. Brown KH, Stallings RY, de Kanashiro HC, de Romaña GL, Black RE. Effects of common illnesses on infants' energy intakes from breast milk and other foods during longitudinal community-based studies in Huascar (Lima), Peru. Am J Clin Nutr. 1990;52:1005-13.

66. Rosenberg IH, Solomons NW, Schneider RE. Malabsorption associated with diarrhea and intestinal infections. Am J Clin Nutr. 1977;30:1248-53.

67. Miller TL, Orav EJ, Martin SR, Cooper ER, McIntosh K, Winter HS. Malnutrition and carbohydrate malabsorption in children with vertically transmitted human immunodeficiency virus 1 infection. Gastroenterology. 1991;100:1296-302.

68. Sivakumar B, Reddy V. Absorption of vitamin A in children during infection. Br J Nutr. 1972;27:299-304.

69. Sivakumar B, Reddy V. Absorption of vitamin A in children with ascariasis. J Trop Med Hyg. 1975;78:114-5.

70. Stephensen CB, Alvarez JO, Kohatsu J, Hardmeier R, Kennedy JI Jr, Gammon B Jr. Vitamin A is excreted in the urine during acute infection. Am J Clin Nutr. 1994;60:388-92.

71. Mitra AK, Alvarez JO, Stephensen CB. Increased urinary retinol loss in children with severe infections. Lancet. 1998;351:1033-4.

72. Knolle P, Löhr H, Treichel U, Dienes HP, Lohse A, Schlaack H, Gerken G. Parenchymal and nonparenchymal liver cells and their interaction in the local immune response. Z Gastroenterol. 1995;33:613-20.

73. Knolle PA, Gerken G. Local control of the immune response in the liver. Immunol Rev. 2000;174:21-34.

74. Desiderio S, Yoo JY. A genome-wide analysis of acute-phase response and its regulation by Stat3β. Ann NY Acad Sci. 2003;987:280-4.

75. Yoo JY, Desiderio S. Innate and acquired immunity intersect in a global view of the acute phase response. Immunol. 2003;100:1157-62.

76. Clyne B, Olshaker JS. The C-reactive protein. J Emerg Med. 1999;17:1019-25.

77. Thurnham DI, McCabe GP, Northrop-Clewes CA, Nestel P. Effects of subclinical infection on plasma retinol concentrations and assessment of prevalence of vitamin A deficiency. Lancet. 2003;362:2052-8.

78. Thompson D, Milford-Ward A, Whicher JT. The value of the acute phase protein measurement in clinical practice. Ann Clin Biochem. 1992;29:123-31.

79. Contreras-Manzano A, Villalpando S, Lamadrid H, Shamah-Levy T. Acute phase proteins are more elevated in acute respiratory infections than in diarrhea. FASEB J. 2013;27:886.3.

80. Mitra AK, Alvarez JO, Wahed MA, Fuchs GJ, Stephensen CB. Predictors of serum retinol in children with shigellosis. Am J Clin Nutr. 1998;68:1088-94.

81. Rosales FJ, Topping JD, Smith JE, Shankar AH, Ross AC. Relation of serum retinol to acute phase proteins in Papua New Guinea children. Am J Clin Nutr. 2000;71:1582-8.

82. Paracha PI, Jamil A, Northrop-Clewes CA, Thurnham DI. Interpretation of vitamin A status in apparently healthy Pakistani children by using markers of subclinical infection. Am J Clin Nutr. 2000;72:1164-9.

83. Beard JL, Murray-Kolb LE, Rosales FJ, Solomons NW, Angelilli ML. Interpretation of serum ferritin concentrations as indicators of total-body iron stores in survey populations: the role of biomarkers for the acute phase response. Am J Clin Nutr. 2006;84:1498-505.

84. Kung'u JK, Wright VJ, Haji HJ, Ramsan M, Goodman D, Tielsch JM, Bickle QD, Raynes JG, Stoltzfus RJ. Adjusting for the acute phase response is essential to interpret iron status indicators among young Zanzibari children prone to chronic malaria and helminth infection. J Nutr. 2009;139:214-31.

85. Brito A, Hertrampf E, Olivares M. Iron status biomarkers and C-reactive protein in children aged 19 to 72 months in Chile. Food Nutr Bull. 2013;34:14-20.

86. Duncan A, Talwar D, McMillan DC, Stafanowicz F, O'Reilly DS. Quantitative data on the magnitude of the systematic inflammatory response and its effects on micronutrient status based on plasma measurements. Am J Clin Nutr. 2012;95:64-71.

87. Stephensen CB, Gildengorin G. Serum retinol, the acute phase response, and the apparent misclassification of vitamin A status in the third National Health and Nutrition Examination Survey. Am J Clin Nutr. 2000;72:1170-8.

88. Dijkhuizen MA, Wieringa FT, West CE, Martuti S, Muhilal. Effects of iron and zinc supplementation in Indonesian infants on micronutrient status and growth. J Nutr. 2001;131:2860-5.

89. Haskell MJ, Lembcke JL, Salazar M, Green MH, Peerson JM, Brown KH. Population-based plasma kinetics of an oral dose of [$^2H_4$]retinyl acetate among preschool-aged, Peruvian children. Am J Clin Nutr. 2003;77:681-6.

90. Northrop-Clewes CA. Interpreting indicators of iron status during an acute phase response – lessons from malaria and human immunodeficiency virus. Ann Clin Biochem. 2008;45:18-32.

91. Cogswell ME, Looker AC, Pfeiffer CM, Cook JD, Lacher DA, Beard JL, Lynch SR, Grummer-Strawn LM. Assessment of iron deficiency in US preschool children and nonpregnancy females of childbearing age: National Health and Nutrition Examination Survey 2003-2006. Am J Clin Nutr. 2009;89:1334-42.

92. Abraham K, Müller C, Grüters A, Whan U, Schweigert FJ. Minimal inflammation, acute phase response and avoidance of misclassification of vitamin A and iron status in infants – importance of a high sensitivity C-reactive protein (CRP) assay. Int J Vitam Nutr Res. 2003;73:423-30.

93. Woloshin S, Schwartz LM. Distribution of C-reactive protein values in the Unites States. New Eng J Med. 2005;352:1161-3.

94. Ferrucci L, Corsi A, Lauretani F, Bandinelli S, Bartali B, Taub DD, Guralnik JM, Longo DL. The origins of age-related proinflammatory state. Blood. 2005;105:2294-9.

95. Reddy V, Bhaskaram P, Raghuramulu N, Milton RC, Rao V, Madhusudan J, Krishna KV. Relationship between measles, malnutrition, and blindness: a prospective study in Indian children. Am J Clin Nutr. 1986;44:924-30.

96. Louw JA, Werbeck A, Louw ME, Kotze TJvW, Cooper R, Labadarios D. Blood vitamin concentrations during the acute-phase response. Crit Care Med. 1992;20:934-41.

97. Brown KH, Lanata CF, Yuen ML, Peerson JM, Butron B, Lönnerdal. Potential magnitude of the misclassification of a population's trace element status due to infection: example from a survey of young Peruvian children. Am J Clin Nutr. 1993;58:549-54.

98. Filteau SM, Morris SS, Abbott RA, Tomkins AM, Kirkwood BR, Arthur P, Ross DA, Gyapong JO, Raynes JG. Influence of morbidity on serum retinol of children in a community-based study in northern Ghana. Am J Clin Nutr. 1993;58:192-7.

99. Salazar-Lindo E, Salazar M, Alvarez JO. Association of diarrhea and low serum retinol in Peruvian children. Am J Clin Nutr. 1993;58:110-3.

100. Das BS, Thurnham DI, Das DB. Plasma α-tocopherol, retinol, and carotenoids in children with Falciparum malaria. Am J Clin Nutr. 1996;64:94-100.

101. Friis H, Ndhlovu P, Kaondera K, Sandström B, Michaelsen KF, Vennervald BJ, Christensen NØ. Serum concentration of micronutrients in relation to schistosomiasis and indicators of infection: a cross-sectional study among rural Zimbabwean schoolchildren. Eur J Clin Nutr. 1996;50:386-91.

102. Das BS, Thurnham DI, Das DB. Influence of malaria on markers of iron status in children: implications for interpreting iron status in malaria-endemic communities. Br J Nutr. 1997;78:751-60.

103. Christian P, Schulze K, Stoltzfus RJ, West KP Jr. Hyporetinolemia, illness symptoms, and acute phase protein in pregnant women with and without night blindness. Am J Clin Nutr. 1998;67:1237-43.

104. Feelders RA, Vreugdenhil G, Eggermont AMM, Kuiper-Kramer PA, van Eijk HG, Swaak AJG. Regulation of iron metabolism in the acute-phase response: interferon γ and tumor necrosis factor α induce hypoferraemia, ferritin production and a decrease in circulating transferrin receptors in cancer patients. Eur J Clin Invest. 1998;28:520-7.

105. Hautvast JLA, Tolboom JJM, Willems JL, Mwela CM, Monnens LAH. Consequences of infections for three-month length increment in young children in rural Zambia. Acta Paediatr. 2000;89:296-301.

106. Stoltzfus RJ, Chwaya HM, Montresor A, Albonico M, Savioli L, Tielsch JM. Malaria, hookworms and recent fever are related to anemia and iron status indicators in 0- to 5-y old Zanzibari children and these relationships change with age. J Nutr. 2000;130:1724-33.

107. Baeten JM, Richardson BA, Bankson DD, Wener MH, Kreiss JK, Lavreys L, Mandaliya K, Bwayo JJ, McClelland RS. Use of serum retinol-binding protein for prediction of vitamin A deficiency: effects of HIV-1 infection, protein malnutrition, and the acute phase response. Am J Clin Nutr. 2004;79:218-25.

108. Duggan C, MacLeod WB, Krebs NF, Westcott JL, Fawzi WW, Premji ZG, Mwanakasale V, Simon JL, Yeboah-Antwi K, Hamer DH, the Zinc Against Plasmodium Study Group. Plasma zinc concentrations are depressed during the acute phase response in children with Falciparum malaria. J Nutr. 2005;135:802-7.

109. Thurnham DI, McCabe LD, Haldar S, Wieringa FT, Northrop-Clewes CA, McCabe GP. Adjusting plasma ferritin concentrations to remove the effects of subclinical inflammation in the assessment of iron deficiency: a meta-analysis. Am J Clin Nutr. 2010;92:546-55.

110. Hotz C, Chileshe J, Siamusantu W, Palaniappan U, Kafwembe E. Vitamin A intake and infection are associated with plasma retinol among pre-school children in rural Zambia. Public Health Nutr. 2012;15:1688-96.

111. Bremner I, Beattie JH. Metallothionein and the trace minerals. Annu Rev Nutr. 1990;10:63-83.

112. Min KS, Terano Y, Onosaka S, Tanaka K. Induction of hepatic metallothionein by nonmetallic compounds associated with acute-phase response in inflammation. Toxicol Appl Pharmacol. 1991;111:152-62.

113. Ilbäck NG, Glynn AW, Wikberg L, Netzel E, Lindh U. Metallothionein is induced and trace element balance changed in target organs of a common viral infection. Toxicol. 2004;199:241-50.

114. Wessling-Resnick M. Iron homeostasis and the inflammatory response. Ann Rev Nutr. 2010;30:105-22.

115. Thurnham DI, McCabe GP. Influence of infection and inflammation on biomarkers of nutritional status with an emphasis on vitamin A and iron. In: World Health Organization. Report: priorities in the assessment of vitamin A and iron status in populations, Panama City, Panama, 15-17 September 2010. Geneva: World Health Organization, 2012.

116. Skikne BS, Flowers CH, Cook JD. Serum transferrin receptor: a quantitative measure of tissue iron deficiency. Blood. 1990;75:1870-6.

117. Beesley R, Filteau S, Tomkins A, Doherty T, Ayles H, Reid A, Ellman T, Parton S. Impact of acute malaria on plasma concentrations of transferrin receptors. Trans R Soc Trop Med Hyg. 2000;94:295-8.

118. Rawat R, Stoltzfus RJ, Ntozini R, Mutasa K, Iliff PJ, Humphrey JH. Influence of inflammation as measured by α-1-acid glycoprotein on iron status indicators among HIV-positive postpartum Zimbabwean women. Eur J Clin Nutr. 2009;63:787-93.

119. Ramdath DD, Simeon DT, Wong MS, Grantham-McGregor SM. Iron status of schoolchildren with varying intensities of *Trichuris trichiura* infection. Parasitology. 1995;110:347-51.

120. Hotz C, Brown KH, editors. International Zinc Consultative Group (IZiNCG) technical document #1: assessment of the risk of zinc deficiency in populations and options for its control. Food Nutr Bull. 2004;25:S91-203.

121. Tanumihardjo SA. Vitamin A: biomarkers of nutrition for development. Am J Clin Nutr. 2011;94:658S-65S.

122. World Health Organization. Serum retinol concentrations for determining the prevalence of vitamin A deficiency in populations. Geneva: World Health Organization; 2011.

123. Willumsen JF, Simmank K, Filteau SM, Wagstaff LA, Tomkins AM. Toxic damage to the respiratory epithelium induces acute phase changes in vitamin A metabolism without depleting retinol stores of South African children. J Nutr. 1997;127:1339-43.

124. Schweigert FJ. Inflammation-induced changes in the nutritional biomarkers serum retinol and carotenoids. Curr Opin Clin Nutr Metab Care. 2001;4:477-81.

125. Tomkins A. Assessing micronutrient status in the presence of inflammation. J Nutr. 2003;133:1649S-55S.

126. Panter-Brick C, Lunn PG, Todd A. Elevated acute-phase protein in stunted Nepali children reporting low morbidity: different rural and urban profiles. Br J Nutr. 2001;85:125-31.

127. United Nations Children's Fund, World Health Organization, World Bank. UNICEF-WHO-World Bank joint childhood malnutrition estimates. New York: United Nations Children's Fund, Geneva: World Health Organization, Washington, D.C.: World Bank; 2012.

128. Pelletier DL, Frongillo EA, Schroeder DG, Habicht JP. The effects of malnutrition on child mortality in developing countries. Bull WHO. 1995;73:443-8.

129. Müller O, Garenne M, Kouyaté B, Becher H. The association between protein-energy malnutrition, malaria morbidity and all-cause mortality in West African children. Trop Med Int Health. 2003;8:507-11.

130. Schneider SM, Veyres P, Pivot X, Soummer AM, Jambou P, Filippi J, van Obberghen E, Hébuterne X. Malnutrition is an independent factor associated with nosocomial infections. Br J Nutr. 2004;92:105-11.

131. Thomas JE, Dale A, Bunn JEG, Harding M, Coward WA, Cole TJ, Weaver LT. Early *Helicobacter pylori* colonization: the association with growth faltering in The Gambia. Arch Dis Child. 2004;89:1149-54.

132. Peters-Golden M, Canetti C, Mancuso P, Coffey MJ. Leukotrienes: underappreciated mediators of innate immune responses. J Immunol. 2004;173:589-94.

133. McDade TW, Beck MA, Kuzawa CW, Adair LS. Prenatal undernutrition and postnatal growth are associated with adolescent thymic function. J Nutr. 2001;131:1225-31.

134. Savino W. The thymus gland is a target in malnutrition. Eur J Clin Nutr. 2002;56:S46-9.

135. Najera O, Gonzalez C, Toledo G, López L, Ortiz R. Flow cytometry study of lymphocyte subsets in malnourished and well-nourished children with bacterial infection. Clin Diagn Lab Immunol. 2004;11:577-80.

136. Moore SE, Goldblatt D, Bates CJ, Prentice AM. Impact of nutritional status on antibody responses to different vaccines in undernourished Gambian children. Acta Paediatr. 2003;92:170-6.

137. Siekmann JH, Allen LH, Watnik MR, Nestel P, Neumann CG, Shoenfeld Y, Peter JB, Patnik M, Ansari AA, Coppel RL, Gershwin ME. Titers of antibody to common pathogens: relation to food-based interventions in rural Kenyan schoolchildren. Am J Clin Nutr. 2003;77:242-9.

138. Doherty JF, Golden MHN, Remick DG, Griffin GE. Production of interleukin-6 and tumour necrosis factor-α *in vitro* is reduced in whole blood of severely malnourished children. Clin Sci. 1994;86:347-51.

139. Sauerwein RW, Mulder JA, Mulder L, Lowe B, Peshu N, Demacker PNM, van der Meet JWM, Marsh K. Inflammatory mediators in children with protein-energy malnutrition. Am J Clin Nutr. 1997;65:1534-9.

140. Dülger H, Arik M, Sekeroglu MR, Tarakçioglu M, Noyan T, Cesur Y, Balahoroglu R. Pro-inflammatory cytokines in Turkish children with protein-energy malnutrition. Mediators Inflamm. 2002;11:363-5.

141. Doherty JF, Golden MHN, Raynes JG, Griffin GE. Acute-phase protein response is impaired in severely malnourished children. Clin Sci. 1993;84:169-175.

142. Reid M, Badaloo A, Forrester T, Morlese JF, Heird WC, Jahoor F. The acute-phase protein response to infection in edematous and nonedematous protein-energy malnutrition. Am J Clin Nutr. 2002;76:1409-15.

143. Manary MJ, Broadhead RL, Yarasheski KE. Whole-body protein kinetics in marasmus and kwashiorkor during acute infection. Am J Clin Nutr. 1998;67:1205-9.

144. Friedland IR. Bacteraemia in severely malnourished children. Ann Trop Paediatr. 1992;12:433-40.

145. Neumann CG, Harrison GC. Onset and evolution of stunting in infants and children. Examples from the Human Nutrition Collaborative Research Support Program. Kenya and Egypt studies. Eur J Clin Nutr. 1994;48:90S-102S.

146. Bwibo NO, Neumann CG. The need for animal food source by Kenyan children. J Nutr. 2003;133:3936S-40S.

147. Assis AMO, Prado MS, Barreto ML, Reis MG, Pinheiro SMC, Parraga IM, Blanton RE. Childhood stunting in Northeast Brazil: the role of *Schistosoma mansoni* infection and inadequate dietary intake. Eur J Clin Nutr. 2004;58:1022-9.

148. Mamiro PS, Kolsteren P, Roberfroid D, Tatala S, Opsomer AS, Van Camp JH. Feeding practices and factors contributing to wasting, stunting, and iron-deficiency anaemia among 3-23-month old children in Kilosa District, rural Tanzania. J Health Popul Nutr. 2005;23:222-30.

149. Olney DK, Kariger PK, Stoltzfus RJ, Khalfan SS, Ali NS, Tielsch JM, Sazawal S, Black R, Allen LH, Pollitt E. Development of nutritionally at-risk young children is predicted by malaria, anemia, and stunting in Pemba, Zanzibar. J Nutr. 2009;139:763-72.

150. United Nations Children's Fund, United Nations University, World Health Organization, Micronutrient Initiative. Preventing iron deficiency in women and children: technical consensus on key issues. Boston, MA: International Nutrition Foundation; 1999.

151. Weiss G. Iron and immunity: a double-edged sword. Eur J Clin Invest. 2002;32:70-8.

152. Schaible UE, Kaufmann SHE. Iron and microbial infection. Nature Rev. 2004;2:946-53.

153. Iannotti LL, Tielsch JM, Black MM, Black RE. Iron supplementation in early childhood: health benefits and risks. Am J Clin Nutr. 2006;84:1261-76.

154. Gera T, Sachdev HPS. Effect of iron supplementation on incidence of infectious illness in children: systematic review. Br Med J. 2002;325:1142-4.

155. Shankar AH, Prasad AS. Zinc and immune function: the biological basis of altered resistance to infection. Am J Clin Nutr. 1998;68:447S-63S.

156. Prasad AS. Effects of zinc deficiency on immune functions. J Trace Elem Exp Med. 2000;13:1-20.

157. Ibs KH, Rink L. Zinc-altered immune function. J Nutr. 2003;133:1452S-6S.

158. Dardenne M. Zinc and immune function. Eur J Clin Nutr. 2002;56:S20-3.

159. Lopez A. Malnutrition and the burden of disease. Asia Pac J Clin Nutr. 2004;13:S7.

160. Sazawal A, Black RE, Jalla S, Mazumdar S, Sinha A, Bhan MK. Zinc supplementation reduces the incidence of acute lower respiratory infections in infants and preschool children: a double-blind, controlled trial. Pediatrics. 1998;102:1-5.

161. Bhutta ZA, Black RE, Brown KH, Gardner JM, Gore S, Hidayat A, Khatun F, Martorell R, Ninh NX, Penny ME, Rosado JL, Roy SK, Ruel M, Sazawal S, Shankar A. Prevention of diarrhea and pneumonia by zinc supplementation in children in developing countries: pooled analysis of randomized controlled trials. J Pediatr. 1999;135:689-97.

162. Brown KH, Peerson JM, Rivera J, Allen LH. Effect of supplemental zinc on the growth and serum zinc concentrations of prepubertal children: a meta-analysis of randomized controlled trials. Am J Clin Nutr. 2002;75:1062-71.

163. Negi R, Dewan P, Shah D, Das S, Bhatnagar S, Gupta P. Oral zinc supplements are ineffective for treating acute dehydrating diarrhea in five to 12-year-olds. Acta Pediatr. *In press.*

164. Stephensen CB. Vitamin A, infection, and immune function. Annu Rev Nutr. 2001;21:167-92.

165. World Health Organization. Guideline: vitamin A supplementation in infants and children 6-59 months of age. Geneva: World Health Organization; 2011.

166. Al-Kubaisy W, Al-Rubaiy MG, Nassief HA. Xerophthalmia among hospitalized Iraqi children. East Mediterr Health J. 2002;8:496-502.

167. World Health Organization. Guideline: vitamin A supplementation for infants 1-5 months of age. Geneva: World Health Organization; 2011.

168. Grubesic RB, Selwyn BJ. Vitamin A supplementation and health outcomes for children in Nepal. J Nurs Scholarship. 2003;35:15-20.

169. Shankar AH, Genton B, Semba RD, Baisor M, Paino J, Tamja S, Adiguma T, Wu L, Rare L, Tielsch JM, Alpers MP, West KP Jr. Effect of vitamin A supplementation on morbidity due to *Plasmodium falciparum* in young children in Papua New Guinea: a randomised trial. Lancet. 1999;354:203-9.

170. Villamor E, Mbise R, Spiegelman D, Hertzmark E, Fataki M, Peterson KE, Ndossi G, Fawzi WW. Vitamin A supplements ameliorate the adverse effects of HIV-1, malaria, and diarrheal infections on growth. Pediatrics. 2002;109:E6.

171. United Nations Children's Fund. Vitamin A supplementation: a decade of progress. New York City, United States: United Nations Children's Fund, 2007.

172. Tanumihardjo SA. Food-based approaches for ensuring adequate vitamin A nutrition. Compr Rev Food Sci Food Saf. 2008;7:373-81.

Table 1 Impact of the acute phase response to infection on energy expenditure and macronutrient metabolism[1]

Metabolic Indicator	Impact of infection
Energy	↓ dietary intake due to appetite suppression, ↑ energy expenditure to produce fever
Macronutrients	
Protein	↑ muscle protein wasting, ↑ hepatic protein synthesis
Carbohydrate	↑ blood glucose due to insulin resistance, glycogenolysis, and gluconeogenesis
Fat	↑ peripheral lipolysis, ↑ hepatic triglyceride and VLDL synthesis, ↓ serum cholesterol

[1]↑, increase; ↓, decrease; VLDL, very low density lipoprotein

Table 2 Effect of inflammation on indicators of micronutrient status[1]

Blood indicator	Impact of inflammation
Iron status	
Ferritin	↑
Serum iron	↓
Transferrin	↓
Transferrin receptors	N to ↓
Hemoglobin	N to ↓
Zinc status	
Serum zinc	↓
Vitamin A status	
Serum retinol	↓
MRDR test	N

[1] ↑, increase; ↓, decrease; N, no change; MRDR, modified relative dose response

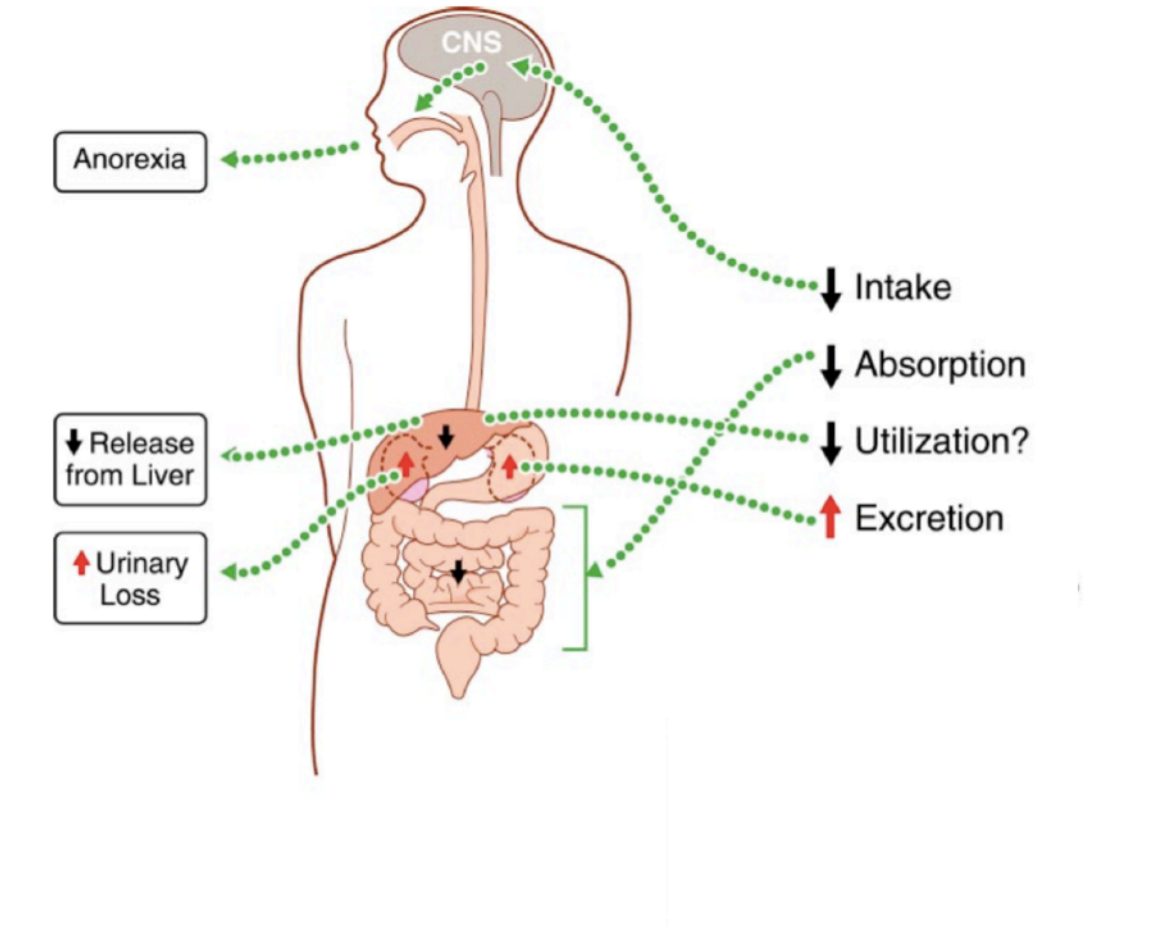

Figure 1 Infections can alter micronutrient status. For example, considering vitamin A, illness can negatively impact status through (i) decreased dietary intake caused by cytokine induced sickness behavior, (ii) reduced intestinal absorption during both enteric and non-enteric infection, and (iii) increased urinary excretion. Infection also suppresses the release of vitamin A from the liver, reducing plasma concentrations and thus, possibly altering utilization of liver stores (reprinted and adapted with permission from 164).

CHAPTER 2

Relative vitamin A value of 9-*cis*- and 13-*cis*-β-carotene do not differ with fed at physiological levels during vitamin A depletion in Mongolian gerbils (*Meriones unguiculatus*)

Kara A. Bresnahan, Christopher R. Davis, and Sherry A. Tanumihardjo

Interdepartmental Graduate Program in Nutritional Sciences

University of Wisconsin, Madison, WI 53706, USA

This manuscript is published in the *British Journal of Nutrition* ©2014

British Journal of Nutrition, page 1 of 8
doi:10.1017/S0007114514000658

Relative vitamin A values of 9-*cis*- and 13-*cis*-β-carotene do not differ when fed at physiological levels during vitamin A depletion in Mongolian gerbils (*Meriones unguiculatus*)

Kara A. Bresnahan, Christopher R. Davis and Sherry A. Tanumihardjo*

Interdepartmental Graduate Program in Nutritional Sciences, University of Wisconsin-Madison, 1415 Linden Drive, Madison, WI 53706, USA

(Submitted 4 November 2013 – Final revision received 28 January 2014 – Accepted 11 February 2014)

Abstract

Provitamin A biofortification of staple crops may decrease the prevalence of vitamin A (VA) deficiency if widely adopted in target countries. To assess the impact of processing methods on the VA value of plant foods, the unique bioefficacies of *cis*-βC isomers (formed during cooking) compared with all-*trans* (a*t*) β-carotene (βC) must be determined. The bioefficacies of 9-*cis* (9*c*)- and 13-*cis* (13*c*)-βC isomers were compared with those of the a*t*-βC isomer and VA positive (VA+) and negative (VA−) controls in VA-depleted Mongolian gerbils (*Meriones unguiculatus*) in two experimental studies (study 1, *n* 56; study 2, *n* 57). A 3- or 4-week depletion period was followed by a 3- or 4-week treatment period in which the groups received oral doses of the 9*c*-, 13*c*- or a*t*-βC isomers in cottonseed oil (study 1, 15 nmol/d; study 2, 30 nmol/d). In study 1, the βC isomers did not maintain baseline liver VA stores in all groups (0·69 (SD 0·20) μmol/liver) except in the VA+ group (0·56 (SD 0·10) μmol/liver) ($P=0·0026$). The βC groups were similar to the VA+ group, but the 9*c*- and 13*c*-βC groups did not differ from the VA− group (0·39 (SD 0·09) μmol/liver). In study 2, the βC isomers maintained baseline liver VA stores in all the βC groups (0·35 (SD 0·13) μmol/liver), and in the VA+ group, the VA supplement (0·54 (SD 0·19) μmol/liver) exceeded the baseline VA status (0·38 (SD 0·15) μmol/liver) ($P<0·0001$); however, the 9*c*-βC group did not differ from the VA− group (0·20 (SD 0·07) μmol/liver). *In vivo* isomerisation of βC was confirmed in both experimental studies. Lower VA bioconversion factor values were obtained for the *cis*-βC isomers in study 2 when compared with study 1, but higher values were obtained for the a*t*-βC isomer. Dose and VA status clearly affect bioconversion factors. In conclusion, the *cis*-βC isomers yielded similar liver VA stores to the a*t*-βC isomer in Mongolian gerbils, and liver VA stores of the 9*c*- and 13*c*-βC groups did not differ when the doses were provided at physiological levels over time in two studies.

Key words: **β-Carotene: Bioavailability: Bioconversion: Isomer effects**

β-Carotene (βC) is one of the most common carotenoids in the human diet[1], and its primary function in the body is provitamin A activity[2]. All-*trans* (a*t*)-βC is the most abundant isomer in fresh plant foods, but thermal processing can increase the proportion of 9-*cis* (9*c*)- and 13-*cis* (13*c*)-βC isomers up to 50 %[3]. Little is known about the vitamin A (VA) efficacies of the *cis*-βC isomers, which have been collectively assigned a bioavailability equivalent to half that of a*t*-βC[4]. Therefore, it is imperative to determine the unique bioefficacies of the *cis*-βC isomers to better understand the impact of thermal processing methods on the VA value of plant foods.

VA deficiency is a global health concern, affecting 190 million children under 5 years of age[5]. The introduction of provitamin A carotenoid-biofortified staple crops is a novel approach to alleviate VA deficiency and prevent issues observed with preformed VA supplementation and fortification, such as cyclical liver stores and hypervitaminosis risk[6]. However, staple crops undergo thermal processing before consumption; thus, the impact of isomerisation may need to be considered when establishing biofortification targets in order to ensure adequate delivery of VA to target populations.

A study by Deming *et al.*[7] has determined the individual bioavailabilities of 9*c*- and 13*c*-βC isomers in gerbils to be 38 and 62 % that of a*t*-βC, respectively, using doses that ranged between 141 and 418 nmol βC/d or 132 and 392 nmol VA/d according to the Institute of Medicine (IOM)'s recommended values of 2 μg a*t*-βC to 1 μg retinol activity equivalents for supplemental βC[4]. These orally administered doses greatly exceeded the utilisation rate (10·8 nmol VA/100 g body weight/d[8]) and the current recommendation of the

Abbreviations: a*t*, all-*trans*; βC, β-carotene; 9*c*, 9-*cis*; 13*c*, 13-*cis*; IOM, Institute of Medicine; VA, vitamin A; VA+, retinyl acetate in cottonseed oil; VA−, vitamin A-free cottonseed oil.

* **Corresponding author:** S. A. Tanumihardjo, fax +1 608 262 5860, email sherry@nutrisci.wisc.edu

British Journal of Nutrition

National Research Council for a typical adult gerbil (15·1 nmol VA/d, assuming an average feed intake of approximately 6 g[9]), and therefore they are not reflective of typical dietary intake that would be achieved from staple crops. Howe *et al.*[10] found that βC-enhanced cassava feeds providing 10·9–27·5 nmol βC/d maintained VA status in gerbils despite 40–50 % *cis*-βC isomer content in cassava. Additionally, in an *in vitro* study using isomeric mixtures, the 9*c*-βC isomer enhanced overall total carotene in solution and resulted in constant or enhanced micellar incorporation[11]. Thus, the objective of the present study was to compare the bioefficacies of the 9*c*-, 13*c*- and a*t*-βC isomers in VA-depleted gerbils using doses reflective of dietary intakes from staple foods. Conversion to retinol was determined using total liver VA stores compared with a positive preformed retinol-supplemented group and a negative VA-depleted group.

Materials and methods

Chemicals and dose preparation

Crystalline a*t*-βC (Sigma-Aldrich), 9*c*-βC and 13*c*-βC (gifts from Hoffmann-La Roche, Inc.) were prepared for purification by dissolution in *n*-hexane. βC isomers were purified on an open column using Al as the stationary phase and *n*-hexane as the mobile phase. The purities of all βC isomers were confirmed as >99 % with HPLC. For stock solutions, purified βC in hexane was condensed to 10 ml under N_2, mixed with 20 ml cottonseed oil in an amber vial, and dried to remove remaining hexane and ensure clear solutions. The removal of hexane was confirmed by repeated weighing until a constant weight was achieved. Using spectrophotometry, concentrations were calculated with $E^{1\%}_{1\,cm} = 2590$ for the a*t*-βC isomer at 450 nm, $E^{1\%}_{1\,cm} = 2550$ for the 9*c*-βC isomer at 445 nm and $E^{1\%}_{1\,cm} = 2090$ for the 13*c*-βC isomer at 443 nm[12]. Specific volumes of stock solution were pipetted into amber vials containing the appropriate amount of oil to achieve the target dose and were mixed by sonication; final doses were validated by dissolution of the dose in hexane followed by spectrophotometry where the reference cell contained an equal amount of oil dissolved in hexane. Doses were divided and stored at −70°C to minimise isomerisation. Lack of isomerisation during dose preparation was confirmed by running an aliquot of the oil dose dissolved in isopropanol on the HPLC system. A new bottle was opened at each study week.

Study design

Male Mongolian gerbils (*Meriones unguiculatus*) (study 1, *n* 56; study 2, *n* 57; Charles River Laboratories), aged 34 d old, were group-housed (*n* 2–3 per cage) throughout the study. Room temperature and humidity were held constant (12 h light–dark cycle). Gerbils were acclimatised to the dosing procedure before the start of the treatments by orally administering 40 μl cottonseed oil/d using a positive displacement pipette. Gerbils were weighed on a daily basis for about 2 weeks and thereafter were weighed twice per week. Animal handling procedures were approved by the College of Agriculture and Life Sciences Animal Care and Use Committee at University of Wisconsin-Madison.

Gerbils consumed *ad libitum* water and VA- and carotenoid-free, semi-purified, powdered feed for the duration of the study. The feed was designed in consultation with a feed nutritionist (Harlan-Teklad) to meet the gerbils' energy and nutrient requirements without any known source of VA or βC (Table 1). Feed intake was determined by measuring the feed in a clean bowl and subtracting the remainder after a 24 h period. After depletion (study 1, 3 weeks; study 2, 4 weeks), a baseline group (study 1, *n* 6; study 2, *n* 7) was killed via exsanguination while under isoflurane anaesthesia to determine initial serum and liver VA concentrations. The remaining cages were sorted into weight-matched treatment groups (*n* 10 per group). To ensure 90 % power with a 5 % significance level, nine gerbils per treatment group were necessary to detect a 30 nmol difference in liver retinol concentration, assuming a standard deviation of 20 nmol. The sample size was increased to ten gerbils per group to account for the animals that may fail to thrive during the experimental period or to mitigate outliers.

In the present study, three groups received daily oral doses of a*t*-, 9*c*- or 13*c*-βC in cottonseed oil, and the other groups received doses of VA-free cottonseed oil (VA−) or retinyl acetate in cottonseed oil (VA+). In study 1, a low βC dose of 15 nmol/d was designed to provide twice the molar equivalent of the daily VA utilisation rate of a 70 g gerbil[8]. In study 2, a moderate βC dose of 30 nmol/d was designed to provide twice the molar equivalent of the current VA recommendation of 2·5 nmol/g feed[9], assuming a typical feed intake of approximately 6 g/d. The retinyl acetate dose provided the proportion of 1 μg retinol:2 μg a*t*-βC in accordance with the current equivalents of the IOM for oil βC supplements[4]. Following a 3-week (study 1) or a 4-week (study 2) treatment period, gerbils were killed. Blood was allowed to clot at room temperature for 30 min and centrifuged for 15 min at 2200 ***g*** at 4°C in vacutainer tubes (Becton Dickinson). Serum and liver samples were stored at −70°C.

Table 1. Composition of a vitamin A (VA)- and carotenoid-free gerbil feed

Ingredients	VA- and carotenoid-free feed (g/kg)
Casein, vitamin-free	200
L-Cys	3·0
Sucrose	360·5
Maltodextrin	120
Maize starch	150
Cottonseed oil	60
Cellulose	60
Mineral mix, AIN-93M-MX	35
Magnesium oxide	1·75
Calcium phosphate, dibasic	2·0
Vitamin mix*	5·0
Vitamin E acetate	0·242
Vitamin D_3	0·004
Choline bitartrate	2·5

* Provided per kg of purified diet: biotin, 0·4 mg; calcium pantothenate, 66·1 mg; folic acid, 2 mg; inositol, 110·1 mg; menadione, 49·6 mg; niacin, 99·1 mg; *p*-aminobenzoic acid, 110·1 mg; pyridoxine-HCl, 22 mg; riboflavin, 22 mg; thiamin-HCl, 22 mg; vitamin B_{12} (0·1 % in mannitol), 29·7 mg; ascorbic acid (97·5 %), 1016·6 mg.

British Journal of Nutrition

British Journal of Nutrition

Tissue extraction procedures

Samples were analysed under gold light to prevent photo-oxidation and isomerisation. C-23 *apo*-carotenol was used as the internal standard in all serum and liver samples. A modified procedure was used for the analysis of serum[13]. In study 1, 500 μl serum was analysed, reconstituted in 100 μl of methanol–dichloroethane (50:50), and 25 μl injected. In study 2, to assess the isomeric profile, 1000 μl serum was analysed, reconstituted in 80 μl of methanol–butanol (70:30), and 50 μl injected.

Liver (approximately 0·7 g) was ground with approximately 4 g sodium sulphate, extracted with dichloromethane, filtered and made up to 50 ml. For retinoid and carotenoid analyses, aliquots of 5 and 15 ml, respectively, were dried under N_2, reconstituted in 100 μl of methanol–dichloroethane (50:50), and 25 or 50 μl injected.

HPLC analyses

βC isomers were separated from the oil doses and liver samples using a C30 YMC carotenoid column (4·6 × 250 mm, 3 μm; Waters, Inc.) at 450 nm, as described previously[14], on a Waters HPLC system consisting of a 1525 binary HPLC pump, a 717 autosampler and a 996 photodiode array detector. The samples were held at 4°C in the autosampler to prevent isomerisation or degradation.

For serum analysis in study 1 and all liver analyses, retinol and retinyl esters were separated using a Resolve C18 column (3·9 × 300 mm, 5 μm; Waters, Inc.) at 325 nm. Solvent A was acetonitrile–water (85:15) with 10 mM-ammonium acetate and solvent B was acetonitrile–methanol–dichloroethane (85:10:5) with 10 mM-ammonium acetate. Gradient elution was performed at 2 ml/min using the following procedure: 100 % A for 3 min, 7 min linear gradient to 100 % B, 15 min hold and 2 min reverse gradient to 100 % A. Identities of βC isomers, retinol and retinyl esters were determined using HPLC-purified standards. Total liver VA stores were calculated by summing retinol and identifiable retinyl esters. For serum analysis in study 2, retinol isomers were separated using a Zorbax ODS C18 column (4·6 × 250 mm, 5 μm; Dupont) at 325 nm with an isocratic mobile phase of methanol–water–butanol (50:27:23) with 10 mM-ammonium acetate run at 1 ml/min. Identities of the retinol isomers were confirmed using the elution order determined by MacCrehan & Schönberger[15] and HPLC spectra. All tissue samples were run on a Waters HPLC system consisting of a Delta 600 quaternary HPLC pump, a 600 controller and a 2487 dual absorbance detector, and the samples were injected manually. C-23 alcohol was used as the internal standard, and standard curves were constructed for the quantification of carotenoids and retinoids.

Statistical analysis

Results are presented as means and standard deviations. Data were analysed using SAS software (version 8.2; SAS Institute). Outcomes of interest (gerbil body and liver weights, serum retinol, and liver retinol and carotenoids) were evaluated using a one-way ANOVA. Differences between groups were determined using the least significant difference test at $\alpha < 0{\cdot}05$. An arcsine-square-root transformation was used to homogenise variance expressed as percentages. Bioconversion factors of βC to retinol were calculated using differences in total liver VA in the βC-treated groups compared directly with the VA+ group after correction for the VA− group.

Results

Gerbil weights

In study 1, the final gerbil weights did not differ between the experimental groups (77·7 (SD 5·15) g) but were found to be higher than that of the baseline group (67·4 (SD 6·62) g, $P=0{\cdot}0043$). The final liver weights did not differ among the groups (3·05 (SD 0·42) g). In study 2, the final gerbil weights (68·8 (SD 5·63) g) and liver weights (2·47 (SD 0·35) g) were found to be similar among all the groups. Feed intakes did not differ within study 1 (6·42 (SD 1·07) g feed/d) or study 2 (5·05 (SD 0·69) g feed/d) (Table 2).

Serum retinol and carotenoids

In both studies, total serum retinol concentration did not differ among the treatment groups, and carotenoids were not detected. In study 1, the range of serum retinol concentrations was 1·43 (SD 0·12) μmol/l in the 13*c*-βC group to 1·70 (SD 0·18) μmol/l in the VA− group. In study 2, the range was found to be 1·19 (SD 0·12) μmol/l in the a*t*-βC group to 1·27 (SD 0·14) μmol/l in the baseline group, and the relative ratio of isomers varied across the treatment groups (Fig. 1). Serum retinol concentrations differed between studies 1 and 2 ($P<0{\cdot}0001$). The composition of 9*c*-retinol in serum differed among the groups ($P=0{\cdot}032$). The proportion of 9*c*-retinol was similar among the 9*c*-βC, 13*c*-βC, a*t*-βC and VA+ groups (1·94 (SD 0·50) %), and the proportion for the baseline and VA− groups (1·59 (SD 0·19) %) was found to be lower than that for the 13*c*-βC and a*t*-βC groups (2·09 (SD 0·51) %), but

Table 2. Gerbil feed consumption in two studies administering low (15 nmol) and moderate (30 nmol) doses of β-carotene (βC) daily
(Mean values and standard deviations)

		Daily feed intake (g/d)	
Treatment	*n*	Mean	SD
Low βC intake			
9*c*-βC	10	5·83	1·15
13*c*-βC	10	6·66	0·73
a*t*-βC	10	6·56	1·03
VA +	10	6·61	1·22
VA −	10	6·44	1·31
Moderate βC intake			
9*c*-βC	10	4·93	0·92
13*c*-βC	10	5·08	0·53
a*t*-βC	10	4·86	0·86
VA +	10	4·93	0·25
VA −	10	5·53	1·16

c, *cis*; a*t*, all-*trans*; VA, vitamin A.

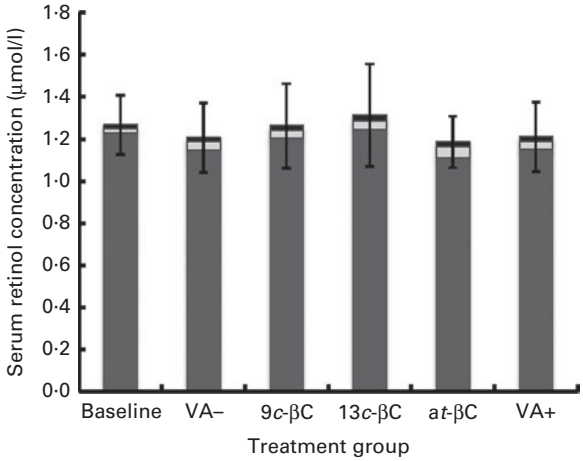

Fig. 1. Serum retinol concentrations in Mongolian gerbils (*Meriones unguiculatus*) fed a vitamin A (VA)- and carotenoid-free feed at baseline after 4 weeks (study 2) of depletion (baseline, *n* 7), or treated with VA-free cottonseed oil (VA−), moderate doses of 30 nmol 9-*cis*-, 13-*cis*- or all-*trans* β-carotene (9*c*- ■, 13*c*- □ or a*t*-βC ■) in cottonseed oil, or retinyl acetate in cottonseed oil (VA+) for an additional 4 weeks. VA intake in the VA+ group was matched to one-half of the theoretical VA intake in the βC groups. Values are means (*n* 10), with standard deviations represented by vertical bars. The treatment groups did not differ significantly ($P>0·05$; ANOVA followed by least significant difference test).

did not differ from each other or the 9*c*-βC and VA+groups (1·77 (SD 0·46)%). The composition of 13*c*-retinol did not differ among the groups (3·16 (SD 1·95)%). The composition of a*t*-retinol differed from baseline (96·7 (SD 0·50)%, $P=0·021$) in the 13*c*-βC and a*t*-βC groups, but did not differ from each other (94·1 (SD 1·94)%) or the VA−, 9*c*-βC and VA+groups (95·1 (SD 1·76)%).

Liver retinol and carotenoids

In study 1, concentrations of liver retinol did not differ among the experimental groups, and the βC isomers did not maintain baseline liver stores in all the groups ($P=0·006$; Fig. 2(A)). Total liver retinol concentrations did not differ among the VA+, 9*c*-βC, 13*c*-βC and a*t*-βC groups, and the βC isomers failed to maintain baseline liver VA stores in all of these groups, except the VA+group ($P=0·0026$; Fig. 2(B)). The 9*c*- and 13*c*-βC groups did not differ from the VA−group. In study 2, the βC isomers maintained baseline liver retinol status in all groups, except the VA−group, which did not differ from the 9*c*- and 13*c*-βC groups ($P=0·0019$; Fig. 3(A)). Total liver retinol stores were found to be highest in the VA+ group and lowest in the VA−group ($P<0·0001$; Fig. 3(B)). The VA−group did not differ from the 9*c*-βC group, but all the βC isomers resulted in similar total liver stores.

Carotenoids were not detected in the liver of the baseline, VA− and VA+groups in either study. In studies 1 and 2, total liver βC concentration was found to be highest in the 9*c*-βC group followed by the a*t*-βC and 13*c*-βC groups (study 1, $P<0·0001$; study 2, $P<0·0001$; Table 3). The presence of the administered βC isomer and the other two isomers in the liver demonstrated *in vivo* isomerisation of the βC doses, and the relative proportions of the βC isomers varied among these groups (Table 3).

Bioconversion factors and utilisation rates

Bioconversion factors for the βC-treated groups were calculated by determining the total liver VA stores above the negative control and by directly comparing this value to that of the VA+group. Bioconversion factors ranged from 115 to 168% and 140 to 175% of the IOM's recommended values in studies 1 and 2, respectively (Table 4). Comparison of the 9*c*- and 13*c*-βC isomers with the a*t*-βC isomer resulted in mass equivalents of 2·9 and 1·6 μg 9*c*-βC and 2·6 and 1·4 μg 13*c*-βC to 1 μg a*t*-βC for studies 1 and 2, respectively. In study 1, the utilisation rate for the VA−group, which was deprived of VA for 21 d beyond baseline, was found to be 5·1 μg/100 g body weight and for the VA+group, which was supplemented cumulatively with 84·6 μg retinol in the form of acetate, was 7·5 μg/100 g body weight. In study 2, the utilisation rate for the VA−group, which was deprived of VA for an additional 14 d in the

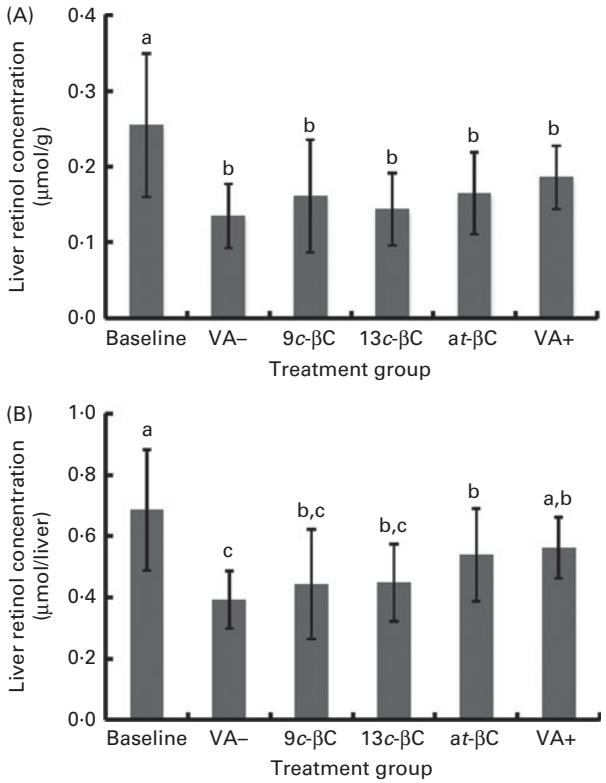

Fig. 2. Liver retinol (A) and total liver retinol (B) concentrations corrected for liver weight in Mongolian gerbils (*Meriones unguiculatus*) fed a vitamin A (VA)- and carotenoid-free feed at baseline after 3 weeks (study 1) of depletion (baseline, *n* 6), or treated with VA-free cottonseed oil (VA−), low doses of 15 nmol 9-*cis*-, 13-*cis*- or all-*trans* β-carotene (9*c*-, 13*c*- or a*t*-βC) in cottonseed oil, or retinyl acetate in cottonseed oil (VA+) for an additional 3 weeks. VA intake in the VA group was given at 1 μg retinol equivalent to 2 μg βC provided to the βC groups. Liver retinol was determined by pooling values for retinol and retinyl esters. Values are means (*n* 10), with standard deviations represented by vertical bars. ^{a,b,c} Mean values with unlike letters were significantly different ($P<0·05$; ANOVA followed by least significant difference test).

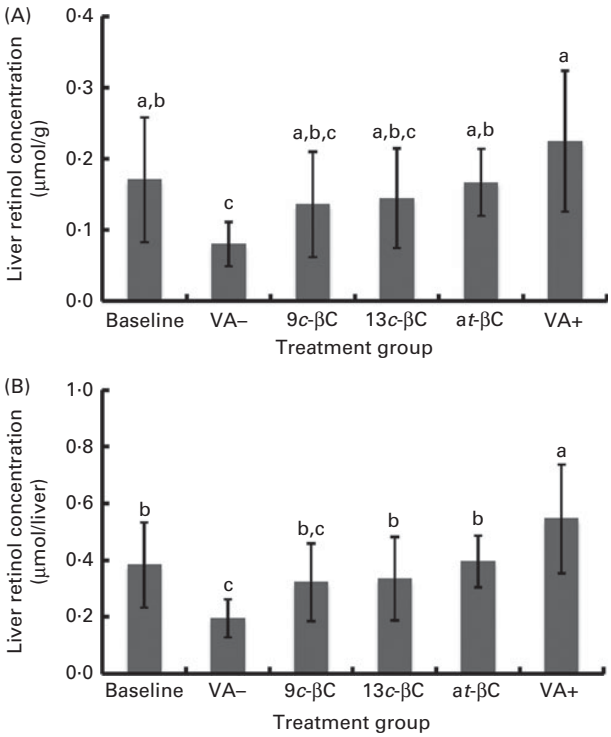

Fig. 3. Liver retinol (A) and total liver retinol (B) concentrations in Mongolian gerbils (*Meriones unguiculatus*) fed a vitamin A (VA)- and carotenoid-free feed at baseline after 4 weeks (study 2) of depletion (baseline, *n* 7), or treated with VA-free cottonseed oil (VA−), moderate doses of 30 nmol 9-*cis*-, 13-*cis*- or all-*trans* β-carotene (9*c*-, 13*c*- or a*t*-βC) in cottonseed oil, or retinyl acetate in cottonseed oil (VA+) for an additional 4 weeks. VA intake in the VA group was given at 1 μg retinol equivalent to 2 μg βC provided to the βC groups. Liver retinol was determined by pooling values for retinol and retinyl esters. Values are means (*n* 10), with standard deviations represented by vertical bars. [a,b,c] Mean values with unlike letters were significantly different ($P<0·05$; ANOVA followed by least significant difference test).

extended depletion and treatment periods, was found to be 2·7 μg/100 g body weight, and for the VA+ group, which received a total of 225·7 μg retinol, was 9·1 μg/100 g body weight.

Discussion

To assess the impact of thermal processing methods on plant foods, it is essential to determine the unique VA bioefficacies of *cis*-βC isomers at physiological levels. To our knowledge, no other studies have investigated the bioefficacies of βC isomers using amounts reflective of dietary intakes in animals with carotenoid metabolism similar to that of humans. In studies 1 and 2, the bioefficacies of 9*c*-, 13*c*- and a*t*-βC in oil at low (15 nmol) and moderate (30 nmol) doses were compared directly with preformed VA supplements. In study 1, the βC isomers did not maintain liver retinol stores; however, in study 2, at higher doses, all βC isomers maintained baseline liver stores and the VA supplement exceeded baseline VA status. Bioconversion factors were 15–75 % less efficient than the current reference values of the IOM. Factors for the supplemental *cis*-βC isomers were similar in each study, with midpoints of 6·4 and 5·3 μg *cis*-βC:1 μg retinol, in studies 1 and 2, respectively. Bioconversion factors for the supplemental a*t*-βC isomer were found to be 2·3 and 3·5 μg a*t*-βC:1 μg retinol in both studies, which are similar to the reported values of 2·5–4·6 in previous gerbil studies[10,13,14,16].

The utilisation rates within and between each study were not the same, but similar to those reported in previous gerbil studies[8,16]. For the VA− group, the utilisation rate in study 2 was almost half of that in study 1, indicating that the gerbils were conserving and recycling more retinol during the period of prolonged deprivation. The higher utilisation of the VA+ group in study 2 probably reflects the more concentrated preformed retinyl acetate dose that they received[17]. The βC doses failed to maintain baseline liver VA stores in study 1, suggesting that the utilisation rates of gerbils exceeded the amount of bioavailable provitamin A provided. Lee *et al.*[8] determined the VA utilisation rate of a VA-deplete adult gerbil to be 3·1 μg/100 g body weight. The difference in utilisation rates between study 1 (i.e. 5·1 μg/100 g body weight) and Lee *et al.*[8] may be due to the gerbils having a higher requirement as they were 16–26 d younger, or due to the variation in the level of

Table 3. Total liver β-carotene (βC) and isomeric compositions in Mongolian gerbils (*Meriones unguiculatus*) administered with low (15 nmol) and moderate (30 nmol) doses of 9-*cis*-, 13-*cis*- or all-*trans* βC (9*c*-, 13*c*- or a*t*-βC)*

(Mean values and standard deviations; *n* 10 per βC group)

	Total liver βC (nmol)		9*c*-βC (%)		13*c*-βC (%)		a*t*-βC (%)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Low βC intake								
9*c*-βC	7·3[a]	1·3	68·6[a]	4·6	10·2	0·9	21·3[c]	3·8
13*c*-βC	2·4[c]	0·46	39·3[b]	5·6	12·3	4·2	48·4[b]	4·4
a*t*-βC	4·9[b]	2·0	31·2[c]	13·0	12·6	2·8	56·2[a]	10·7
P	<0·0001		<0·0001		NS		<0·0001	
Moderate βC intake								
9*c*-βC	5·0[a]	0·6	21·6[c]	4·1	6·2[b]	1·2	72·2[a]	3·4
13*c*-βC	2·2[c]	0·5	57·1[b]	6·0	9·6[a]	1·8	33·2[b]	6·6
a*t*-βC	3·2[b]	0·5	69·6[a]	3·6	9·1[a]	2·9	21·3[c]	2·3
P	<0·0001		<0·0001		0·0029		<0·0001	

[a,b,c] Mean values within a column with unlike superscript letters were significantly different ($P<0·05$).

* The limit of detection for βC was <0·007 nmol.

Table 4. Bioconversion factors in Mongolian gerbils (*Meriones unguiculatus*) administered with low (15 nmol) or moderate (30 nmol) doses of β-carotene (βC)*

	μmol βC: 1 μmol retinol	μg βC: 1 μg retinol	IOM reference (μg βC:1 μg retinol)†	Percentage of IOM value	Percentage of a*t*-βC bioefficacy‡
Low βC intake					
9*c*-βC	3·6	6·7	4·0	168	34
13*c*-βC	3·2	6·1	4·0	153	37
a*t*-βC	1·2	2·3	2·0	115	100
Moderate βC intake					
9*c*-βC	3·0	5·6	4·0	140	63
13*c*-βC	2·7	5·0	4·0	125	70
a*t*-βC	1·9	3·5	2·0	175	100

IOM, Institute of Medicine; a*t*, all-*trans*; *c*, *cis*.
* *n* 10 per βC group.
† The IOM reference value for supplemental *cis*-βC isomers was based on the current retinol activity equivalents from food, which assumes a bioavailability that is equivalent to 50 % that of a*t*-βC for all *cis*-βC isomers.
‡ Determined by direct comparison with bioconversion factors for the a*t*-βC isomer in studies 1 and 2.

depletion[16,17]. In study 2, the depletion period was extended to reach deficiency, which resulted in a similar utilisation rate (i.e. 2·7 μg/100 g body weight) to that of the Lee *et al.*'s value, and doses were increased to allow for adequate VA storage in the βC-treated groups.

It is important to consider the impact of VA status on bioconversion factors. The utilisation rate slows and recycling becomes more efficient during the state of deficiency and the converse occurs when the status is sufficient[17,18]. This departure in utilisation rates between the VA-deplete and VA-replete groups can therefore lead to the inflation of conversion factors, which may partially explain the higher conversion factors in study 1 compared with study 2 for the *cis*-βC isomers. The VA− group had higher VA status in study 1 than in study 2 where the depletion and treatment periods were extended. Furthermore, bioconversion of βC to retinol in humans and gerbils is dependent on dose size[19,20] and is inversely related to VA status[21,22], which may explain the difference in bioconversion factors for the a*t*-βC isomer between the two studies because the dose was doubled in study 2.

In study 2, the relative VA values of the 9*c*- and 13*c*-βC isomers were similar with bioefficacies of 63 and 70 % that of the a*t*-βC isomer, respectively. In previous studies, the relative VA values of the 9*c*- and 13*c*-βC isomers compared with that of the a*t*-βC isomer were 33–61 and 48–74 %, respectively, in rats[23–25] and 38 and 62 %, respectively, in gerbils[7]. The variations in the VA values determined in the previous studies may be due to the differences in the amount (0·6–224·4 μg βC/d) and duration (7–28 d) of dosing, the depletion period (20–56 d) or the basis of calculation (i.e. body-weight gain *v.* liver VA)[7,23–25]. The gerbil provides a more advantageous model for studying carotenoid metabolism compared with the rat because, like humans, they are capable of cleaving provitamin A carotenoids to retinal and absorbing physiological doses intact[8,26,27]. Although rats are efficient cleavers of βC in the small intestine, they only absorb βC intact under conditions of great dietary excess[28–30]. The present study demonstrates more efficient conversion of the *cis*-βC isomers, particularly 9*c*-βC, in gerbils than that found by Deming *et al.*[7], which, again, is likely attributable to the larger dose size used in that study.

The observation of higher total liver βC concentration found in the 9*c*-βC group suggests that the 9*c*-βC isomer may be more efficiently delivered to the liver. *In vitro* studies have suggested that the *cis*-βC isomers are similarly or more efficiently incorporated into micelles during digestion[11,31–33], but data are inconsistent regarding the efficiency of *cis*-βC isomer uptake by intestinal cells[31–33]. Based on accumulation in human serum, the a*t*-βC isomer appears to be absorbed and/or packaged into the chylomicra more efficiently than the *cis*-βC isomers, with 13*c*-βC being the predominant *cis*-βC isomer[34–37]. Additionally, in human subjects fed supplements or *Dunaliella bardawil* containing an a*t*-βC:9*c*-βC ratio of approximately 50:50, the a*t*-βC isomer appears to be preferentially incorporated into the chylomicra and VLDL[38–40]. However, *in vivo* interconversion of βC isomers, documented previously in human subjects[41] and gerbils[37] and confirmed in the present study, may have a positive impact on the VA values of the *cis*-βC isomers, and result in inaccurate interpretations of their bioavailabilities when confirmed solely by the presence of the isomer of interest. More work is necessary to elucidate common *in vivo* patterns for and mechanisms of βC isomer interconversion as none is apparent from the isomeric composition found in the gerbil liver in these studies. Furthermore, enzymatic isomerisation of *cis*-βC isomers to a*t*-retinal by β-carotene 15,15′-mono-oxygenase 1 was observed in rats, with 9*c*-βC converting to a mixture of 9*c*-, 13*c*- and a*t*-retinal and 13*c*- and a*t*-βC converting primarily to a*t*-retinal[42]. In a previous study, the composition of retinol isomers in the liver of gerbils was determined to be similar among groups dosed with βC isomers and the negative control, with *cis*-βC isomers constituting approximately 10 % of total liver VA[37]. Similarly, the isomeric composition of serum retinol in study 2 was found to be 3·3–6·5 % of *cis*-retinol isomers, which supports the *in vivo* isomerisation of βC and/or retinol. Together, these findings demonstrate that the isomeric compositions of liver and serum retinol do not reflect the dietary intake of βC, and that the a*t*-retinol isomer appears to be preferable for the storage and circulation of VA.

The lack of the difference observed in liver VA stores among the 9*c*-, 13*c*- and a*t*-βC groups and bioefficacies of the *cis*-βC isomers >60 % that of the a*t*-βC isomer contradicts the idea that the *cis*:*trans* ratio significantly decreases the VA value of

British Journal of Nutrition

British Journal of Nutrition

plant foods. These findings support the conclusion made by Howe *et al.*[10] that regular, moderate intakes of *cis*-βC isomers from plant foods allow for efficient absorption and bioconversion to VA in gerbils. The cassava feeds used by Howe *et al.* contained 41–47 % of *cis*-βC isomers; by using a bioefficacy of *cis*-βC isomers equivalent to two-thirds that of the a*t*-βC isomer, the VA value may have been decreased by only 14–16 %. In fact, Howe *et al.*[10] demonstrated a conversion factor of 3·7 μg βC:1 μg retinol, which is similar to the values from plant foods containing much lower concentrations of *cis*-βC isomers[43,44]. Similar bioconversion factors for thermally processed provitamin A-biofortified cassava have recently been determined in women, ranging from 4·2–4·5 μg βC:1 μg retinol depending on oil content[45]. Additionally, the matrix of certain plant foods can have a negative impact on the VA value[46], in which case thermal processing can increase βC bioavailability[33,47,48]. It is therefore important for nutritionists and plant breeders to holistically consider the impacts of processing on matrix effects and carotenoid concentrations when establishing provitamin A biofortification targets in staple crops.

Liver is the primary storage site of retinol in mammals and is considered the best measure of VA status[18]. Serum retinol concentrations do not provide an effective measure of status because of homeostatic regulation over a wide range of liver stores[18], as demonstrated by similar concentrations among all the treatment groups within both studies. In that regard, the difference between serum retinol concentrations between studies 1 and 2 is interesting, considering that the gerbils in study 2 received twice the supplemental doses and liver VA concentrations did not differ between the two studies. Subclinical deficiency has recently been defined as liver stores $< 0{\cdot}1$ μmol retinol/g liver[18]; according to this definition, the VA− group in study 1 was nearing deficiency and the VA− group in study 2 was deficient, but serum retinol concentrations were not low (i.e. concentrations were $>0{\cdot}7$ μmol/l). Additionally, the absence of carotenoids in serum is expected because the lipid profile of gerbils differs from that of humans in that the major circulating lipoprotein is HDL and carotenoids are predominantly associated with LDL during fasting[49].

In summary, the 9*c*-, 13*c*- and a*t*-βC isomers prevented retinol deficiency in gerbils when provided in amounts reflective of moderate dietary intake, and the *cis*-βC isomers demonstrated bioefficacies >60 % that of the a*t*-βC isomer when baseline liver stores were maintained. The results of these studies suggest that mild thermal processing modestly decreases the VA value of plant foods through the generation of the *cis*-βC isomers. Furthermore, the currently assigned bioavailability of the *cis*-βC isomers, equivalent to 50 % that of the a*t*-βC isomer[4], may need to be clarified as being dependent on dose size and VA status. More research is needed to elucidate the mechanism of *in vivo* isomerisation of *cis*-βC to a*t*-βC and to assess the bioavailabilities of *cis*-βC isomers from plant foods in human subjects.

Acknowledgements

We thank Peter Crump, Senior Information Processing Consultant at the University of Wisconsin-Madison College of Agriculture and Life Sciences Statistical Consulting Service, for providing statistical assistance.

The present study was supported by USDA Hatch WIS01528, a College of Agricultural and Life Sciences Honors Scholarship (to K. A. B), a gift from Standard Process, Inc., and HarvestPlus. HarvestPlus (www.harvestplus.org) is a global alliance of agriculture and nutrition research institutions working to increase the micronutrient density of staple food crops through biofortification. The views expressed herein do not necessarily reflect those of HarvestPlus. The funders had no role in the design, analysis or writing of this article.

The authors' contributions are as follows: K. A. B. conducted the study; C. R. D. conducted the analyses; K. A. B. and S. A. T. analysed the data, designed the research and wrote the manuscript. All authors read and approved the final version of the paper.

Part of this work was presented at Experimental Biology Meetings in 2011 and 2013.

The authors have no conflicts of interest to declare.

References

1. Britton G, Liaaen-Jensen S & Pfander H (2008) *Carotenoids*. Basel, Switzerland: Birkhäuser.
2. Grune T, Lietz G, Palou A, *et al.* (2010) β-Carotene is an important vitamin A source for humans. *J Nutr* **140**, 2268S–2285S.
3. Chandler LA & Schwartz SJ (1987) HPLC separation of *cis–trans* carotene isomers in fresh and processed fruits and vegetables. *J Food Sci* **52**, 669–672.
4. Institute of Medicine (2006) *Dietary Reference Intakes: The Essential Guide to Nutrient Requirements*. Washington, DC: National Academies Press.
5. World Health Organization (2011) *Guideline: Vitamin A Supplementation in Infants and Children 6–59 Months of Age*. Geneva, Switzerland: World Health Organization.
6. Tanumihardjo SA (2008) Food-based approaches for ensuring adequate vitamin A nutrition. *Compr Rev Food Sci Food Safety* **7**, 373–381.
7. Deming DM, Baker DH & Erdman JW Jr (2002) The relative vitamin A value of 9-*cis* β-carotene is less and that of 13-*cis* β-carotene may be greater than the accepted 50 % that of all-*trans* β-carotene in gerbils. *J Nutr* **132**, 2709–2712.
8. Lee CM, Lederman JD, Hofmann NE, *et al.* (1998) The Mongolian gerbil (*Meriones unguiculatus*) is an appropriate animal model for evaluation of the conversion of β-carotene to vitamin A. *J Nutr* **128**, 280–286.
9. National Research Council (1995) *Nutrient Requirements in Laboratory Animals*, 4th ed. Washington, DC: National Academy Press.
10. Howe JA, Maziya-Dixon B & Tanumihardjo SA (2009) Cassava with enhanced β-carotene maintains adequate vitamin A status in Mongolian gerbils (*Meriones unguiculatus*) despite substantial *cis*-isomer content. *Br J Nutr* **102**, 342–349.
11. Levin G & Mokady S (1995) Incorporation of all-*trans*- or 9-*cis*-β-carotene into mixed micelles *in vitro*. *Lipids* **30**, 177–179.
12. Schierle J, Hardi W, Faccin N, *et al.* (1995) Example 8: Geometrical isomers of β,β-carotene. In *Carotenoids: Isolation and Analysis*, vol. 1A, pp. 265–272 [G Britton, S Liaaen-Jensen and H Pfander, editors]. Basel, Switzerland: Birkhäuser.
13. Tanumihardjo SA & Howe JA (2005) Twice the amount of α-carotene isolated from carrots is as effective as β-carotene

in maintaining the vitamin A status of Mongolian gerbils. *J Nutr* **135**, 2622–2626.
14. Howe JA & Tanumihardjo SA (2006) Carotenoid-biofortified maize maintains adequate vitamin A status in Mongolian gerbils. *J Nutr* **136**, 2562–2567.
15. MacCrehan WA & Schönberger E (1987) Reversed-phase high performance liquid chromatographic separation and electrochemical detection of retinol and its isomers. *J Chromatogr* **417**, 65–78.
16. Davis C, Jing H, Howe JA, *et al.* (2008) β-Cryptoxanthin from supplements or carotenoid-enhanced maize maintains liver vitamin A in Mongolian gerbils (*Meriones unguiculatus*) better than or equal to β-carotene supplements. *Br J Nutr* **100**, 786–793.
17. Green MH, Green JB & Lewis KC (1987) Variation in the retinol utilisation rate with vitamin A status in the rat. *J Nutr* **117**, 694–703.
18. Tanumihardjo SA (2011) Vitamin A: biomarkers of nutrition for development. *Am J Clin Nutr* **94**, 658S–665S.
19. Tang G, Qin J, Dolnikowski GG, *et al.* (2000) Vitamin A equivalence of β-carotene in a woman as determined by a stable isotope reference method. *Eur J Nutr* **39**, 7–11.
20. Simon PW, Tanumihardjo SA, Clevidence BA, *et al.* (2008) Role of color and pigments in breeding, genetics, and nutritional improvements of carrots. In *Color Quality of Fresh and Processed Foods*, pp. 151–164 [CA Culver and RE Wrolstad, editors]. Washington, DC: American Chemical Society.
21. Ribaya-Mercado JD, Solon FS, Solon MA, *et al.* (2000) Bioconversion of plant carotenoids to vitamin A in Filipino school-age children varies inversely with vitamin A status. *Am J Clin Nutr* **72**, 455–465.
22. Haskell MJ, Jamil KH, Hassan F, *et al.* (2004) Daily consumption of Indian spinach (*Basella alba*) or sweet potatoes has a positive effect on total-body vitamin A stores in Bangladeshi men. *Am J Clin Nutr* **80**, 705–714.
23. Duel HJ, Johnston CH, Meserve ER, *et al.* (1945) Stereochemical configuration and provitamin A activity IV. Neo-α-carotene and neo-β-carotene. *Arch Biochem* **7**, 247–255.
24. Johnson RM & Baumann CA (1947) Storage and distribution of vitamin A in rats fed certain isomers of carotene. *Arch Biochem* **14**, 361–367.
25. Sweeny JP & Marsh AC (1973) Liver storage of vitamin A in rats fed carotene stereoisomers. *J Nutr* **103**, 20–25.
26. Pollack J, Campbell JM, Potter SM, *et al.* (1994) Mongolian gerbils (*Meriones unguiculatus*) absorb β-carotene intact from a test meal. *J Nutr* **124**, 869–873.
27. House WA, Apgar J & Smith JC (1997) The gerbil: a model for studying the metabolism of β-carotene and minerals. *Nutr Res* **17**, 1293–1302.
28. Ribaya-Mercado JD, Holmgren SC, Fox JG, *et al.* (1988) Dietary β-carotene absorption and metabolism in ferrets and rats. *J Nutr* **119**, 665–668.
29. Biesalski HK & Weiser H (1993) β-Carotene supplements cannot meet all vitamin A requirements of vitamin A-deficient rats. *NY Acad Sci* **69**, 216–219.
30. Grolier P, Agoudave S & Azais-Braesco V (1995) Comparative bioavailability of diet-, oil-, and emulsion-based preparations of vitamin A and β-carotene in the rat. *Nutr Res* **15**, 1507–1516.
31. Ferruzzi MG, Lumpkin JL, Schwartz SJ, *et al.* (2006) Digestive stability, micellarization, and uptake of β-carotene isomers by Caco-2 human intestinal cells. *J Agric Food Chem* **54**, 2780–2785.
32. Thakkar SK, Maziya-Dixon B, Dixon AGO, *et al.* (2007) β-Carotene micellarization during *in vitro* digestion and uptake by Caco-2 cells is directly proportional to β-carotene content in different genotypes of cassava. *J Nutr* **137**, 2229–2233.
33. Aherne SA, Daly T, Jiwan MA, *et al.* (2010) Bioavailability of β-carotene isomers from raw and cooked carrots using an *in vitro* digestion model coupled with a human intestinal Caco-2 cell model. *Food Res Int* **43**, 1449–1454.
34. Rushin WG, Catignani GL & Schwartz SJ (1990) Determination of β-carotene and its *cis* isomers in serum. *Clin Chem* **36**, 1986–1989.
35. Gaziano JM, Johnson EJ, Russell RM, *et al.* (1995) Discrimination in absorption or transport of β-carotene isomers after oral supplementation with either all-*trans*- or 9-*cis*-β-carotene. *Am J Clin Nutr* **61**, 1248–1252.
36. Johnson EJ, Krinsky NI & Russell RM (1996) Serum response of all-*trans* and 9-*cis* isomers of β-carotene in humans. *J Am Coll Nutr* **15**, 620–624.
37. Deming DM, Teixeria SR & Erdman JW Jr (2002) All-*trans* β-carotene appears to be more bioavailable than 9-*cis* or 13-*cis* β-carotene in gerbils given single oral doses of each isomer. *J Nutr* **132**, 2700–2708.
38. Stahl W, Schwarz W & Sies H (1993) Human serum concentrations of all-*trans* β- and α-carotene but not 9-*cis* β-carotene increase upon ingestion of a natural isomer mixture obtained from *Dunaliella salina* (Betatene). *J Nutr* **123**, 847–851.
39. Stahl W, Schwarz W, von Laar J, *et al.* (1995) All-*trans* β-carotene preferentially accumulates in human chylomicrons and very low density lipoproteins compared with the 9-*cis* geometrical isomer. *J Nutr* **125**, 2128–2133.
40. Ben-Amotz A & Levy Y (1996) Bioavailability of a natural isomer mixture compared with synthetic all-*trans* β-carotene in human serum. *Am J Clin Nutr* **63**, 729–734.
41. You CS, Parker RS, Goodman KJ, *et al.* (1996) Evidence of *cis–trans* isomerization of 9-*cis*-β-carotene during absorption in humans. *Am J Clin Nutr* **64**, 177–183.
42. Nagao A & Olson JA (1994) Enzymatic formation of 9-*cis*, 13-*cis*, and all-*trans* retinals from isomers of β-carotene. *FASEB J* **8**, 968–973.
43. Mills JP, Simon PW & Tanumihardjo SA (2007) β-Carotene from red carrots maintains vitamin A status, but lycopene bioavailability is lower relative to tomato paste in Mongolian gerbils. *J Nutr* **137**, 1395–1400.
44. Arscott SA, Howe JA, Davis CR, *et al.* (2010) Carotenoid profiles in provitamin A-containing fruits and vegetables affect the bioefficacy in Mongolian gerbils. *Exp Biol Med* **235**, 839–848.
45. La Frano MR, Woodhouse LR, Burnett DJ, *et al.* (2012) Biofortified cassava increases β-carotene and vitamin A concentrations in the TAG-rich plasma layer of American women. *Br J Nutr* **110**, 310–320.
46. Tanumihardjo SA, Palacios N & Pixley KV (2010) Provitamin A carotenoid bioavailability: what really matters? *Int J Vitam Nutr Res* **80**, 336–350.
47. Rock CL, Lovalvo JL, Emenhiser C, *et al.* (1998) Bioavailability of β-carotene is lower in raw than in processed carrots and spinach in women. *J Nutr* **128**, 913–916.
48. Bresnahan KA, Arscott SA, Khanna H, *et al.* (2012) Cooking enhances but the degree of ripeness does not affect provitamin A carotenoid bioavailability from bananas in Mongolian gerbils. *J Nutr* **142**, 2097–2104.
49. Dosti MP, Mills JP, Simon PW, *et al.* (2006) Bioavailability of β-carotene (βC) from purple carrots is the same as typical orange carrots while high-βC carrots increase βC stores in Mongolian gerbils (*Meriones unguiculatus*). *Br J Nutr* **96**, 258–267.

British Journal of Nutrition

CHAPTER 3

Cooking Enhances but the Degree of Ripeness Does Not Affect Provitamin A Carotenoid Bioavailability from Bananas in Mongolian Gerbils

Kara A. Bresnahan[1], Sara A. Arscott[1], Harjeet Khanna[2], Geofrey Arinaitwe[3], James Dale[2], Wilberforce Tushemereirwe[3], Stephanie Mondloch[1], Jacob P. Tanumihardjo[1], Fabiana F. De Moura[4], and Sherry A. Tanumihardjo[1]

[1]Indepartmental Graduate Program in Nutritional Sciences, University of Wisconsin, Madison, WI 53706, USA

[2]Center for Tropical Crops and Biocommodities, Science and Engineering Faculty, Queensland University of Technology, Brisbane, Queensland, Australia

[3]National Agriculture Research Laboratories, National Agricultural Research Organization, Kawanda, Kampala, Uganda

[4]HarvestPlus, c/o International Food Policy Research Institute, Washington, DC, USA

This manuscript is published in the *Journal of Nutrition* ©2012

The Journal of Nutrition. First published ahead of print October 24, 2012 as doi: 10.3945/jn.112.167544.

The Journal of Nutrition
Nutrient Physiology, Metabolism, and Nutrient-Nutrient Interactions

Cooking Enhances but the Degree of Ripeness Does Not Affect Provitamin A Carotenoid Bioavailability from Bananas in Mongolian Gerbils[1–4]

Kara A. Bresnahan,[5] Sara A. Arscott,[5] Harjeet Khanna,[6] Geofrey Arinaitwe,[7] James Dale,[6] Wilberforce Tushemereirwe,[7] Stephanie Mondloch,[5] Jacob P. Tanumihardjo,[5] Fabiana F. De Moura,[8] and Sherry A. Tanumihardjo[5]*

[5]Interdepartmental Graduate Program in Nutritional Sciences, University of Wisconsin, Madison, WI; [6]Centre for Tropical Crops and Biocommodities, Science and Engineering Faculty, Queensland University of Technology, Brisbane, Queensland, Australia; [7]National Agriculture Research Laboratories, National Agricultural Research Organization, Kawanda, Kampala, Uganda; and [8]HarvestPlus, c/o International Food Policy Research Institute, Washington, DC

Abstract

Banana is a staple crop in many regions where vitamin A deficiency is prevalent, making it a target for provitamin A biofortification. However, matrix effects may limit provitamin A bioavailability from bananas. The retinol bioefficacies of unripe and ripe bananas (study 1A), unripe high-provitamin A bananas (study 1B), and raw and cooked bananas (study 2) were determined in retinol-depleted Mongolian gerbils (n = 97/study) using positive and negative controls. After feeding a retinol-deficient diet for 6 and 4 wk in studies 1 and 2, respectively, customized diets containing 60, 30, or 15% banana were fed for 17 and 13 d, respectively. In study 1A, the hepatic retinol of the 60% ripe Cavendish group (0.52 ± 0.13 μmol retinol/liver) differed from baseline (0.65 ± 0.15 μmol retinol/liver) and was higher than the negative control group (0.39 ± 0.16 μmol retinol/liver; $P < 0.0065$). In study 1B, no groups differed from baseline (0.65 ± 0.15 μmol retinol/liver; $P = 0.20$). In study 2, the 60% raw Butobe group (0.68 ± 0.17 μmol retinol/liver) differed from the 60% cooked Butobe group (0.87 ± 0.24 μmol retinol/liver); neither group differed from baseline (0.80 ± 0.27 μmol retinol/liver; $P < 0.0001$). Total liver retinol was higher in the groups fed cooked bananas than in those fed raw ($P = 0.0027$). Body weights did not differ even though gerbils ate more green, ripe, and raw bananas than cooked, suggesting a greater indigestible component. In conclusion, thermal processing, but not ripening, improves the retinol bioefficacy of bananas. Food matrix modification affects carotenoid bioavailability from provitamin A biofortification targets. J. Nutr. doi: 10.3945/jn.112.167544.

Introduction

Banana is a staple crop in many tropical and subtropical regions where vitamin A (VA)[9] deficiency (VAD) is present (1,2). Biofortification of staple crops with micronutrients is an agricultural approach to improve human health (3). Provitamin A enhancement of bananas may provide a novel and sustainable approach to alleviate VAD in several populations and should be considered a prime biofortification target, because >90% of bananas harvested are consumed locally in many countries (4,5). Recent research has located several high-provitamin A bananas to use in traditional and genetically modified biofortification efforts (6–9).

When assessing the impact of biofortification, it is essential to elucidate matrix effects on provitamin A bioavailability. For example, bananas contain considerable amounts of resistant starch, an indigestible matrix component that is thought to limit bioavailability (3,10,11). In human small intestine, raw banana starch was >80% resistant to digestion (12,13). Ripening and thermal processing reduce resistant starch concentrations and may positively affect bioavailability (14,15).

In a recent study assessing the bioefficacies of provitamin A-equalized fruit diets, processed banana flour was less effective than other fruits in maintaining the liver reserves of Mongolian gerbils (16). The bioconversion factor for the banana diet was

[1] Supported by the Grand Challenges in Global Health (no. 9), an initiative of the Bill and Melinda Gates Foundation through a grant to the International Food Policy Research Institute; also funded by USDA Hatch WIS01528.
[2] Presented at Experimental Biology 2011.
[3] Author disclosures: K. A. Bresnahan, S. A. Arscott, H. Khanna, G. Arinaitwe, J. Dale, W. Tushemereirwe, S. Mondloch, J. P. Tanumihardjo, F. F. De Moura, and S. A. Tanumihardjo, no conflicts of interest.
[4] Supplemental Table 1 is available from the ''Online Supporting Material'' link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
[9] Abbreviations used: BC, Butobe cooked; βCE, β-carotene equivalent; BR, Butobe raw; CB, control banana; CG, Cavendish green; CR, Cavendish ripe; KG, Kluai Khai Bong green; KK, Kluai Khai; LG, Lady Finger green; LR, Lady Finger ripe; MC, M9 cooked; MR, M9 raw; NC, Nakinyika cooked; NR, Nakinyika raw; SN, Sukali Ndizi; VA, vitamin A; VA+, positive control; VA−, negative control; VAD, vitamin A deficiency.
* To whom correspondence should be addressed. E-mail: sherry@nutrisci.wisc.edu.

Manuscript received July 30, 2012. Initial review completed August 21, 2012. Revision accepted September 27, 2012.
doi: 10.3945/jn.112.167544.

much higher than for the other fruits, implying that a higher provitamin A concentration would be needed to support adequate VA status (16). To determine matrix effects, we investigated provitamin A carotenoid bioefficacy in unripe compared with ripe bananas, naturally occurring high-provitamin A bananas, and raw compared with cooked bananas. We hypothesized that ripening and thermal processing would decrease resistant starch and increase VA bioefficacy. Mongolian gerbils are capable of metabolizing provitamin A carotenoids in a manner similar to humans (17) and were therefore used as the model.

Materials and Methods

Banana preparation. In study 1, bananas were from Queensland DPI South Johnstone Research Station in Australia: Cavendish, Lady Finger, and Kluai Khai (KK) Bong. In study 2, bananas were from the National Banana Research Program Germplasm Collection at Kwanda, Uganda: Butobe, Nakinyika, Hybrid M9, and Sukali Ndizi (SN). An exception was the banana used as control in study 1, which was procured locally. None of the bananas were biofortified but were typical cultivars.

Bananas were harvested at the same time and level of ripeness. Nakinyika, Hybrid M9, and SN were selected because they are biofortification targets, and Cavendish and Lady Finger were selected because they are being used for development of biofortification technology within the Bill and Melinda Gates Foundation's program for Uganda. Cultivars Butobe and KK Bong were included, because they have relatively high carotenoid levels. Bananas were harvested at the full-green stage, which is ~15 wk after bunch emergence when the fruits develop round edges. Green bananas are typically used to make "matooke" (steamed in banana leaves) and are used in flour production (18). Cavendish and Lady Finger were ripened for 1 wk, which is when they are normally consumed fresh. Ripeness was determined by the peel turning yellow. Peels were removed by hand and fingers were sliced into pieces and freeze-dried for 1 wk in a Cuddon 1015 Floral Dryer. Samples were shipped to the U.S. the day after freeze-drying. Upon arrival, bananas were further freeze-dried, processed into flour, and stored at −70°C until mixed into feeds.

Bunches for all cooking bananas (i.e., Butobe, Nakinyika, Hybrid M9) were harvested at the same time in Uganda. Eighteen hours after harvest, bananas were peeled, divided into 2 samples (steaming and raw), sliced into diagonal pieces, wrapped in banana leaves, and placed in stainless-steel pans. After covering with more leaves, they were steamed for 1 h using firewood (as done traditionally), allowed to cool, and placed in containers for storage at −20°C. For SN bananas (usually eaten ripe), the peels were removed and the bananas were sliced into pieces and stored at −20°C. Samples were frozen for 1 mo, freeze-dried for 3 d, ground, and sealed in airtight containers at −20°C until shipped.

Study design. Male 34- to 40-d-old Mongolian gerbils (n = 97/study; Charles River Laboratories) were group housed (n = 3/cage) during depletion and individually housed during treatment. Room temperature and humidity were held constant (12-h-light/-dark cycle). Gerbils were acclimated to the procedures by administering 40 μL/d cottonseed oil with a positive displacement pipette. Gerbils were weighed daily until all were thriving and were thereafter weighed every other day. All animal handling procedures were approved by the College of Agriculture and Life Sciences Animal Care and Use Committee at the University of Wisconsin-Madison.

Gerbils consumed ad libitum VA- and carotenoid-free, semipurified, powdered feed. In consultation with a feed nutritionist (Harlan-Teklad), we designed the feeds to provide isoenergetic and isonitrogenous diets. The feeds were stored at −20°C (**Supplemental Table 1**). Macronutrients and fiber (USDA Nutrient Database) were equalized depending on percent banana by adjusting casein, sucrose, oil, and cellulose. After depletion (study 1, 6 wk; study 2, 4 wk), a baseline group (n = 7) was killed via exsanguination while under isoflurane anesthesia to determine the initial serum and liver concentrations. The remaining gerbils were sorted into weight-matched groups (n = 10/group) and fed customized diets with 60, 30, or 15% banana. Following a 17-d (study 1A and B) or 13-d (study 2) treatment, the gerbils were killed (**Fig. 1**). Blood was centrifuged for 15 min at 2200 × g in Vacutainer tubes (Becton Dickinson). Livers were excised and stored at −70°C.

To assess the effect of ripening, study 1A compared the retinol bioefficacy of green (unripe) and ripe dessert bananas and study 1B compared unripe dessert bananas with unripe, high-carotenoid bananas at a lower diet percentage. Study 2 compared the retinol bioefficacy of raw compared with cooked bananas. Both studies employed positive (VA+) and negative (VA−) control groups, which were fed VA-free basal feed. The VA+ group was given a daily dose of retinyl acetate dissolved in cottonseed oil. Study 1 also included a VA-free customized diet with low-carotenoid bananas [control banana (CB)] and a daily retinyl acetate dose that matched the VA+ group. All other treatment groups received matched oil doses. The daily theoretical retinol intake was calculated based on mean feed consumption, measured daily, with the assumption of 100% bioefficacy (i.e., 1 mol β-carotene provides 2 mol retinol and 1 mol α-carotene provides 1 mol retinol). In study 1, one-half of the previous day's mean theoretical retinol intake based on the intakes of the 60% Lady Finger [ripe (LR) and green (LG)] and 60% Cavendish [ripe (CR) and green (CG)] groups was used to determine the retinyl acetate dose administered to the VA+ and CB groups. In study 2, because of the large variations in provitamin A content, an intermediate dose was used

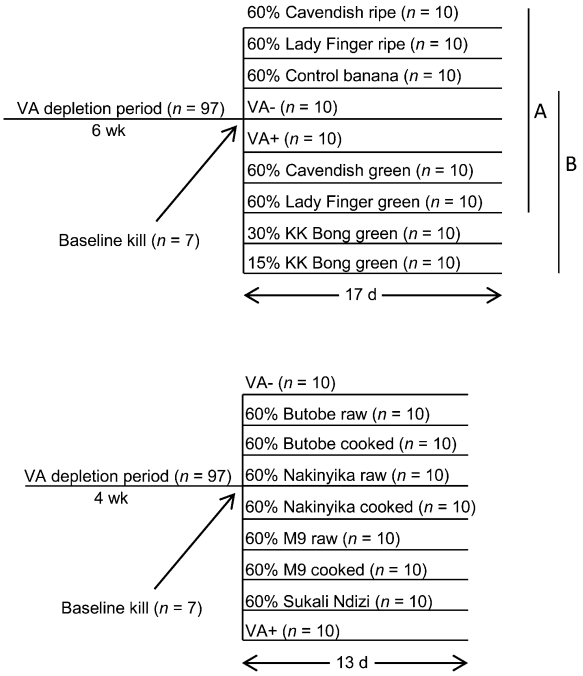

FIGURE 1 Experimental timeline for 3 studies that determined VA bioefficacy of provitamin A carotenoids from bananas. After a retinol depletion period, treatments included VA− and carotenoid-free diets with negative and positive controls (VA− and VA+); 60% CB with VA supplement (study 1A); 60% CR (study 1A) and CG (studies 1A and 1B); 60% LR (study 1A) and LG (studies 1A and 1B); and 30% and 15% KG (study 1B). In addition to controls, study 2 included 60% BR or BC; 60% NR or NC; 60% MR or MC; and 60% SN raw. Retinyl acetate doses for the VA+ and control banana groups were matched to one-half of the mean theoretical intake of the banana diets in study 1 and a mid-level in study 2. All treatments included daily doses of cottonseed oil with the volume matched to the VA+ oil intake. BC, Butobe cooked; BR, Butobe raw; CB, control banana; CG, Cavendish green; CR, Cavendish ripe; KG, Kluai Khai Bong green; LG, Lady Finger green; LR, Lady Finger ripe; MC, M9 cooked; MR, M9 raw; NC, Nakinyika cooked; NR, Nakinyika raw; SN, Sukali Ndizi; VA, vitamin A.

based on the intakes of the 60% Butobe [cooked (BC) and raw (BR)], Nakinyika [cooked (NC) and raw (NR)], and M9 [cooked (MC) and raw (MR)] groups.

Diet analyses. Diets were analyzed weekly in triplicate for carotenoids to assess degradation and correct theoretical retinol values (19). Samples (0.4 g) were ground with a mortar and pestle, saponified with 250 μL 50% KOH in water (wt:v), kept on ice to minimize degradation, and mixed with a vortex every 5 min for 15 min. Hexane extractions (3 mL) were repeated 3 times, pooled, dried under nitrogen, and reconstituted in 100 μL 50:50 methanol:dichloroethane (v:v); then 50 μL was injected into the HPLC (Waters). β-8′-*Apo*-carotenal, added postsaponification, was used as an internal standard. HPLC-purified lutein, α-carotene, and β-carotene were used for quantification. Chromatograms were generated at 450 nm.

Neutral detergent fiber and resistant starch contents were determined for all diets in studies 1 and 2 using previously published procedures (20,21).

Tissue analyses. Samples were analyzed under gold light to prevent photo-oxidation and isomerization. A modified procedure was used for analysis of 500 μL serum (22). C-23 *apo*-carotenol was the internal standard and 25 μL sample was injected. Chromatograms were generated at 325 and 450 nm.

Liver (~0.5 g) was ground with ~4 g sodium sulfate, extracted with dichloromethane, and filtered to 25 mL volume. C-23 *apo*-carotenol was the internal standard; 5 mL was dried under nitrogen and reconstituted in 100 μL 50:50 methanol:dichloroethane and 25 μL was injected. A Waters photodiode array HPLC system with a guard column and Resolve C18 column (5 μm, 3.9 × 300 mm) was used. Solvent A was 87.5:12.5 acetonitrile:H_2O (v:v) and solvent B was 70:20:10 acetonitrile:dichloroethane:methanol; both contained 10 mmol/L ammonium acetate. Gradient elution was at 1 mL/min: *1*) 15-min hold at 100% A; *2*) 10-min linear gradient to 100% B; *3*) 23-min hold at 100% B; *4*) 5-min reverse gradient to 100% A; and *5*) 10-min hold. Chromatograms were generated at 311, 325, and 450 nm. The quantification of retinol, retinyl esters, α-retinol, α-retinyl esters, lutein, α-carotene, and β-carotene was determined using HPLC-purified standards. The liver retinol concentration and total retinol reflect retinol and esters.

Stool fat analysis. In study 1, feces were randomly collected upon the observance of abnormal specimens, which occurred when the animals were switched to the high-percentage banana diets. In study 2, feces were collected twice weekly from the same gerbils across treatment groups to account for the natural variance in fat between animals (23). Samples (~0.2 g) were ground with ~1 g sodium sulfate, extracted into a 50-mL, round-bottom flask, dried using a rotary evaporator, reconstituted in 3 mL dichloromethane, and transferred to a tared test tube. The extract was dried under nitrogen, placed in a vacuum-sealed desiccator overnight, and weighed for difference.

Statistical analysis. Values are means ± SD. Data were analyzed using SAS software (SAS Institute, 8.2). Outcomes of interest were evaluated using 1-way and 2-way ANOVA or *t* tests. Differences between groups were determined using least significant differences at $\alpha < 0.05$. We calculated β-Carotene equivalents (βCEs) to retinol bioconversion factors using differences in total liver retinol equivalents in treatment groups compared directly with the VA+ group after correction for the control group. A correction factor was utilized for the difference in theoretical retinol and feed intakes between groups (22).

Results

Carotenoid, fiber, and resistant starch concentrations. In study 1, α- and β-carotene concentrations were highest for KK Bong green (KG) followed by LR, CR, CG, LG, and CB. In study 2, α- and β-carotene concentrations were highest in BR followed by NC, BC, NR, MR, MC, and SN (**Table 1**). The theoretical retinol based on 100% bioefficacy of all banana diets, with the exception of CB and SN (**Table 2**), exceeded gerbil requirements (2.45 nmol retinol/g diet) (24). The theoretical retinol intake was calculated as retinol in feed multiplied by daily intake.

Fiber content did not differ in study 1 ($P = 0.89$). In study 2, the 60% MR and SN diets contained lower fiber than the VA-free diet ($P < 0.0068$) (Table 2) and cooking increased fiber ($P = 0.0137$). Resistant starch concentrations differed in all studies ($P \leq 0.0007$) (Table 2) and none was detected in the VA-free diet. In study 1A, ripening decreased resistant starch ($P < 0.0001$). In study 1B, resistant starch concentrations were highest in the 60% LG diet followed by the 60% CG and 30% KG diets. In study 2, the cooked banana diets had lower resistant starch than the matched raw diets; cultivar, cooking, and an interaction between these variables affected resistant starch (all $P < 0.0001$).

Weights, intakes, and stool fat. Gerbil weights did not differ among treatments within studies (study 1A, 71.9 ± 1.4 g, $P = 0.23$; study 1B, 72.7 ± 1.6 g, $P = 0.36$; study 2, 66.1 ± 1.0 g, $P = 0.89$). Final liver weights differed in study 1A ($P = 0.0042$) and study 2 ($P = 0.0334$). In study 1A, the 60% CB group liver weight (2.77 ± 0.39 g) was higher than the VA−, 60% CG, 60% LR, and 60% LG groups (2.25 ± 0.31 to 2.40 ± 0.22 g). In study 2, liver weights were higher for the VA−, 60% BC, 60% SN, and VA+ groups (2.72 ± 0.35 to 3.02 ± 0.04 g) but did not differ in study 1B (2.42 ± 0.38 g; $P = 0.09$). Feed intake differed among groups within studies (study 1A, $P = 0.0003$; study 1B, $P = 0.0007$; study 2, $P < 0.0001$) (Table 2). Feed intake was higher in study 1A in the 60% CR, 60% CG, 60% LG, and 60% CB groups; in the 60% CG and 60% LG groups in study 1B; and in the 60% BR, 60% NR, and 60% MR groups in study 2. Daily theoretical retinol intake differed among treatments (all $P < 0.0001$) (**Table 3**). Stool fat concentrations did not differ within

TABLE 1 Carotenoid concentrations in freeze-dried components used to prepare Mongolian gerbil feeds in provitamin A carotenoid bioefficacy studies[1]

Component	Lutein	α-Carotene	β-Carotene[2]
		nmol/g	
Study 1			
CR	4.91 ± 0.24	5.41 ± 0.25	1.66 ± 0.11
CG	3.58 ± 0.02	5.04 ± 0.52	1.68 ± 0.19
LR	3.96 ± 0.01	7.78 ± 0.24	3.60 ± 0.12
LG	2.54 ± 0.19	3.65 ± 0.04	2.13 ± 0.08
KG	2.27 ± 0.05	21.7 ± 0.72	21.3 ± 0.67
CB	1.88 ± 0.14	1.33 ± 0.08	0.68 ± 0.03
Study 2			
BR	0.0061 ± 0.0008	4.55 ± 0.10	8.46 ± 1.29
BC	0.014 ± 0.007	3.17 ± 0.14	2.10 ± 0.60
NR	0.0030 ± 0.0009	3.30 ± 0.20	1.58 ± 0.32
NC	0.020 ± 0.002	3.64 ± 0.14	3.38 ± 0.31
MR	0.00078 ± 0.00004	1.23 ± 0.11	2.38 ± 0.35
MC	0.022 ± 0.003	1.67 ± 0.02	1.65 ± 0.17
SN	0.029 ± 0.004	0.24 ± 0.003	0.47 ± 0.26

[1] Values are means ± SD, $n \geq 3$ replicates. BC, Butobe cooked; BR, Butobe raw; CB, control banana; CG, Cavendish green; CR, Cavendish ripe; KG, Kluai Khai Bong green; LG, Lady Finger green; LR, Lady Finger ripe; MC, M9 cooked; MR, M9 raw; NC, Nakinyika cooked; NR, Nakinyika raw; SN, Sukali Ndizi.
[2] Includes all-*trans*-, 9-*cis*-, and 13-*cis*-β-carotene isomers.

TABLE 2 Provitamin A carotenoid concentrations, fiber and resistant starch content of feeds, feed intake, and theoretical VA intake for Mongolian gerbils (studies 1 and 2)[1]

Component	α-Carotene	β-Carotene	Theoretical VA	Neutral detergent fiber	Resistant starch	Feed intake	Daily theoretical VA intake[2]
	nmol/g feed			*%*		*g/d*	*nmol/d*
Study 1A							
P value				0.69	<0.0001	0.0003	<0.0001
VA−	ND[3]	ND	ND	12.5 ± 1.30	ND	6.77 ± 0.74^{c}	0
60% CR	2.02 ± 0.17	1.22 ± 0.31	4.46 ± 0.79	11.3 ± 0.37	4.65 ± 0.84^{c}	8.99 ± 1.89^{a}	37.5 ± 6.57^{b}
60% CG	1.51 ± 0.14	0.90 ± 0.36	3.31 ± 0.86	11.6 ± 1.43	8.05 ± 0.20^{b}	9.16 ± 2.66^{a}	32.5 ± 6.85^{c}
60% LR	2.49 ± 0.19	2.00 ± 0.41	6.49 ± 1.01	11.6 ± 0.37	4.88 ± 0.80^{c}	7.67 ± 1.95^{bc}	48.8 ± 8.54^{a}
60% LG	1.09 ± 0.08	1.27 ± 0.24	3.63 ± 0.56	11.6 ± 0.32	9.10 ± 0.46^{a}	8.28 ± 2.48^{ab}	29.7 ± 5.84^{c}
60% CB	0.31 ± 0.07	0.69 ± 0.12	1.69 ± 0.31	11.7 ± 0.34	1.13 ± 0.17^{d}	8.12 ± 1.58^{ab}	30.4 ± 3.10^{c}
VA+	ND	ND	ND	12.5 ± 1.30	ND	6.64 ± 0.94^{c}	19.1 ± 2.93^{d}
Study 1B							
P value				0.13	0.0007	0.0007	<0.0001
VA−	ND	ND	ND	12.5 ± 1.30	ND	6.77 ± 0.74^{c}	0
60% CG	1.51 ± 0.14	0.90 ± 0.36	3.31 ± 0.86	11.6 ± 1.43	8.05 ± 0.20^{b}	9.16 ± 2.66^{a}	32.5 ± 6.85^{c}
60% LG	1.09 ± 0.08	1.27 ± 0.24	3.63 ± 0.56	11.6 ± 0.32	9.10 ± 0.46^{a}	8.28 ± 2.48^{ab}	29.7 ± 5.84^{c}
30% KG	2.98 ± 0.74	5.40 ± 1.29	13.8 ± 3.32	12.1 ± 0.29	6.11 ± 0.66^{c}	7.61 ± 2.00^{bc}	104 ± 21.0^{a}
15% KG	1.43 ± 0.29	2.97 ± 0.6	7.37 ± 1.49	NM	NM	7.55 ± 1.84^{bc}	56.2 ± 13.3^{b}
VA+	ND	ND	ND	12.5 ± 1.30	ND	6.64 ± 0.94^{c}	19.1 ± 2.93^{d}
Study 2							
P value				0.0068	<0.0001	<0.0001	<0.0001
VA−	ND	ND	ND	12.5 ± 1.30^{a}	ND	6.73 ± 1.07^{c}	0
60% BR	4.79 ± 0.31	3.47 ± 0.20	11.7 ± 0.72	10.3 ± 2.35^{ab}	13.1 ± 0.53^{a}	9.60 ± 2.83^{ab}	112 ± 25.1^{a}
60% BC	2.75 ± 0.56	2.31 ± 0.48	7.37 ± 1.53	11.8 ± 1.92^{a}	3.37 ± 0.40^{d}	8.18 ± 0.88^{bc}	60.2 ± 11.1^{c}
60% NR	3.56 ± 0.35	2.72 ± 0.25	9.00 ± 0.85	10.4 ± 0.67^{ab}	10.8 ± 2.03^{b}	9.71 ± 3.04^{ab}	86.7 ± 14.9^{b}
60% NC	3.28 ± 0.48	2.78 ± 0.40	8.83 ± 1.29	12.9 ± 1.78^{a}	0.57 ± 0.21^{e}	6.69 ± 1.69^{c}	59.0 ± 12.1^{c}
60% MR	1.25 ± 0.03	0.86 ± 0.01	2.97 ± 0.05	7.38 ± 0.32^{b}	4.77 ± 0.60^{c}	10.5 ± 2.98^{a}	31.1 ± 6.24^{d}
60% MC	1.40 ± 0.35	0.89 ± 0.27	3.17 ± 0.89	12.0 ± 0.16^{a}	3.36 ± 0.36^{d}	6.79 ± 2.25^{c}	22.1 ± 7.66^{d}
60% SN	0.26 ± 0.025	0.32 ± 0.00	0.90 ± 0.03	7.59 ± 1.13^{b}	0.19 ± 0.10^{e}	8.87 ± 3.29^{c}	7.99 ± 2.93^{d}
VA+	ND	ND	ND	12.5 ± 1.30^{a}	ND	6.41 ± 1.63^{c}	11.6 ± 1.80^{d}

[1] Values are means ± SD, $n \geq 3$ replicates (diet analysis) and $n = 5$ (feed intake and daily theoretical VA intake). Means in a row with superscripts without a common letter differ, $P < 0.05$. BC, Butobe cooked; BR, Butobe raw; CB, control banana; CG, Cavendish green; CR, Cavendish ripe; KG, Kluai Khai Bong green; LG, Lady Finger green; LR, Lady Finger ripe; MC, M9 cooked; MR, M9 raw; NC, Nakinyika cooked; ND, not detected; NM, not measured; NR, Nakinyika raw; SN, Sukali Ndizi; VA, vitamin A; VA−, negative control; VA+, positive control.

[2] Daily theoretical retinol intake assumes 100% bioefficacy (i.e., 1 mol β-carotene provides 2 mol retinol and 1 mol α-carotene provides 1 mol retinol). This calculation accounts for losses in provitamin A concentrations over time.

[3] The limit of detection for carotenoids is <0.007 nmol and that for resistant starch is 0.08%.

studies (study 1, 10.9 ± 7.6 mg fat/g stool, $P = 0.73$; study 2, 9.90 ± 5.46 mg fat/g stool, $P = 0.55$).

Tissue concentrations. Serum retinol concentrations did not differ among groups (study 1, $P = 0.35$; study 2, $P = 0.43$). In study 1, serum retinol ranged from 1.53 ± 0.40 μmol/L in the 60% CG group to 2.06 ± 0.40 μmol/L in the 15% KG group. In study 2, serum retinol ranged from 0.97 ± 0.16 μmol/L in the 60% NR group to 1.14 ± 0.16 μmol/L in the 60% NC group. Retinyl esters and carotenoids were not detected.

In study 1A, liver retinol concentrations did not differ from baseline (0.26 ± 0.07 μmol retinol/g liver; $P = 0.07$) for any group. For total retinol, the groups that differed from baseline (0.65 ± 0.15 μmol retinol/liver; $P = 0.0065$) were 60% CR (0.52 ± 0.13 μmol retinol/liver) and VA− (0.39 ± 0.16 μmol retinol/liver) (**Fig. 2***A*). The bioconversion of α-carotene was confirmed by quantifying α-retinol. α-Retinol concentrations were higher in the ripe banana groups than green banana groups ($P < 0.0001$); ripeness ($P < 0.0001$) and an interaction between cultivar and ripeness ($P = 0.0281$) affected α-retinol concentration (Fig. 2*B*). In study 1B, the retinol concentration and total hepatic levels did not differ among groups ($P = 0.06$; $P = 0.20$) (**Fig. 3***A*). Hepatic α-retinol was higher in the 30% and 15% KG groups ($P < 0.0001$) (Fig. 3*B*). α-Retinol was correlated with the α-carotene content of the diet ($r = 0.86$).

Liver provitamin A carotenoids reflected those in the diets. α-Carotene ranged from 1.84 ± 1.03 nmol/liver in the 60% CB group to 10.6 ± 4.6 nmol/liver in the 60% LR group in study 1A and from 1.84 ± 1.03 nmol/liver in the 60% CB group to 11.9 ± 6.1 nmol/liver in the 30% KG group in study 1B. β-Carotene was highest in the 60% LG group (0.90 ± 0.80 nmol/liver) in study 1A and in the 30% KG group (10.4 ± 9.8 nmol/liver) in study 1B.

In study 2, all groups maintained a baseline concentration and total retinol (0.24 ± 0.08 μmol retinol/g liver; 0.80 ± 0.27 μmol retinol/liver; both $P < 0.0001$) except the VA−, VA+, and 60% SN groups, which did not differ from each other (0.14 ± 0.06 to 0.16 ± 0.06 μmol retinol/g liver) (**Fig. 4***A*). Interestingly, liver retinol in the VA+ group did not differ from the VA− group on a concentration or hepatic level, which indicates differential utilization rates (25). For both concentration and total retinol, the 60% BR group (0.20 ± 0.05 μmol retinol/g liver; 0.68 ± 0.17 μmol retinol/liver) was lower than the 60% BC group (0.26 ± 0.07 μmol retinol/g liver; 0.87 ± 0.24 μmol retinol/liver) (Fig. 4*A*).

TABLE 3 Bioconversion factors for provitamin A carotenoid equivalents (βCE) from banana diets fed to Mongolian gerbils (studies 1 and 2)[1]

	Conversion factor	
	μg βCE to μg retinol	μmol βCE to μmol retinol
Study 1		
60% CR	2.3	1.2
60% CG	1.3	0.7
60% LR	2.1	1.1
60% LG	1.7	0.9
30% KG	3.0	1.6
15% KG	2.3	1.2
Study 2		
60% BR	4.1	2.2
60% BC	1.1	0.6
60% NR	2.4	1.3
60% NC	1.1	0.6
60% MR	1.0	0.5
60% MC	0.5	0.2
60% SN	0.9	0.5

[1] βCE is equivalent to 1 μg β-carotene and 2 μg α-carotene. BC, Butobe cooked; βCE, β-carotene equivalent; BR, Butobe raw; CG, Cavendish green; CR, Cavendish ripe; KG, Kluai Khai Bong green; LG, Lady Finger green; LR, Lady Finger ripe; MC, M9 cooked; MR, M9 raw; NC, Nakinyika cooked; NR, Nakinyika raw; SN, Sukali Ndizi.

An effect of cooking was found for concentration and total retinol (both $P = 0.0036$). A t test comparing total hepatic retinol of all cooked compared with raw banana treatments showed that cooking enhanced retinol stores ($P = 0.0027$).

Hepatic α-retinol concentrations were higher in the cooked groups than in their raw compliments ($P < 0.0001$) (Fig. 4*B*), and α-retinol in the cooked banana groups' livers had a strong correlation with α-carotene content in the feed ($r = 0.98$). An effect of cooking ($P < 0.0001$) and cultivar ($P = 0.0383$) on hepatic α-retinol concentrations was observed. α-Carotene ranged from 0.18 ± 0.30 nmol/liver in the 60% SN group to 19.4 ± 12.5 nmol/liver in the 60% BC group; β-carotene ranged from 3.0 ± 2.5 nmol/liver in the 60% NR group to 11.1 ± 10.6 nmol/liver in the 60% BC group. No α-retinol or carotenoids were detected in the baseline, VA−, or VA+ groups.

Bioconversion factors. Due to study design based on the percentage of banana, the retinol intake of the banana groups varied from the daily retinol intake administered by the supplements and therefore adjustments were made based on intake differences. Bioconversion factors in study 1 varied from 1.3 to 3.0 μg βCE to 1 μg retinol and from 0.5 to 4.1 μg βCE to 1 μg retinol in study 2 (Table 3).

Discussion

Provitamin A biofortification of bananas may provide a sustainable alternative to periodic retinyl ester supplements in countries where banana is a staple. In these studies, the retinol bioefficacy of green and ripe dessert bananas, green high-provitamin A bananas, and raw and cooked bananas was directly compared with VA supplements. In the LG, LR, CG, and KG diets at 2 levels (15 and 30%), the BC, NR, NC, MR, and MC bananas maintained liver retinol stores. With few exceptions, these bananas prevented VAD in gerbils. Additionally, the conversion factors for all banana types were efficient (i.e., 0.5–4.1 μg βCE to 1 μg retinol). Theoretically, the lowest conversion factor is ~0.9 μg βCE to 1 μg retinol based on the addition of oxygen to the hydrocarbon. Only 1 of the 13 banana preparations was below this value, which may indicate that these gerbils were using the α-retinol formed for partial VA activity (26), their utilization rate had slowed (25), or that they were recycling retinol at a faster rate (27) than other groups.

The primary retinol storage site is the liver, which is considered a gold standard (28). Deficiency was recently defined as reserves <0.1 μmol retinol/g liver, at which point animals begin to store more retinol as esters and below which *apo*-retinol binding protein begins to accumulate (28); the VA− groups in both studies were nearing deficiency. In study 1, all groups were able to prevent deficiency and in study 2 all groups except SN prevented the degree of depletion in the VA− group. In study 1, CR maintained the baseline retinol concentration but not the hepatic retinol, suggesting lower bioavailability. Additionally, conversion factors were numerically higher in ripe banana compared with green, contradicting the hypothesis that ripening would improve bioavailability. The ripening process results in the breakdown of insoluble starches, thought to limit provitamin A bioavailability, to soluble sugars (3,29–32). Green banana accounts for ~80% of banana intake in Uganda (F.F. De Moura, unpublished results).

Like fiber, raw, uncooked banana starch is resistant to enzyme-catalyzed degradation during digestion, which limits micellarization and carotenoid absorption in the small intestines

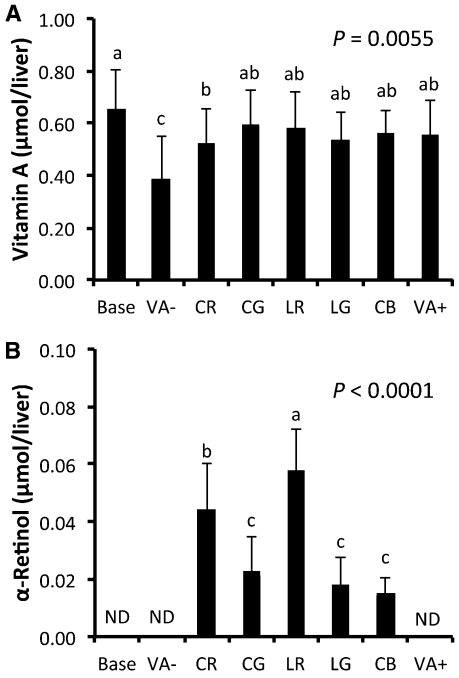

FIGURE 2 Total liver VA (*A*) and α-retinol (*B*) in Mongolian gerbils at baseline after 6 wk of being fed a VA-depleted diet (Base, $n = 7$); fed VA and carotenoid-free control diet (VA−); fed VA and carotenoid-free diet containing 60% CR or CG, or LR or LG; fed 60% CB and supplemented with VA as retinyl acetate in oil; or fed a VA and carotenoid-free diet supplemented with VA as retinyl acetate in oil (VA+) for 17 additional days (study 1A). VA intake in the VA group was matched to one-half of the mean theoretical intake of the CR and CG groups and the LR and LG groups. Values are means ± SD, $n = 10$. Means without a common letter differ, $P < 0.05$. The detection limits for retinol and α-retinol were 0.0012 and 0.0007 μg, respectively. CB, control banana; CG, Cavendish green; CR, Cavendish ripe; LG, Lady Finger green; LR, Lady Finger ripe; ND, not detected; VA, vitamin A; VA−, negative control; VA+, positive control.

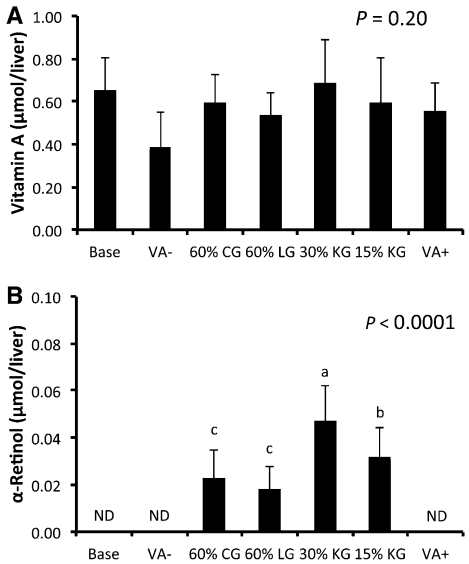

FIGURE 3 Total liver VA (*A*) and α-retinol (*B*) in Mongolian gerbils at baseline after 6 wk of being fed a VA-depleted diet (Base, $n = 7$); fed VA and carotenoid-free control diet (VA−); fed VA and carotenoid-free diet containing 60% CG, 60% LG, or 30 or 15% KG; or fed a VA and carotenoid-free diet supplemented with VA as retinyl acetate in oil (VA+) for 17 additional days (study 1B). Values are means ± SD, $n = 10$. Means without a common letter differ, $P < 0.05$. The detection limits for retinol and α-retinol were 0.0012 and 0.0007 μg, respectively. CG, Cavendish green; KG, Kluai Khai Bong green; LG, Lady Finger green; ND, not detected; VA, vitamin A; VA−, negative control; VA+, positive control.

through the binding of bile acids and lipids (33–35). Little information is available on the effects of freeze-drying on the banana matrix. However, freeze-drying increases resistant starch concentrations in potato (36). Dehydration is a common method to preserve bananas in Africa, Latin American, and Asia, where banana flour is used in a variety of foods (18). When preservation of provitamin A carotenoids is paramount, freeze-drying is preferential to heat-drying to prevent degradation (37). The resistant starch concentration of the CR treatment was lower than that of CG, but the hepatic retinol concentration of the CG treatment did not differ from baseline. The LG and LR treatments did not differ at the retinol hepatic or concentration level, but the ripe treatment had a lower resistant starch concentration. This is particularly of interest, because both the CR and LR treatments provided a greater daily theoretical retinol intake compared with the green treatments. Fiber concentrations did not differ between treatments and the resistant starch reduction in ripe bananas did not appear to improve provitamin A bioavailability. Longer term studies to elucidate resistant starch effects might include unripe and ripe banana paired-feeding with equalized resistant starch.

In study 1B, all treatments maintained baseline retinol. Gerbils receiving the 15 and 30% KG treatments consumed ~2–3 times higher daily theoretical retinol compared with those fed the CG and LG treatments, which were fed at 60%. Thus, a lower dietary intake from a higher provitamin A-containing banana can maintain VA stores and complementary consumption of biofortified banana may be effective.

In study 2, the bioconversion factors for cooked bananas were at least twice as efficient as raw bananas. The BC treatment was more effective than BR; thus, cooking positively affected provitamin A bioavailability, suggesting thermal processing improves bioavailability from bananas. The lack of difference observed between the VA− and VA+ groups suggests that the VA+ group had a higher retinol utilization rate compared with the VA− and baseline groups as noted previously when VA supplements were administered (25).

Thermal processing increases starch solubility at temperatures >60°C; however, thermal processing can also result in the generation of thermal-resistant starch in bananas (15,38). The impact of specific types of resistant starch on provitamin A bioavailability has yet to be determined. Muranga et al. (39) found that mice fed cooked starch consumed less and were better able to maintain body mass than mice fed raw banana starch, suggesting that the raw starch contained a greater indigestible fraction. A similar intake pattern was observed in studies 1A and 2, when gerbils fed the green or raw banana feeds had higher intakes than gerbils fed the ripe or cooked feeds but equal body weights. This suggests that the green and raw banana feeds had a greater indigestible component compared with the ripe and cooked feeds, which increased intake to compensate for the reduced energy availability. The observation of indigestible components in the banana matrix is also supported by the intake patterns in study 1B, because diets with greater proportions of banana also had higher feed intakes. Stool fat content was used to investigate matrix effects on fat absorption as a possible

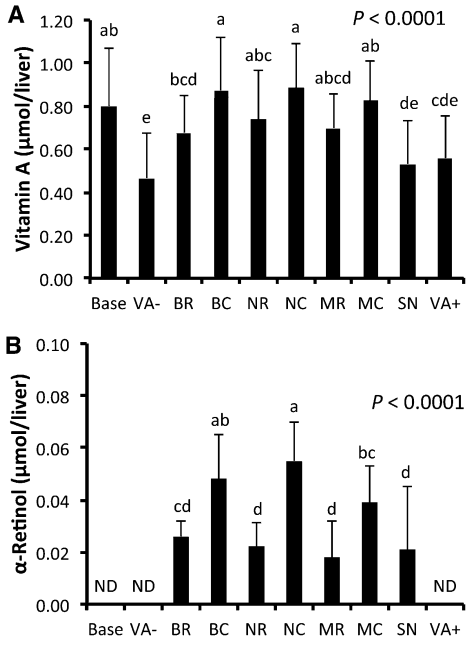

FIGURE 4 Total liver VA (*A*) and α-retinol (*B*) in Mongolian gerbils at baseline after 4 wk of being fed a VA-depleted diet (Base, $n = 7$); fed VA and carotenoid-free control diet (VA−); fed VA and carotenoid-free diet containing 60% BR or BC, NR or NC, MR or MC, or SN raw banana; or fed VA and carotenoid-free diet supplemented with vitamin A as retinyl acetate in oil (VA+) for 13 additional days (study 2). Diets were equalized to contain 60% banana. VA intake in the VA group was an intermediate-dose amount, because there was variance among the cultivars and processing methods. Values are means ± SD, $n = 10$. Means without a common letter differ, $P < 0.05$. The detection limits for retinol and α-retinol were 0.0012 and 0.0007 μg, respectively. BC, Butobe cooked; BR, Butobe raw; MC, M9 cooked; MR, M9 raw; NC, Nakinyika cooked; ND, not detected; NR, Nakinyika raw; SN, Sukali Ndizi; VA−, negative control; VA+, positive control.

explanation for feed intake differences. Fiber and resistant starch have previously demonstrated increased fecal content of bile acids and lipids in rats (40,41), but fecal fat did not differ within these studies. Due to the high variability of fecal fat content, more precise methodology may be required to determine differences.

High-provitamin A bananas contain α-carotene ranging from 0.20 to 28 nmol/g banana (6,7,42,43). α-Carotene concentrations in this experiment ranged from 0.26 to 4.79 nmol/g feed and bioconversion was demonstrated with hepatic α-retinol concentrations ranging from 15 to 55 nmol/liver. This finding supports previous work that demonstrated twice the amount of α-carotene was as effective as β-carotene in maintaining the VA status of gerbils (22), proving α-carotene to be an important contributor to retinol stores.

In summary, all bananas prevented retinol depletion, with the exception of SN. This study demonstrated the bioavailability of α- and β-carotene from bananas; however, limiting matrix effects were observed that were decreased through cooking. Thus, banana, particularly cooked, can be an effective provitamin A biofortification target used as part of a food-based intervention to alleviate VAD. The selection of appropriate banana cultivars and education on preparation techniques may support the success of biofortification efforts, because carotenoid concentrations, matrix effects, and thermal processing affect bioavailability. More research is warranted to fully understand the impact of resistant starch and other matrix effects on provitamin A carotenoid bioavailability from foods.

Acknowledgments

The authors thank Peter Crump, University of Wisconsin-Madison College of Agriculture and Life Sciences Statistical Consulting Service, for statistical analysis consultation. K.B. was responsible for conducting research, tissue analysis, resistant starch and fiber analysis, input into statistical analysis, and writing the paper; S.A.A. provided assistance in gerbil procurement and data analysis; H.K. and J.D. provided bananas from Australia and organized procurement from Uganda; G.A. and W.T. prepared and shipped the bananas from Uganda; S.M. analyzed liver samples; J.P.T. performed stool analysis; H.K., F.F.D.M., and S.A.T. designed research; and S.A.T. provided input into data analysis and revised the paper. All authors read and approved the final manuscript.

Literature Cited

1. WHO. Global prevalence of vitamin A deficiency. MDIS Working Paper No. 2. Geneva: WHO; 1995.
2. Jones DR. Introduction to banana, abaca and enset. In: Jones DR, editor. Diseases of banana, abaca and enset. Wallingford (CT): CABI; 2000. p. 1–36.
3. Tanumihardjo SA, Palacios N, Pixley KV. Provitamin A carotenoid bioavailability: what really matters? Int J Vitam Nutr Res. 2010;80:336–50.
4. Frison E, Sharrock S. Banana weevil: ecology pest status and prospects for integrated control with emphasis on East Africa. In: Saini SK, editor. Proceedings of a symposium on biological control in tropical habitats: Third International Conference on Tropical Entomology. Nairobi: ICIPE; 1998. p. 49–74.
5. Tanumihardjo SA. Food-based approaches for ensuring adequate vitamin A nutrition. Compr Rev Food Sci Food Safety. 2008;7:373–81.
6. Englberger L, Aalbersberg W, Ravi P, Bonnin E, Marks GC, Fitzgerald MH, Elymore J. Further analysis on Micronesian banana, taro, breadfruit and other foods for provitamin A carotenoids and minerals. J Food Compost Anal. 2003;16:219–36.
7. Englberger L, Schierle J, Marks GC, Fitzgerald MH. Micronesian banana, taro, and other foods: newly recognized sources of provitamin A and other carotenoids. J Food Compost Anal. 2003;16:3–19.
8. Arvanitoyannis IS, Mavromatis AG, Grammatikaki-Avegli G, Sakellariou M. Banana: cultivars, biotechnological approaches and genetic transformation. Int J Food Sci Technol. 2008;43:1871–9.
9. Fungo R, Kikafunda JK, Pillay M. β-Carotene, iron and zinc content in Papua New Guinea and East African Highland Bananas. Afr J Food Agric Nutr Dev. 2010;10:2629–43.
10. Zhang P, Whistler RL, BeMiller JN, Hamaker BR. Banana starch: production, physicochemical properties, and digestibility: a review. Carbohydr Polym. 2005;59:443–58.
11. Menezes EW, Tandini CC, Tribess TB, Zuleta A, Binaghi J, Pak N, Vera G, Dan MC, Bertolini AC, Cordenunsi BR, et al. Chemical composition and nutritional value of unripe banana flour (*Musa acuminata*, var. Nanicão). Plant Foods Hum Nutr. 2011;66:231–7.
12. Faisant N, Buléon A, Colonna A, Molis C, Lartigue S, Galmiche JP, Champ M. Digestion of raw banana starch in the small intestine of healthy humans: structural features of resistant starch. Br J Nutr. 1995;73:111–23.
13. Faisant N, Gallant DJ, Bouchet B, Champ M. Banana starch breakdown in the human small intestine studied by electron microscope. Eur J Clin Nutr. 1995;49:98–104.
14. Ketiku AO. Chemical composition of unripe (green) and ripe plantain (*Musa paradisiaca*). J Sci Food Agric. 1973;24:703–7.
15. Waliszewski KN, Aparicio MA, Bello LA, Monroy JA. Changes of banana starch by chemical and physical modification. Carbohydr Polym. 2003;52:237–42.
16. Arscott SA, Howe JA, Davis CR, Tanumihardjo SA. Carotenoid profiles in provitamin A-containing fruits and vegetables affect the bioefficacy in Mongolian gerbils. Exp Biol Med (Maywood). 2010;235:839–48.
17. Lee CM, Lederman JD, Hofmann NE, Erdman JW Jr. The Mongolian gerbil (*Meriones unguiculatus*) is an appropriate animal model for evaluation of the conversion of β-carotene to vitamin A. J Nutr. 1998; 128:280–6.
18. Narayana CK, Pillay M. Postharvest processed products from banana. In: Pillay M, editor. Banana breeding progress and challenges. Boca Raton (FL): Taylor & Francis Group; 2011. p. 269–84.
19. Howe JA, Tanumihardjo SA. Carotenoid-biofortified maize maintains adequate vitamin A status in Mongolian gerbils. J Nutr. 2006;136: 2562–7.
20. Champ M. Determination of resistant starch in foods and food products: interlaboratory study. Eur J Clin Nutr. 1992;46 Suppl 2:S51–62.
21. Mertens DR. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing beakers or crucibles: collaborative study. J AOAC Int. 2002;85:1217–40.
22. Tanumihardjo SA, Howe JA. Twice the amount of α-carotene isolated from carrots is as effective as β-carotene in maintaining the vitamin A status of Mongolian gerbils. J Nutr. 2005;135:2622–6.
23. McOrist AL, Miller RB, Bird AR, Keogh JB, Noakes M, Topping DL, Conlon MA. Fecal butyrate levels vary widely among individuals but are usually increased by a diet high in resistant starch. J Nutr. 2011;141: 883–9.
24. NRC. Nutrient requirements of laboratory animals. 4th ed. Washington, DC: National Academy Press; 1995.
25. Davis C, Jing H, Howe JA, Rocheford T, Tanumihardjo SA. β-Cryptoxanthin from supplements or carotenoid-enhanced maize maintains liver vitamin A in Mongolian gerbils (*Meriones unguiculatus*) better than or equal to β-carotene supplements. Br J Nutr. 2008;100:786–93.
26. Sneider WD, Rosso GC, Rogers AE, Wolf G, Dowling JE, Callahan MJ. The "partial" vitamin A function of α-retinol. J Nutr. 1974;104:1662–8.
27. Green MH, Green JB, Lewis KC. Variation in the retinol utilization rate with vitamin A status in the rat. J Nutr. 1987;117:694–703.
28. Tanumihardjo SA, Vitamin A: biomarkers of nutrition for development. Am J Clin Nutr. 2011;94:S658–65.
29. Marriott J, Robinson M, Karikari SK. Starch and sugar transformation during the ripening of plantains and bananas. J Sci Food Agric. 1981; 32:1021–6.
30. Prabha TN, Bhagyalakshmi N. Carbohydrate metabolism in ripening banana fruit. Phytochemistry. 1998;48:915–20.
31. Chen CR, Ramaswamy HS. Color and texture change kinetics in ripening banana. Lebenson Wiss Technol. 2002;35:415–9.

32. Adao RC, Gloria BA. Bioactive amines and carbohydrate changes during ripening of 'Prata' banana (*Musa acuminate* x *M. balbisiana*). Food Chem. 2004;90:705–11.
33. Farness PL, Schneeman BO. Effects of dietary cellulose, pectin, and oat bran on the small intestine in the rat. J Nutr. 1982;112:1315–9.
34. Lairon D. Dietary fibres: effects on lipid metabolism and mechanisms of action. Eur J Clin Nutr. 1996;50:125–33.
35. Harrison EH. Mechanisms of digestion and absorption of dietary vitamin A. Annu Rev Nutr. 2005;25:87–103.
36. Mishra S, Monro J, Hedderley D. The effect of processing on slowly digestible starch and resistant starch in potato. Starch. 2008;60:500–7.
37. Thane C, Reddy S. Processing of fruit and vegetables: effect on carotenoids. Nutr Food Sci. 1997;97:58–65.
38. Lehmann U, Jacobasch G, Schmiedl D. Characterization of resistant starch type III from banana (*Musa acuminata*). J Agric Food Chem. 2002;50:5236–40.
39. Muranga FI, Sampath H, Marlett JA, Ntambi JM. Impact of processing technique on the apparent bioavailability of cooking banana (*matooke*) starch. African J Biochem Res. 2007;1:72–7.
40. Vahouny GV, Khalafi R, Satchithanandam S, Watkins DW, Story JA, Cassidy MM, Kritchevsky D. Dietary fiber supplementation and fecal bile acids, neutral steroids and divalent cations in rats. J Nutr. 1987;117:2009–15.
41. Kleessen B, Stoof G, Proll J, Schmiedl D, Noack J, Blaut M. Feeding resistant starch affects fecal and cecal microflora and short-chain fatty acids in rats. J Anim Sci. 1997;75:2453–62.
42. Blades BL, Dufficy L, Englberger L, Daniells JW, Coyne T, Hamill S, Wills RB. Bananas and plantains as a source of provitamin A. Asia Pac J Clin Nutr. 2003;12:S36.
43. Englberger L, Wills RBH, Blades B, Dufficy L, Daniells JW, Coyne T. Carotenoid content and flesh color of selected banana cultivars growing in Australia. Food Nutr Bull. 2006;27:281–91.

CHAPTER 4

Quantification of food and nutrient intakes in Zambian children with and without malaria under controlled feeding conditions

Kara A. Bresnahan[1], Justin Chileshe[2], and Sherry A. Tanumihardjo[1]

[1]Interdepartmental Graduate Program in Nutritional Sciences, University of Wisconsin, Madison, WI 53706, USA

[2]Tropical Disease Research Center, Ndola, Zambia

This manuscript is published in *Experimental Biology and Medicine* ©2014

Original Research

Quantification of food and nutrient intakes in Zambian children with and without malaria under controlled feeding conditions

Kara A Bresnahan[1], Justin Chileshe[2] and Sherry A Tanumihardjo[1]

[1]Interdepartmental Graduate Program in Nutritional Sciences, University of Wisconsin-Madison, Madison, WI 53706, USA
[2]Tropical Diseases Research Center, Ndola, Zambia
Corresponding author: Sherry A Tanumihardjo. Email: sherry@nutrisci.wisc.edu
Presented as a poster at the 2013 Experimental Biology meeting.

Abstract

Vitamin A supplementation improves status, which may protect against malarial infection. Provitamin A carotenoid biofortified staple crops may provide a more sustainable approach to alleviate vitamin A deficiency than supplementation, but the impact of febrile illness on food intake must be considered in malaria endemic regions. Morbidity data and food logs from a three-month efficacy trial on provitamin A biofortified (orange) maize in preschool Zambian children ($n = 181$, age 3–5 years) were systematically analyzed over time to determine the impact of malaria on food intake. Nutrients examined included macronutrients, iron, zinc, and vitamin A. Comparisons based on individual intakes in healthy and malarial states over three-day intervals were made including children from both the orange and white maize groups ($n = 100$). Malaria prevalence did not differ overall or between treatment groups over time (all $P > 0.05$). Lower nutrient intakes were observed for all variables during malaria outbreaks (food 289 ± 412 g; energy 248 ± 346 kcal; carbohydrate 42 ± 62 g; protein 8 ± 12 g; fat 5 ± 7 g; iron 1 ± 2 mg; zinc 1 ± 1 mg; vitamin A 58 ± 100 retinol activity equivalents; all $P < 0.05$). No differences were observed between nutrient decreases in orange and white maize groups ($P > 0.05$). Considering the impact of malaria on food and nutrient intakes and increased vitamin A utilization and excretion due to the acute phase response, biofortification targets for provitamin A carotenoids may need to be elevated in malaria endemic regions.

Keywords: Biofortification, carotenoids, maize, malaria, vitamin A

Experimental Biology and Medicine **2014; 239: 45–51. DOI: 10.1177/1535370213510661**

Introduction

Malaria and micronutrient deficiencies are widespread health issues in Africa and are of particular concern in children.[1,2] Malaria is the most common reason for hospitalization in African children under five years of age,[3] and increased malaria morbidity and mortality are associated with micronutrient deficiency in this population.[4] In fact, certain micronutrient deficiencies appear to exacerbate malarial illness[4,5] epitomizing the importance of nutrition interventions in initiatives aimed at malaria prevention and treatment.

Vitamin A is essential for healthy growth, immune function, vision, and reproduction. Vitamin A deficiency compromises the immune function in 40% of children under five years of age, resulting in 1 million deaths annually.[6] Stürchler *et al.*[7] found low serum retinol concentrations to be associated with increased risk of malarial illness, and an inverse association between serum retinol and malarial parasitemia has been documented in subsequent studies.[8–12] Vitamin A supplementation reduced the frequency of malarial episodes by 30% in young children in Papua New Guinea[13] and promoted ponderal growth and protection against malaria-related death in Tanzanian children.[14]

The efficiency of vitamin A supplement distribution has doubled since 2000 with at least 80% of preschool-aged children in least developed countries receiving coverage with two vitamin A doses in 2011.[15] However, the introduction of provitamin A biofortified crops provides a potentially effective method for alleviating vitamin A deficiency in the rural poor, who are disproportionally missed by supplementation interventions.[16] Additionally, provitamin A carotenoid biofortified staple crops may provide a more feasible and long-term approach to alleviate vitamin A deficiency without the risk of hypervitaminosis A attributed to fortification with preformed vitamin A.[17,18] Plant sources of provitamin A carotenoids maintained total body vitamin A stores in Chinese and Filipino children,[19,20] demonstrating that dietary approaches to ameliorate vitamin A deficiency

are efficacious. However, malaria suppresses appetite and growth,[21–23] and therefore it is important to consider its potential impact on the effectiveness of biofortified crops in malaria endemic regions.

A recent study assessing the adaptation of children to biofortified maize was conducted in the Eastern Province of Zambia,[24] a region where malaria is >75% endemic.[25,26] In order to determine the impact of malaria on food and micronutrient intake in Zambian children during a three-month intervention, we systematically analyzed food intake and morbidity data as a function of time and then compared food and micronutrient intakes between healthy and malarial states from children receiving either biofortified orange or white maize.

Materials and methods

Subjects and maize

The feeding protocol used in this trial was previously published by Nuss *et al*.[24] and is briefly summarized below. All procedures involving human subjects were approved by the Tropical Diseases Research Center (TDRC) Ethics Review Committee in Zambia and by the Health Sciences Human Subjects Institutional Review Board at the University of Wisconsin-Madison. Written informed consent was obtained from parents or guardians of all participating children. The feeding trial was conducted from March to June of 2010 in the Nyimba District of the Eastern Province of Zambia with children 3–5 years of age ($n = 189$ initial participatory enrollment, Table 1). The target for study size was set to conservatively accommodate a 10% dropout rate and provided enough feeding observations for greater than 90% power to detect a difference between groups in the adaptation to orange maize.[24] Six study sites were chosen based on interest expressed by local communities during sensitization programs related to orange maize conducted by the National Food and Nutrition Commission of Zambia (Lusaka, Zambia) in 2009. Enrollment at each site was approximately 30 participants, who were randomly assigned to either orange or white maize treatments. Participants were screened and excluded from the study for severe anemia (hemoglobin <7.0 g/dL) and malnutrition (<−3 SD weight-for-age or height-for-age).[27]

Participating children were fed breakfast, lunch, and snack six days per week for a total of 70 days of feeding, excluding Sundays. Orange maize used in the trial was developed from conventional breeding programs that selected for high provitamin A kernel content; typically consumed white maize was sourced locally.

Project menu, food intake measurement, and environment

The project menu was designed to contain common Zambian foods and cooking methods as outlined by a national food intake survey.[28] A six-day rotation of traditional menu items with standardized serving quantities supplied similar energy and nutrient amounts to the orange and white maize treatment groups, with the exception of vitamin A, which was of primary interest. Caloric content and nutrient quantities, including carbohydrate, protein, fat, vitamin A, iron, and zinc were determined using food labels of locally procured ingredients and Nutritionist Pro™ (version 4.6.0). Vitamin A content of maize was determined using published methods.[29,30] Food intake was determined by subtracting uneaten food at the end of the meal from the initial serving using digital kitchen scales (My-weigh KD7000, China). Mealtime attendance was recorded so that zero food waste was not mistaken as complete consumption of the meal.

Table 1 Baseline and final anthropometric and biochemical measurements by treatment group (mean ± SD)

Measurement	Orange maize ($n = 95$)	White maize ($n = 86$)	Total ($n = 181$)
Baseline age (months)	52.9 ± 10.2	55.1 ± 11.3	54.0 ± 10.7
Weight (kg)*			
Baseline	14.8 ± 1.9	14.7 ± 2.1	14.8 ± 2.0
Final	15.8 ± 1.9	15.6 ± 2.0	15.7 ± 2.0
Height (cm)*			
Baseline	98.3 ± 7.0	98.5 ± 7.2	98.4 ± 7.0
Final	100.5 ± 6.9	100.9 ± 6.5	100.7 ± 6.7
Weight-for-height z-score†			
Baseline	0.11 ± 0.84	−0.10 ± 0.88	0.008 ± 0.86
Final	0.11 ± 0.84	−0.10 ± 0.88	0.010 ± 0.87
Baseline malaria prevalence	17.6%	24.1%	20.8%
Parasite load >0	14.3%	20.7%	17.4%
Parasite load >1000	3.3%	3.4%	3.4%
Final malaria prevalence	8.8%	16.1%	12.4%
Parasite load >0	8.8%	13.8%	11.2%
Parasite load >1000	0.0%	2.3%	1.2%

*Weight and height increases were seen in orange and white maize treatment groups and total study population ($P < 0.05$).
†World Health Organization Child Growth Standards, ages 2–5 years.[35]

Feeding practices were standardized across all six feeding sites. The orange and white maize groups were fed in separate rooms to avoid between-group influences that may have impaired acceptance of the orange maize. Each participant's identification number was confirmed at every meal to ensure accurate data collection. Mealtimes were monitored to prevent food sharing and all subjects were equally encouraged to finish their meals. All ingredients for the meals were measured and prepared on-site by Zambian nutritionists and locally recruited women.

Weekly intake

For weekly food and nutrient intake, days of feeding were converted into weeks of feeding to account for the six-day rotating menu. A ''week'' in this analysis includes six days of project feeding, excluding Sunday. Food and nutrient intakes were calculated for both orange and white maize groups. Due to staggered initial and final feeding days, weeks 1 and 12 were not matched in terms of feed intake across all study sites and were excluded from all analyses.

Malaria diagnosis

All blood collection and analysis were conducted by TDRC in Zambia. Blood was collected for malaria parasite identification and quantification at baseline and final measurements. Thick blood smear slides were prepared on-site, air-dried, and transported in slide boxes for analysis. Blood slides for malaria parasites were prepared in accordance with standard protocols using a Giemsa stain and compared under oil immersion by microscope against 200 white blood cells (Olympus CX31 IRBSFA).[31,32] A slide was termed positive for malaria if parasites were observed and negative if no parasites were observed in 100 fields. Quality control procedures included the re-examination of approximately 10% of the slides by a second laboratory technologist and the use of a third technologist if disparities existed between the first and second reading.

Malaria was diagnosed throughout the study on a weekly basis by local clinicians who were trained by TDRC to diagnose malaria based on symptoms of fever and general malaise. A standardized morbidity questionnaire was used to diagnose malaria during the previous week by day and was completed by clinicians based on reports from the participants' parents or guardians. Malaria treatment was provided to participants according to WHO recommendations and guidelines from the Ministry of Health in Zambia.

Malaria prevalence and association with food intake

Baseline and final malaria prevalence and parasite load quantification were determined from biochemical analysis of blood smears. To determine weekly malaria prevalence, morbidity records were analyzed and participants diagnosed with malaria for at least one day of the week were included in calculations. Prevalence calculations for weeks 4 and 5 were adjusted to account for missing morbidity logs from one of the study sites.

A total of 100 participants, 50 each from the orange and white maize groups, were randomly selected in order to establish an association between malaria and food intake. In order to account for increasing food intake over time,[22] 10 participants with malaria from the orange and white treatments ($n = 5$/group) were selected from each week and each participant was selected only once during the intervention phase of the study. The duration of malaria symptoms during an attack in young children is approximately 3–4 days.[33] In order to elucidate the relationship between malaria and food intake, the same three-day time intervals were compared from a week when the participant was experiencing a malaria episode and the preceding week when the participant was in a healthy, non-febrile state. For week 2 or if a participant was absent during one of the healthy state comparison days, food and nutrient intake values from the week following the malaria episode were used for comparison. Dietary intakes were compared between a malarial state to a previous healthy state for the following reasons: (1) the incubation period for malaria, during which *Plasmodium* reproduces in the liver, can vary with the earliest onset of symptoms occurring between 5 and 12 days,[34] so it is unlikely that participants displayed symptoms the week before the outbreak was identified by clinicians; (2) malaria attacks reoccur every 2–3 days, when the parasites reproduce in blood cells,[34] so participants may have experienced symptoms the week following the initial outbreak; and (3) participants were provided a course of antimalarial medication once diagnosed with fever, which may produce side effects the following week.[34]

Statistical analysis

SAS statistical package (version 9.2) was used to analyze data. To investigate differences between treatments and malaria prevalence across weeks, repeated measures ANOVA followed by least square difference of variance testing was used to account for autocorrelated errors due to repeated measures on each participant with a first-order autoregressive structure. For malaria prevalence, an arcsine square root transformation was used to homogenize variance. Paired *t*-test was used for comparison of baseline and final anthropometric measurements and for in-group comparisons of food and nutrient intake between a malarial and healthy state. Independent *t*-test was implemented for between-group comparison of intake differences. All tests were two-sided, and $P \leq 0.05$ was considered significant. Values for food and nutrient intake are rounded to the nearest unit to reflect the precision of the field scales.

Results

Subject data

Baseline and final anthropometric data and malaria prevalence based on blood smear are given individually for orange and white maize groups and all study subjects in Table 1. Although no differences were observed in weight-for-height z-score ($P > 0.05$), increases were observed in weight and height for each treatment and overall ($P < 0.05$). No differences were observed in these

measurements between treatments ($P > 0.05$). Malaria prevalence in the white maize group was approximately 5% higher than the orange maize group at the beginning and end of the study; prevalence decreased in both treatments by approximately 9%. Eight children dropped out of the study (4%). Primary reasons for discontinuing study participation included non-compliance with study requirements and severe illness.

Comparison of weekly intakes

No differences were observed in food or energy intake reflecting equal acceptance of the project menu; participants consumed 90% of the food and the total kcal served each week. Of the total amount served each week, participants consumed 91% of carbohydrates, 89% of protein, 92% of fat, 92% of vitamin A, 90% of iron, and 75% of zinc. As expected, weekly vitamin A intake was higher in the orange maize group ($P < 0.05$). Intakes of food and all nutrients, except vitamin A, increased by week ($P < 0.05$), supporting previous findings by Nuss *et al.*,[24] which reported increasing intakes of menu items by week and similar intakes of all menu items after week 2 of the study.

Comparison of weekly malaria prevalence

During weeks 2 through 11 of the study, 94.5% of all study participants were diagnosed with at least one case of malaria. Malaria prevalence did not differ by maize treatment or week, and no interactions were observed between these variables (Figure 1, $P > 0.05$). The mean weekly malaria prevalence was 25.6%. Comparison of malaria prevalence at weeks 2–11 demonstrated a significant decrease from the beginning to the end of the examined study period ($P < 0.05$), which corresponded to the end of the rainy season.

Intake patterns associated with malarial status

Malarial status was associated with decreased intake of food, energy, and all nutrients ($P < 0.05$), which did not differ between treatments (Table 2). Food and kcal intake decreased during a malaria episode by 11%. Carbohydrate, protein, and fat intake decreased by 10%, 11%, and 10%, respectively, in children with malaria. During a three-day period with malaria, vitamin A intake decreased by 58 ± 100 retinol activity equivalents (9% decrease), iron intake decreased by 1 ± 2 mg (13% decrease), and zinc intake decreased by 1 ± 1 mg (14% decrease).

Discussion

Appetite suppression is a common symptom of malaria, which may impact the deliverance of micronutrients using biofortified staple crops to target populations where the infection is endemic. The findings presented in this study are important, as little work has been done to estimate the impact of malarial infection on dietary intake. In this study, the impact of malarial infection on food, energy, and nutrient intake was examined in rural Zambian children during a three-month feeding trial with provitamin A carotenoid biofortified maize. Intakes of food and all examined nutrients were suppressed during malarial infection in these children. The amount of decrease in food, energy, and nutrient intake was similar in the orange and white maize groups, which is expected because overall dietary intakes were similar and malaria prevalence did not differ between groups. Vitamin A intake was suppressed by 9.5% during the three-day malarial intervals assessed. This equates to a vitamin A loss of 19 ± 33 retinol activity equivalents/day or a 5–6% of the Recommended Dietary Allowance (RDA) in the study population due to malaria-related appetite suppression.

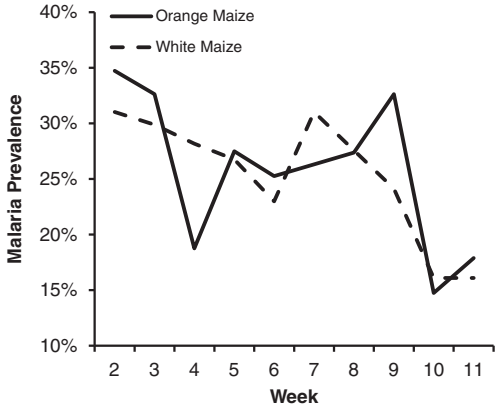

Figure 1 Weekly malaria prevalence by treatment group in rural Zambian children. No overall differences were observed in malaria prevalence by treatment group or week and no interactions were observed (*all* $P > 0.05$). A decrease in total malaria prevalence was found when comparing week 2 to week 11, reflecting seasonal effects ($P < 0.05$)

Ninety percent of the maize crop produced in Africa is white carotenoid-poor maize, which accounts for >70% of daily calories consumed in the Eastern Province of Zambia.[36–39] Poorly diversified diets can lead to subclinical micronutrient deficiencies, wasting, and stunting,[40,41] which can increase risk of infection and weaken immune response. Micronutrient deficiencies are associated with increased malaria morbidity and mortality in children.[4] Additionally, energy and protein intakes and weight gain are inversely proportional to febrile infections in Guatemalan and Bangladeshi children.[42,43] Stunting, a reflection of protein energy malnutrition, can be predicted by malaria in rural African settings and is associated with more frequent malarial infection and greater related morbidity.[43] Because of this, integration of nutrition and malaria initiatives may prove effective in malaria endemic regions. According to WHO criteria, the children in this study were in the healthy weight-for-height range and not malnourished (>−2 SD weight-for-height z-score)[27]; however, significant increases in weight and height during the three-month study period confirm that the nutritious study menu was beneficial to the children despite a weekly malaria prevalence ≥14%.

Malaria is endemic in the Eastern Province of Zambia with seasonal effects from December to April.[25,26,45–47] This study took place from March to June of 2010, during the transition out of ''malaria season.'' Although no overall

Table 2 Three-day comparison of nutrient intakes during a malaria episode and healthy state in rural Zambian children (mean ± SD)*

Total population studied	Food (g)	Energy (kcal)	Carbohydrate (g)	Protein (g)	Fat (g)	Vitamin A (RAE)	Iron (mg)	Zinc (mg)
Healthy state	2597 ± 321	2263 ± 274	395 ± 45	67 ± 12	48 ± 8	608 ± 84	9 ± 1	8 ± 1
Malarial state	2308 ± 467	2015 ± 380	352 ± 68	59 ± 15	43 ± 8	550 ± 113	8 ± 2	7 ± 1
Difference	289 ± 412	248 ± 346	42 ± 62	8 ± 12	5 ± 7	58 ± 100	1 ± 2	1 ± 1

*$n = 100$ comparisons between healthy and malarial states. Data for both treatments were pooled because no differences were observed between malaria-related decreases in food, energy, and nutrient intakes for orange and white groups ($P > 0.05$). Decreases were observed in all food, energy, and nutrient intakes during a malarial state when compared to a healthy state ($P < 0.05$).

differences were observed in weekly malaria prevalence throughout the study, a comparison of the extremes revealed a decrease in malaria prevalence from weeks 2 to 11 due to the seasonal effect. With the exception of baseline and final biochemical analysis to determine malaria parasitemia, clinicians diagnosed malaria symptomatically throughout the study. Without confirmation of parasitemia, it is possible that misdiagnosis of malaria occurred, inflating or underestimating weekly malaria prevalence. In fact, malaria is commonly misdiagnosed in resource-poor clinics for other febrile illnesses, such as pneumonia.[48,49] The implementation of malaria-specific rapid diagnostic tests can improve the proper diagnosis of malaria where microscopy is not available; however, these tests are costly and do not provide information on parasite density.[48,50] Nonetheless, the children were ill with fever, which does not negate our findings.

Malaria exacerbates vitamin A deficiency in at-risk populations.[51] The acute phase response to infections leads to a drop in serum retinol that is typically restored after the infection resolves.[52,53] This results in serum retinol measurements that do not accurately reflect vitamin A status during infection. For example, Thurnham and Singkamani[54] found Thai patients suffering from malarial infection to have serum retinol values indicating deficiency, although the patients had no clinical signs and a diet high in provitamin A carotenoids. It is paramount to measure and adjust for elevated positive acute phase proteins in the serum to avoid misclassification of vitamin A deficiency during infection when more reliable measurements, such as the modified relative dose response test or retinol isotope dilution, are not possible.[55,56]

The decrease in serum retinol during the acute phase response results from the suppression of retinol binding protein and transthyretin (prealbumin) in order to increase vitamin A availability to tissues.[9,57] Increased vitamin A utilization during malarial infection is also supported by reductions in serum provitamin A carotenoids.[54,58,59] Furthermore, significant urinary retinol losses occur during infection.[60,61] This increase in vitamin A utilization coupled with appetite suppression and urinary loss may result in increased vitamin A requirements in malaria endemic regions. Based on other febrile disease states and illnesses, we quantified the projected loses of vitamin A in the urine during a malaria episode. In children with infections that cause fever, up to 500 μg retinol can be lost per day,[60] for a total of 1.5 mg for a three-day malarial attack. Up to 1.7 mg has been estimated to be lost in children with a severe infection that lasts for 10 days.[61] Therefore, a single malarial episode could result in urinary losses that are greater than the weekly intake of vitamin A from foods as quantified in this controlled study. This implies that vitamin A requirements may be higher in areas with endemic febrile illness.

In conclusion, provitamin A biofortified staple crops can increase vitamin A intake in regions where deficiency is prevalent. However, as malarial infection negatively impacts vitamin A status through decreased food intake, urinary loss, and increased utilization, biofortification targets may need to be elevated to meet vitamin A requirements in endemic regions. The provitamin A biofortified maize target level is currently set at 15–17 μg β-carotene equivalents/g dry weight, which is projected to meet 50% of the estimated average requirement for vitamin A or 140 μg/day for a child.[62] Raising the target level by 3–5 μg/g in countries with endemic malaria and high maize intakes could act as a buffer to assist in maintaining adequate vitamin A stores during febrile episodes. This target level, which has already been surpassed in some experimental maize genotypes, is achievable with traditional breeding techniques (SAT personal observations). Considering that maize intakes of preschool children in Zambia are 150–200 g dry weight/day, a 3-μg/g increase in target level would provide approximately 100 extra retinol activity equivalents during a three-day malaria episode, which would cover the loss from appetite suppression found in this study. The introduction of provitamin A biofortified staple crops in coordination with supplementation programs may be particularly beneficial to children in malaria endemic regions.

Author contributions: KAB was responsible for training staff, facilitating field research, data entry and input into statistical analysis, and writing the paper; JC was responsible for coordinating blood sample collection and storage, analyzing blood slides for malaria parasitemia, and training local clinicians for collection of morbidity data; and SAT designed research, provided input into data analysis, and revised the paper. All authors read and approved the final manuscript.

ACKNOWLEDGEMENTS

The authors thank Peter Crump, University of Wisconsin-Madison College of Agriculture and Life Sciences Statistical

Consulting Service, for statistical analysis consultation. The authors also thank Sara Arscott, Emily Nuss, and Rebecca Surles for assisting with fieldwork and data collection in Zambia. This work was supported by Global Health funds at University of Wisconsin-Madison during the manuscript preparation and HarvestPlus contract number 8217 for the fieldwork. This work was part of the Global Health certification process for KAB. HarvestPlus (www.harvestplus.org) is a global alliance of agriculture and nutrition research institutions working to increase the micronutrient density of staple food crops through biofortification. The views expressed do not necessarily reflect those of HarvestPlus.

REFERENCES

1. World Health Organization. *Malaria: high risk groups*, http://www.who.int/malaria/high_risk_groups/en/. (2012, accessed 14 August 2012)
2. World Health Organization. *Micronutrient deficiencies: vitamin A deficiency*, http://www.who.int/nutrition/topics/vad/en/. (2010, accessed 13 August 2012)
3. Roca-Feltrer A, Carneiro I, Armstrong Schellenberg JRM. Estimates of the burden of malaria morbidity in Africa in children under the age of 5 years. *Trop Med Int Health* 2008;**13**:771–83
4. Caufield LE, de Onis M, Blôssner M, Black RE. Undernutrition as an underlying cause of child deaths associated with diarrhea, pneumonia, malaria, and measles. *Am J Clin Nutr* 2004;**80**:193–8
5. Shankar AH. Nutritional modulation of malaria morbidity and mortality. *J Infect Dis* 2000;**182**:S37–53
6. Micronutrient Initiative/United Nations International Children's Emergency Fund. *Vitamin and mineral deficiency: a global progress report*, http://www.micronutrient.org/CMFiles/PubLib/VMd-GPR-English1KWW-3242008-4681.pdf. (2004, accessed 15 August 2012)
7. Strüchler D, Tanner M, Hanck A, Betschart B, Gautschi K, Weiss N, Burnier E, Del Giudice G, Degrémont A. A longitudinal study on relation of retinol with parasitic infections and the immune response in children of Kikwawila village, Tanzania. *Acta Trop* 1987;**44**:213–27
8. Galan P, Samba C, Luzeau R, Amedee-Manesme O. Vitamin A deficiency in pre-school age Congolese children during malarial attacks. Part 2: impact of parasitic disease on vitamin A status. *Int J Vitam Nutr Res* 1990;**60**:224–8
9. Tabone MD, Muanza K, Lyagoubi M, Jardel C, Pied S, Amedee-Manesme O, Grau GE, Mazier D. The role of interlukin-6 in vitamin A deficiency during *Plasmodium falciparum* malaria and possible consequences for vitamin A supplementation. *Immunology* 1992;**75**:553–4
10. Filteau SM, Morris SS, Abbott RA, Tomkins AM, Kirkwood BR, Arthur P, Ross DA, Gyapong JO, Raynes JG. Influence of morbidity on serum retinol of children in a community-based study in northern Ghana. *Am J Clin Nutr* 1993;**58**:192–7
11. Friis H, Mwaniki D, Omondi B, Muniu E, Magnussen P, Geissler W, Thiong'o F, Michaelsen KF. Serum retinol concentrations and *Schistosoma mansoni*, intestinal helminths, and malarial parasitemia: a cross-sectional study in Kenyan preschool and primary school children. *Am J Clin Nutr* 1997;**66**:665–71
12. Hautvast JL, Tolboom JJ, West CE, Kafwembe EM, Sauerwein RW, van Staveren WA. Malaria is associated with reduced serum retinol levels in rural Zambian children. *Int J Vitam Nutr Res* 1998;**68**:384–8
13. Shankar AH, Genton B, Semba RD, Baisor M, Paino J, Tamja S, Adiguma T, Wu L, Rare L, Tielsch JM, Alpers MP, West KP Jr. Effect of vitamin A supplementation on morbidity due to *Plasmodium falciparum* in young children in Papua New Guinea: a randomised trial. *Lancet* 1999;**354**:203–9
14. Villamor E, Mbise R, Spiegelman D, Hertzmark E, Fataki M, Peterson KE, Ndossi G, Fawzi WW. Vitamin A supplements ameliorate the adverse effect of HIV-1, malaria, and diarrheal infections on child growth. *Pediatrics* 2002;**109**:E6
15. UNICEF. *Vitamin A deficiency: progress*, http://www.childinfo.org/vitamina_progress.html. (2013, accessed 27 August 2013)
16. UNICEF. *Vitamin A supplementation: a decade of progress*, http://www.unicef.org/immunization/files/Vitamin_A_Supplementation.pdf. (2007, accessed 27 August 2013)
17. Ribaya-Mercado JD, Solomons NW, Medrano Y, Bulux J, Dolnikowski GG, Russell RM, Wallace CB. Use of the deuterated-retinol-dilution technique to monitor the vitamin A status of Nicaraguan schoolchildren 1 y after initiation of the Nicaraguan national program of sugar fortification with vitamin A. *Am J Clin Nutr* 2004;**80**:1291–8
18. Tanumihardjo SA. Food-based approaches for ensuring adequate vitamin A nutrition. *Compr Rev Food Sci Food Saf* 2008;**7**:373–81
19. Tang G, Gu X, Hu S, Xu Q, Qin J, Dolnikowski GG, Fjeld CR, Gao X, Russell RM, Yin S. Green and yellow vegetables can maintain body stores of vitamin A in Chinese children. *Am J Clin Nutr* 1999;**70**:1069–76
20. Ribaya-Mercado JD, Maramag CC, Tengco LW, Dolnikowski GG, Blumberg JB, Solon FS. Carotene-rich plant foods ingested with minimal dietary fat enhance the total-body vitamin A pool size in Filipino schoolchildren as assessed by stable-isotope-dilution methodology. *Am J Clin Nutr* 2007;**85**:1041–9
21. Rowland MGM, Cole TJ, Whitehead RG. A quantitative study into the role of infection in determining nutritional status in Gambian village children. *Br J Nutr* 1977;**37**:441–50
22. Snow RW, Molyneux CS, Njeru EK, Omumbo J, Nevill CG, Muniu E, Marsh K. The effects of malaria control on nutritional status in infancy. *Acta Trop* 1997;**65**:1–10
23. Harrison NE, Odunukwe NN, Ijoma CK, Mafe AG. Current clinical presentation of malaria in Enugu, Nigeria. *Niger Postgrad Med J* 2004;**11**:240–5
24. Nuss ET, Arscott SA, Bresnahan K, Pixley KV, Rocheford T, Hotz C, Siamusantu W, Chileshe J, Tanumihardjo SA. Comparative intake of white- versus orange-colored maize by Zambian children in the context of promotion of biofortified maize. *Food Nutr Bull* 2012;**33**:63–71
25. Craig MH, Snow RW, le Sueur D. A climate-based distribution model of malaria transmission in Sub-Saharan Africa. *Parasitol Today* 1999;**15**:105–11
26. Mapping Malaria Risk in Africa. *Zambia: distribution of endemic malaria*, http://www.mara.org.za/. (2005, accessed 15 August 2012)
27. de Onis M, Blössner M. *WHO global database on child growth and malnutrition*. Geneva: World Health Organization, 1997, http://whqlibdoc.who.int/hq/1997/WHO_NUT_97.4.pdf. (accessed 1 July 2012)
28. Hotz C, Chileshe J, Siamusantu W, Palaniappan W, Kafwembe E. Vitamin A intake and infection are associated with plasma retinol among pre-school children in rural Zambia. *Public Health Nutr* 2012;**23**:1–9
29. Kurlich AC, Juvick JA. Simultaneous quantification of carotenoids and tocopherols in corn kernel extracts by HPLC. *J Liq Chromatogr Relat Technol* 1999;**22**:2925–34
30. Howe JA, Tanumihardjo SA. Carotenoid-biofortified maize maintains adequate vitamin A status in Mongolian gerbils. *J Nutr* 2006;**136**:2562–7
31. Trape JF. Rapid evaluation of malaria parasite density and standardization of thick smear examination for epidemiological investigations. *Trans R Soc Trop Med Hyg* 1985;**79**:181–4
32. Sazawal S, Black RE, Ramsan M, Chwaya HM, Stoltzfus RJ, Dutta A, Dhingra U, Kabole I, Deb S, Othman MK, Kabole FM. Effects of routine prophylactic supplementation with iron and folic acid on admission to hospital and mortality in preschool children in a high malaria transmission setting: community-based, randomized, placebo-controlled trial. *Lancet* 2006;**367**:133–43
33. Snow RW, Newton CRJC, Craig MH, Steketee RW. *The public health burden of Plasmodium falciparum malaria in Africa: deriving the numbers*. Working Paper no. 11, Disease Control Priorities Project. Bethesda, MD: Fogarty International Center, National Institutes of Health, 2003
34. World Health Organization. *Guidelines for treatment of malaria, second edition*, http://whqlibdoc.who.int/publications/2010/9789241547925_eng.pdf. (2010, accessed 10 September 2013)
35. World Health Organization. *Child growth standards: weight-for-height for boys and girls ages 2-5 yr*, http://www.who.int/childgrowth/standards/weight_for_height/en/index.html. (2011, accessed 1 July 2012)

36. Kumar SK. *Adoption of hybrid maize in Zambia: effects on gender roles, food consumption, and nutrition*. Washington, DC: International Food Policy Research Institute, 1994
37. McCann JC. Africa and the world ecology of maize. In: McCann JC (ed.). *Maize and grace: Africa's encounter with a New World crop, 1500–2000*. Cambridge, MA: Harvard University Press, 2005:1–22
38. Pfeiffer WH, McClafferty B. HarvestPlus: breeding crops for better nutrition. *Crop Sci* 2007;**47**:S88–105
39. Nuss ET, Tanumihardjo SA. Maize: a paramount staple crop in the context of global nutrition. *Compr Rev Food Sci Food Saf* 2010;**9**:417–36
40. Tanumihardjo SA, Palacios N, Pixley KV. Provitamin A carotenoid bioavailability: what really matters? *Int J Vitam Nutr Res* 2010;**80**:336–50
41. Mamiro PS, Kolsteren P, Roberfroid D, Tatala S, Opsomer AS, Van Camp JH. Feeding practices and factors contributing to wasting, stunting, and iron-deficiency anaemia among 3-23-month old children in Kilosa district, rural Tanzania. *J Health Popul Nutr* 2005;**23**:222–30
42. Mata LJ, Kromal RA, Urrutia JJ, Garcia B. Effect of infection on food intake and the nutritional state: perspectives as viewed from the village. *Am J Clin Nutr* 1977;**30**:1215–27
43. Becker S, Black RE, Brown KH. Relative effects of diarrhea, fever, and dietary energy intake on weight gain in rural Bangladeshi children. *Am J Clin Nutr* 1991;**53**:1499–503
44. Friedman JF, Kwena AM, Mirel LB, Kariuki SK, Terlouw DJ, Phillips-Howard PA, Hawley WA, Nahlen BL, Shi YP, Kuile FOT. Malaria and nutritional status among pre-school children: results from cross-sectional surveys in western Kenya. *Am J Trop Med Hyg* 2005;**73**:698–704
45. Mapping Malaria Risk in Africa. *Zambia: first month of the malaria transmission season*, http://www.mara.org.za/. (2001, accessed 15 August 2012)
46. Mapping Malaria Risk in Africa. *Zambia: last month of malaria transmission season*, http://www.mara.org.za/. (2001, accessed 15 August 2012)
47. Transer FC, Sharp B, le Sueur D. Potential effect on climate change on malaria transmission in Africa. *Lancet* 2003;**362**:1792–8
48. Amexo M, Tolhurst R, Barnish G, Bates I. Malaria misdiagnosis: effects on the poor and vulnerable. *Lancet* 2004;**364**:1896–8
49. Källander K, Nungwa-Sabiiti J, Peterson S. Symptom overlap for malaria and pneumonia-policy implications for home management strategies. *Acta Trop* 2004;**90**:211–4
50. World Health Organization. *Making rapid diagnosis work*, http://www2.wpro.who.int/sites/rdt/. (2005, accessed 20 August 2012)
51. Sanjoaquin MA, Molyneux ME. Malaria and vitamin A deficiency in African children: a vicious circle? *Malaria J* 2009;**8**:134–9
52. Coutsoudis A, Coovadia HM, Broughton M, Salisbury RT, Elson I. Micronutrient utilization during measles treated with vitamin A or placebo. *Int J Vitam Nutr Res* 1991;**61**:199–204
53. Willumsen JF, Simmank K, Filteau SM, Wagstaff LA, Tomkins AM. Toxic damage to the respiratory epithelium induces acute phase changes in vitamin A metabolism without depleting retinol stores of South African children. *J Nutr* 1997;**127**:1339–43
54. Thurnham DI, Singkamani R. The acute phase response and vitamin A status in malaria. *T Roy Soc Trop Med H* 1991;**85**:1994–9
55. Thurnham DI, McCabe GP, Northrop-Clewes CA, Nestel P. Effects of subclinical infection on plasma retinol concentrations and assessment of prevalence of vitamin A deficiency: meta-analysis. *Lancet* 2003;**362**:2052–8
56. Tanumihardjo SA. Vitamin A: biomarkers of nutrition for development. *Am J Clin Nutr* 2011;**94**:658S–65S
57. Gruys E, Toussaint MJM, Niewold TA, Koopmans SJ. Acute phase reaction and acute phase proteins. *J Zhejiang Univ Sci* 2005;**6B**:1045–56
58. Das BS, Thurnham DI, Das DB. Plasma α-tocopherol, retinol, and carotenoids in children with falciparum malaria. *Am J Clin Nutr* 1996;**64**:94–100
59. Nussenblatt V, Mukasa G, Metzger A, Ndeezi G, Eisinger W, Semba RD. Relationship between carotenoids and anaemia during acute uncomplicated *Plasmodium falciparum* malaria in children. *J Health Popul Nutr* 2002;**20**:205–14
60. Stephensen CB, Alvarez JO, Kohatsu J, Hardmeier R, Kennedy JI Jr, Gammon RB Jr. Vitamin A is excreted in the urine during acute infection. *Am J Clin Nutr* 1994;**60**:388–92
61. Mitra AK, Alvarez JO, Stephensen CB. Increased urinary retinol loss in children with severe infection. *Lancet* 1998;**351**:1033–4
62. Bouis HE, Hotz C, McClafferty B, Meenakshi JV, Pfeiffer WH. Biofortification: a new tool to reduce micronutrient malnutrition. *Food Nutr Bull* 2011;**32**:S31–40

(Received May 12, 2013, Accepted September 26, 2013)

CHAPTER 5

The Acute Phase Response Affected Traditional Measures of Micronutrient Status in Rural Zambian Children during a Randomized, Controlled Feeding Trial

Kara A. Bresnahan[1], Justin Chileshe[2], Sara Arscott[1], Emily Nuss[1], Rebecca Surles[1], Cassim Masi[3], Emmanuel Kafwembe[2], and Sherry A Tanumihardjo[1]

[1]Interdepartmental Graduate Program in Nutritional Sciences, University of Wisconsin, Madison, WI 53706, USA

[2]Tropical Disease Research Center, Ndola, Zambia

[3]National Food and Nutritional Commission of Zambia, Lusaka, Zambia

This manuscript is published in the *Journal of Nutrition* ©2014

The Journal of Nutrition. First published ahead of print April 9, 2014 as doi: 10.3945/jn.114.192245.

The Journal of Nutrition
Community and International Nutrition

The Acute Phase Response Affected Traditional Measures of Micronutrient Status in Rural Zambian Children during a Randomized, Controlled Feeding Trial[1,2]

Kara A. Bresnahan,[3] Justin Chileshe,[4] Sara Arscott,[3,6] Emily Nuss,[3] Rebecca Surles,[3] Cassim Masi,[5] Emmanuel Kafwembe,[4] and Sherry A. Tanumihardjo[3]*

[3]Interdepartmental Graduate Program in Nutritional Sciences, University of Wisconsin-Madison, Madison, WI; [4]Tropical Diseases Research Center, Ndola, Zambia; and [5]National Food and Nutrition Commission of Zambia, Lusaka, Zambia

Abstract

The acute phase response (APR) to infection can alter blood-based indicators of micronutrient status. Data from a 3-mo randomized, controlled feeding trial in rural Zambian children (n = 181, aged 3–5 y) were used to determine the impact of the APR on indicators of vitamin A and iron status using baseline and final blood samples. Concentrations of acute phase proteins were categorized as raised C-reactive protein (CRP; >5 and >10 mg/L) only, both raised CRP and α_1–acid glycoprotein (AGP; >1.2 g/L), raised AGP only, and neither CRP nor AGP raised to identify the respective stages of infection: incubation, early convalescence, convalescence, and healthy state. Data were insufficient to examine the incubation stage of infection. A CRP concentration of >5 mg/L was an effective elevation cutoff point in this population to show impact on micronutrient markers. Time did not affect hemoglobin, serum ferritin, or serum retinol concentrations ($P > 0.05$). During early convalescence, hemoglobin decreased (14–16%; $P \leq 0.05$), serum ferritin increased (279–356%; $P \leq 0.05$), and serum retinol decreased (20–30%; $P \leq 0.05$). Serum retinol concentrations did not change during convalescence; however, hemoglobin remained depressed (4–9%) and serum ferritin was elevated (67–132%) (both $P \leq 0.05$). Modified relative dose response values were unaffected by the APR ($P > 0.05$) but increased between time points (16%; $P \leq 0.05$), indicating a decrease in liver vitamin A reserves on the background of a semiannual vitamin A supplementation program. The observed prevalence of anemia and vitamin A deficiency assessed by serum retinol concentration was higher during the APR ($P \leq 0.05$). It is important to consider the impact of infection on dietary interventions and to adjust for acute phase proteins when assessing iron status or vitamin A status by serum retinol concentration alone in children. J. Nutr. doi: 10.3945/jn.114.192245.

Introduction

In public health assessments, micronutrient status is commonly determined using concentrations in blood as a surrogate for total body status. Blood concentrations of micronutrients can be altered by the acute phase response (APR)[7] to infection (1,2). Whether this change is reflective of a redistribution of the micronutrient within the body, a negative shift in total stores, or a combination of both factors is a complex issue. Micronutrient demands tend to be increased during infection, and deficiency can increase susceptibility to, and be exacerbated by, chronic or recurrent episodes of febrile illness (3–6). However, substantial changes in micronutrient status do not likely occur as early as decreases in blood concentrations suggest during the APR. Additionally, blood concentrations are typically restored when the infection resolves (7–10), supporting measurements taken during the APR as inadequate reflections of micronutrient status. Therefore, it is paramount to consider the impact of the APR on blood-based micronutrient status indicators in populations with a high or unknown prevalence of febrile infection.

[1] Supported by Global Health funds at the University of Wisconsin-Madison during manuscript preparation and HarvestPlus contract number 8217 for the fieldwork. HarvestPlus is a global alliance of agriculture and nutrition research institutions working to increase the micronutrient density of staple food crops through biofortification. The views expressed in this manuscript do not necessarily reflect those of HarvestPlus. This work will be presented at Experimental Biology 2014.
[2] Author disclosures: K. A. Bresnahan, J. Chileshe, S. Arscott, E. Nuss, R. Surles, C. Masi, and S. A. Tanumihardjo, no conflicts of interest. E. Kafwembe, deceased 17 May 2010.
[6] Present address: Standard Process Inc., 1200 W. Royal Lee Dr., Palmyra, WI 53156.
* To whom correspondence should be addressed. E-mail: sherry@nutrisci.wisc.edu.

[7] Abbreviations used: AGP, α_1–acid glycoprotein; APR, acute phase response; C−A−, neither CRP nor AGP raised; C−A+, AGP raised only; C+A−, CRP raised only; C+A+, both CRP and AGP raised; CRP, C-reactive protein; MRDR, modified relative dose response.

Manuscript received February 11, 2014. Initial review completed March 10, 2014. Revision accepted March 18, 2014.
doi: 10.3945/jn.114.192245.

The APR is a systemic reaction to disruptions in the body's homeostasis that is characterized by inflammatory mechanisms aimed at mitigating infection or trauma (11). Positive acute phase proteins that increase in the blood during infection can be used to assess the stage and severity of the response; C-reactive protein (CRP) and α_1–acid glycoprotein (AGP) are commonly measured in nutrition interventions. CRP concentrations rise within 6 to 8 h of infection, reach maximal 10- to 1000-fold increases in concentration around 48 h, and normalize rapidly as infection resolves (12,13). AGP concentrations become elevated 24 to 48 h after initial infection with a maximum 4-fold increase in concentration around 5 to 6 d and can remain raised weeks after the infection convalesces (11,14). Both of these acute phase proteins are also raised during low-grade chronic inflammation, such as heart disease (15,16). Using these proteins, it is possible to identify different stages of infection including incubation (CRP only raised), early convalescence (both CRP and AGP raised), convalescence (AGP only raised), and return to a healthy state (neither CRP nor AGP raised) (17).

We investigated the impact of the APR on blood-based indicators of vitamin A and iron status using data from baseline and final assessments of a 70-d maize feeding trial in rural Zambian children. Treatment arms included high provitamin A–biofortified (orange) and white maize. The effect of the APR was estimated for each micronutrient indicator investigated, and the consequence of infection on deficiency prevalence was determined. Prevalence of CRP elevation cutoffs of 5 and 10 mg/L are commonly reported in the literature (15,17–23); therefore, both values were examined. For AGP, 1.2 g/L was used as the raised cutoff point (21,24).

Participants and Methods

Study design and location. The feeding protocol used in this trial was previously published (25,26) and is summarized herein. All procedures involving human participants were approved by the Tropical Diseases Research Center Ethics Review Committee in Zambia and by the Health Sciences Human Subjects Institutional Review Board at the University of Wisconsin-Madison. Children 3–5 y of age (n = 181 at final enrollment) were recruited to participate in a randomized, controlled feeding trial with traditional white and high provitamin A carotenoid–biofortified (orange) maize. The mean participant age was 4.5 ± 0.9 y. Written informed consent was obtained from guardians of all participating children. Participants were screened and excluded from the study for severe anemia (hemoglobin <70 g/L) and malnutrition (<−3 SD weight-for-age or height-for-age Z-scores) (27). The feeding trial was carried out in 6 villages in the Eastern Province of Zambia from March to June of 2010.

After being randomly assigned to the treatment groups, participating children were fed a standardized menu including breakfast, lunch, and snack 6 d/wk for a total of 70 d of feeding, excluding Sundays. The only difference between the treatment groups was the type of maize used in the standardized menu. The orange maize contained 3.0 to 6.3 μg of theoretical vitamin A/g during the 70-d study (25). Overall, dietary intakes of macro- and micronutrients were similar between the white and orange maize groups (26). Data on the adaptation of children to orange maize (25) and on the impact of malaria on dietary intake are published (26).

Blood samples were collected at baseline and final time points. Zambia has an active, high-dose vitamin A supplementation program for children <5 y of age as recommended by the WHO (28). Therefore, the entire trial occurred after a high-dose supplement was consumed and finished a few days before administration of the next semiannual supplement. Children were only enrolled if they had consumed a supplement.

Modified relative dose response test and serum retinol concentrations. For the modified relative dose response (MRDR) test, a single oral dose of 5.3-μmol 3,4-didehydroretinyl acetate in corn oil was administered via positive displacement pipet to participants followed by consumption of peanut butter and bread to ensure adequate fat for efficient dose absorption. Blood samples were drawn 4 to 6 h after dosing using 5-mL Vacutainer tubes (Becton Dickinson). Samples were placed on ice until serum could be separated by centrifugation; serum was transported in nitrogen vapor in a dry shipper and then stored at −70°C until analysis. Samples were analyzed simultaneously for MRDR value and serum retinol concentration using a standardized method (29). Serum (200 μL) was treated with 250-μL ethanol and extracted twice with hexane; retinyl acetate served as the internal standard. Pooled hexanes were dried under nitrogen and reconstituted in 40-μL 75:25 methanol:dichloroethane for injection (35 μL) onto an HPLC system equipped with a Waters Resolve C18 column (3.9 × 150 mm, 5 μm). The mobile phase of 89:11 methanol:water (0.73-g/L triethylamine) was run at a flow rate of 1 mL/min. The definition for vitamin A deficiency using the MRDR test was considered a serum molar ratio of 3,4-didehydroretinol: retinol ≥0.060 (29,30).

Hemoglobin, serum ferritin, and markers of infection. At the time of blood draw, hemoglobin concentrations of whole blood were determined using a portable hemoglobinometer (Hemocue). Serum ferritin concentrations were measured using a commercially available ELISA kit (Ramco). The absorbance of all samples was verified at 510 and 630 nm on a Tecan Sunrise ELISA reader (Model A 5080; Tecan Group). Calibration graphs for serum ferritin were created using supplied standards.

CRP and AGP concentrations were measured using radial immunodiffusion kits (Kent Laboratory). The assays quantified the concentration of the acute phase proteins using agar plates containing either CRP- or AGP-specific antibodies. The diameters of the resultant precipitin complexes were determined to the nearest 0.1 mm using a plate reader (Nidek 2743 Calibration Viewer; Transdyne General Corporation). CRP and AGP standards were used to create calibration curves for the conversion of precipitin complex diameters to serum concentrations. Methods for the determination of malaria parasitemia are published (26).

Statistical analysis. Values are means ± SDs. Data were analyzed using SAS software (SAS Institute). Serum ferritin concentrations were transformed via logarithm before statistical analysis. Baseline and final anthropometric and biochemical data were compared within and between white and orange maize groups using paired and independent t tests, respectively. Correlations between markers of micronutrient status concentrations and acute phase proteins were assessed with Pearson's correlation. Separately investigating elevation cutoffs for CRP of >5 and >10 mg/L, differences in micronutrient status among participants with neither CRP nor AGP raised (C−A−), CRP raised only (C+A−), both CRP and AGP raised (C+A+), and AGP raised only (C−A+) were analyzed by time using 2-factor ANOVA tests. Differences between groups were determined using least-square differences at $\alpha = 0.05$. The effect of gender on hemoglobin and serum ferritin values was investigated and excluded because it was not significant (both $P > 0.05$). The C+A− group was excluded because the sample size ($n \leq 4$) did not yield enough observations to justify analysis. Therefore, the total population included in the overall and 2-factor ANOVA tests was decreased by a total of 5 and 1 observations, respectively, for CRP elevation cutoffs of >5 and >10 mg/L, respectively. Differences in the prevalence of micronutrient deficiency were determined within a time point by directly comparing groups with elevated acute phase proteins to the group without raised acute phase proteins using Fisher's exact test. Only participants with a complete data set for markers of infection (n = 178) at initial and final time points were included in ANOVA and Fisher's exact tests.

Sample size calculations were based upon a projected change in the most sensitive marker of vitamin A status used, the MRDR test. A sample size of 80 was needed to measure a difference of 0.02 in the MRDR value, assuming a SD of 0.04 in each group at a 5% significance level and 90% power. This sample size was used in a trial in South African children for a sweet potato intervention, and the MRDR showed an intervention effect (30). To account for dropouts during the trial, we aimed to recruit 100 children in each group.

Results

Participant data. Overall, 14 children dropped out of the study (7%; **Fig. 1**). Primary reasons for discontinuing study participation included noncompliance with study requirements and severe illness. Data were pooled by time point for white and orange maize groups because no differences were observed between groups with the exception of initial serum ferritin, which was higher in the orange maize group (198 ± 232 μg/L) than the white maize group (128 ± 131 μg/L) (**Table 1**). No change was seen in the weight-for-height Z-score; however, weight and height increased by 6.1% and 2.3%, respectively, during the study. Malaria prevalence decreased by 8.4%, likely because of the transition out of malaria season that occurred during the trial (26). Serum CRP concentration did not change; the prevalence of elevated CRP decreased by 2.8% and 0.5% using the >5 and >10 mg/L cutoffs, respectively. Serum AGP concentration decreased by 17.6%, which corresponded to a 27.0% decrease in the prevalence of elevated AGP at the final assessment. Of participants with malaria (n = 37 at baseline; n = 22 at final), 78.4% and 59.1% had raised acute phase proteins using a CRP cutoff of 5 mg/L, and 78.4% and 54.6% using a CRP cutoff of 10 mg/L at initial and final time points, respectively.

Comparison of micronutrient status concentrations or values across infection stage. Similar correlations between markers of interest were found at initial and final time points (**Table 2**). Both hemoglobin and serum retinol concentrations correlated negatively with CRP and AGP concentrations. A positive correlation was found between serum ferritin and acute phase protein concentration. No correlational relation was found between MRDR value and acute phase protein concentration. CRP and AGP were positively correlated. Hemoglobin and serum ferritin shared a negative association, suggesting a shift in iron distribution during infection.

The overall test revealed differences among infection groups in all investigated micronutrient indicators (all $P \leq 0.05$) (**Table 3**). The 2-factor ANOVA analysis revealed a significant effect of infection stage, but not a time effect or an interaction between infection stage and time for hemoglobin, serum ferritin, and serum retinol concentrations. Similar patterns of significance were also discovered using both CRP elevation cutoffs of >5 and >10 mg/L for all micronutrient indicators investigated. Compared with the C−A− group, hemoglobin measures were suppressed in both the C+A+ and C−A+ groups. Serum ferritin concentrations were markedly higher in groups with elevated acute phase proteins; the C+A+ group was 349% and 353% elevated, and the C−A+ group was 67% and 132% elevated at initial and final time points, respectively, when compared with the C−A− group for the CRP cutoff of 5 mg/L. Serum retinol concentration was lower in the C+A+ group, but the C−A+ group did not differ from the C−A− group. For the MRDR value, a significant effect of time was observed but no effect of infection stage or an interaction. MRDR values were higher at the final assessment point when compared with the initial assessment, which indicates a decrease in vitamin A liver reserves over time on the background of semiannual high-dose vitamin A supplementation. The mean MRDR value indicated

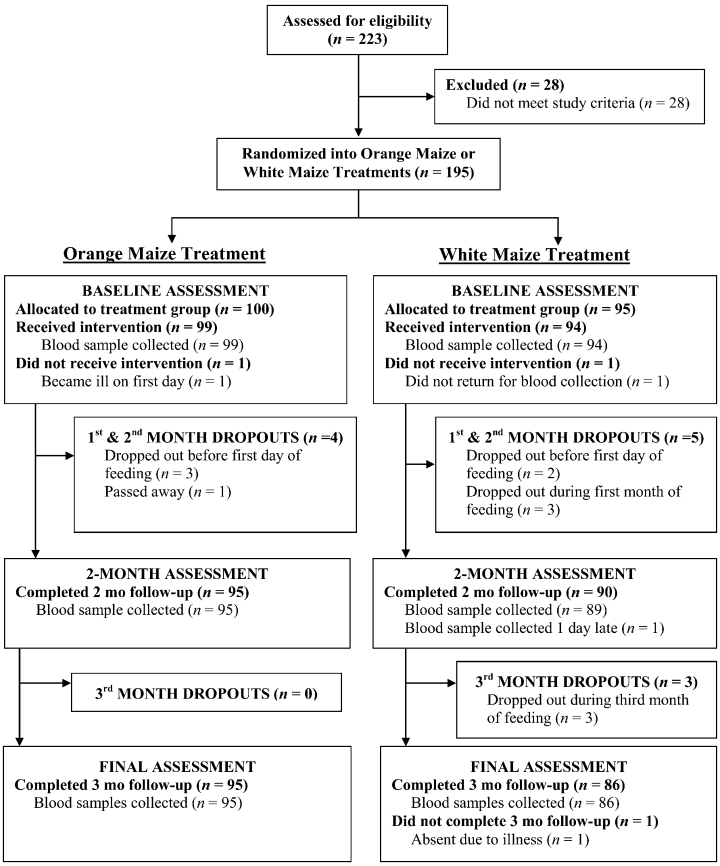

FIGURE 1 Enrollment and participant retention during a 3-mo randomized, controlled feeding trial with high provitamin A–biofortified (orange) maize in rural Zambian children aged 4.5 ± 0.9 y. Initial enrollment included 195 participants, 181 of whom completed all 3 assessment points in the study. Primary reasons for discontinuing study participation included noncompliance with study requirements and severe illness.

TABLE 1 Baseline and final anthropometric and biochemical measurements in Zambian children during a 70-d randomized, controlled feeding trial[1]

Measurements	Initial	Final	*P*
Weight, *kg*	14.8 ± 1.9	15.7 ± 2.0	<0.0001
Height, *cm*	98.4 ± 6.9	100.7 ± 6.7	<0.0001
Weight-for-height Z-score[2]	0.008 ± 0.86	0.010 ± 0.87	0.98
Markers of infection			
Malaria prevalence,[3] *%*	20.8	12.4	—
Parasite load >0[4]	17.4	11.2	—
Parasite load >1000	3.4	1.2	—
CRP,[5] *mg/L*	2.88 ± 3.47	3.04 ± 4.30	0.67
Elevated >5, *%*	15.7	12.9	—
Elevated >10, *%*	6.7	6.2	—
AGP,[5] *g/L*	1.42 ± 0.53	1.17 ± 0.50	<0.0001
Elevated >1.2, *%*	65.2	38.2	—

[1] Values are means ± SDs or percentages; $n = 181$. The data for orange and white maize groups were combined because no differences were observed (all $P > 0.05$), with the exception of initial serum ferritin, which was higher in the orange maize group (198 ± 232 μg/L) than the white group (128 ± 131 μg/L) ($P = 0.015$). Zambian children were aged 53.7 ± 10.2 mo at baseline. AGP, α_1–acid glycoprotein; CRP, C-reactive protein.
[2] WHO Child Growth Standards, ages 2–5 y (31).
[3] Overall malaria prevalence did not differ by treatment group or study week ($P > 0.05$); a decrease in total malaria prevalence was found when comparing the extremes ($P < 0.041$) (26).
[4] Parasite counts per 200 white blood cells.
[5] Raised serum concentrations of acute phase proteins were defined as CRP >5 or >10 mg/L and AGP >1.2 g/L (15,18–24).

adequate concentrations of liver vitamin A at both initial and final time points (<0.060; $P < 0.0001$), but the prevalence of children above this cutoff increased at the end of the intervention (**Table 4**).

Comparison of deficiency prevalence across infection stage. Similar patterns of significance were discovered for both CRP cutoffs of 5 and 10 mg/L. Anemia prevalence at the initial time point was elevated in the C+A+ group. At the final measurement, the C+A+ and C−A+ groups were found to have inflated estimations of anemia. The prevalence of iron deficiency anemia was low, and no difference was found among groups when using either CRP cutoff. Vitamin A deficiency, as determined by serum retinol concentration, was inflated in the C+A+ group and C−A+ group at the initial assessment. At the final time point, only the group with both acute phase proteins elevated, i.e., C+A+, was found to have an increased estimation of vitamin A deficiency by serum retinol concentrations. Estimations of insufficient vitamin A liver stores by the MRDR test were lower in the C−A+ group at initial assessment and higher in the C+A+ group at final assessment.

TABLE 2 Correlations between markers of micronutrient status and acute phase proteins in Zambian children at initial and final time points of a 70-d randomized, controlled feeding trial[1]

	Serum ferritin	Serum retinol	MRDR	CRP	AGP
Initial					
Hemoglobin	−0.298*	0.0210	0.124	−0.346*	−0.355*
Serum ferritin	**	−0.158†	−0.0510	0.465*	0.438*
Serum retinol	**	**	−0.148†	−0.225†	−0.149†
MRDR	**	**	**	−0.0270	−0.0932
CRP	**	**	**	**	0.478*
Final					
Hemoglobin	−0.353*	0.116	−0.189†	−0.264**	−0.436*
Serum ferritin	**	−0.156†	0.0584	0.392*	0.370*
Serum retinol	**	**	−0.334*	−0.292*	−0.169†
MRDR	**	**	**	0.133	0.114
CRP	**	**	**	**	0.446*

[1] Pearson's correlations were used to assess significant correlations between variables: *$P < 0.0001$, **$P < 0.001$, and †$P \leq 0.05$. AGP, α_1–acid glycoprotein; CRP, C-reactive protein; MRDR, modified relative dose response.

Discussion

The APR alters blood-based micronutrient status indicators, making it crucial to consider the impact of infection on the outcomes of nutrition interventions. This phenomenon was described in previous studies; however, a limited body of work focuses on the quantification of infection's impact on micronutrient status indicators, especially in areas with endemic malaria. The current study adds to the pool of data available for the development of standardized methods for the adjustment of micronutrient status indicators during the APR. In this study, the effect of the APR on hemoglobin, serum ferritin, serum retinol, and MRDR value was quantified in rural Zambian children at baseline and final time points of a 70-d feeding trial. Data were insufficient to investigate the impact of the incubation stage of infection (C+A−) because most children had elevated AGP living in this rural environment. The observed prevalence of anemia and vitamin A deficiency assessed by serum retinol concentration was significantly higher during the APR. Iron deficiency anemia prevalence was very low (1.0–4.1%), likely due in part to the marked inflation of 67 to 353% in serum ferritin values during infection. The MRDR value was unaffected by the APR, which is in agreement with one former report of this observation (21). The APR should be considered when measuring micronutrient status indicators that are reactive to it.

Blood-based micronutrient indicators are affected during the APR through the hepatic suppression of transport proteins (e.g., retinol-binding protein and transthyretin) and an increase in serum ferritin, itself a positive acute phase protein that assists iron sequestration (2,35,36). The magnitude of the APR varies by time stage and severity of infection. As reported in this study and by others, serum retinol and hemoglobin concentrations are negatively, and serum ferritin is positively, correlated with CRP and AGP concentration in children (10,18,24,37,38). Additionally, Duncan et al. (39) recently demonstrated that for serum retinol, and several other micronutrients assessed in the blood, the extent of alteration corresponded to the magnitude of the APR when assessed by CRP concentration in adults.

Serum retinol and ferritin concentrations typically return to preinfection values as the APR resolves (7–10) but are affected differently by the time stage of infection. Louw et al. (8) found changes in serum retinol concentrations to correspond to CRP concentration in patients who underwent orthopedic surgery. Serum ferritin concentrations remain elevated after CRP values normalized and AGP was still raised (9,21,40). Similar patterns of alteration during the APR were found for serum retinol and ferritin concentrations in the present study. Hemoglobin was suppressed in the current study and was also discovered to be so in rural British and Zanzibari children during the APR (38,41). Additionally, hemoglobin was previously, and in this work, found to be inversely related to serum ferritin (38), supporting its role as a reactive iron status indicator. However, Wieringa et al. (21) reported no effect of the APR on hemoglobin, and Das

TABLE 3 Initial and final markers of micronutrient status in Zambian children with various stages of infection as determined by the acute phase proteins during a 70-d randomized, controlled trial[1]

	CRP >5 mg/L					CRP >10 mg/L				
Markers of infection	*n*	Hemoglobin	Serum ferritin	Serum retinol	MRDR value	*n*	Hemoglobin	Serum ferritin	Serum retinol	MRDR value
		g/L	*μg/L*	*μmol/L*			*g/L*	*μg/L*	*μmol/L*	
Initial										
Total	178	102 ± 14	164 ± 193	0.96 ± 0.34	0.036 ± 0.038	178	102 ± 14	163 ± 193	0.96 ± 0.34	0.036 ± 0.038
C−A−	61	106 ± 13^{ab}	86.2 ± 79.0^{c}	1.01 ± 0.27^{a}	0.038 ± 0.025^{b}	62	106 ± 13^{ab}	94.4 ± 102^{c}	1.01 ± 0.26^{a}	0.038 ± 0.025^{b}
C+A−[2]	1	95	600	0.97	0.020	0	—	—	—	—
C+A+[2]	27	91 ± 14^{d}	387 ± 302^{a}	0.80 ± 0.30^{b}	0.034 ± 0.23^{b}	12	90 ± 15^{d}	431 ± 272^{a}	0.75 ± 0.31^{bc}	0.042 ± 0.027^{ab}
C−A+[2]	89	102 ± 13^{bc}	144 ± 148^{b}	0.98 ± 0.39^{a}	0.036 ± 0.047^{b}	104	101 ± 14^{bc}	174 ± 197^{b}	0.96 ± 0.38^{a}	0.035 ± 0.044^{b}
Final										
Total	178	105 ± 15	155 ± 277	0.96 ± 0.34	0.050 ± 0.028	178	105 ± 15	155 ± 277	0.96 ± 0.34	0.050 ± 0.028
C−A−	106	110 ± 12^{a}	86.7 ± 127^{c}	1.02 ± 0.31^{a}	0.045 ± 0.015^{ab}	109	110 ± 12^{a}	92.6 ± 139^{c}	1.02 ± 0.32^{a}	0.045 ± 0.015^{ab}
C+A−[2]	4	112 ± 16	266 ± 309	0.73 ± 0.15	0.050 ± 0.014^{a}	1	101	160	0.72	0.071
C+A+[2]	19	92 ± 18^{d}	392 ± 463^{a}	0.71 ± 0.32^{b}	0.055 ± 0.018^{a}	10	98 ± 16^{dc}	351 ± 327^{a}	0.68 ± 0.29^{c}	0.057 ± 0.013^{a}
C−A+[2]	49	100 ± 14^{c}	201 ± 355^{b}	0.95 ± 0.36^{a}	0.058 ± 0.046^{a}	58	98 ± 16^{c}	238 ± 405^{b}	0.92 ± 0.37^{ab}	0.057 ± 0.043^{a}
P values										
Overall	—	<0.0001	<0.0001	0.0008	0.0016	—	<0.0001	<0.0001	0.0052	0.0012
Infection effect	—	<0.0001	<0.0001	<0.0001	0.39	—	<0.0001	<0.0001	<0.0001	0.33
Time effect	—	0.61	0.49	0.37	<0.0001	—	0.20	0.23	0.62	0.0057
Interaction	—	0.22	0.60	0.69	0.17	—	0.078	0.47	0.90	0.17

[1] Values for micronutrient status are means ± SDs. Groups with $n \leq 4$ were excluded from the ANOVA. Serum ferritin was analyzed using logarithmic transformation. Letters within a column, excluding values for total population, indicate significant differences determined by least square difference. AGP, α_1–acid glycoprotein; C−A−, neither CRP nor AGP raised; C−A+, AGP raised only; C+A−, CRP raised only; C+A+, both CRP and AGP raised; CRP, C-reactive protein; MRDR, modified relative dose response.
[2] Raised serum concentrations of acute phase proteins were defined as CRP >5 or >10 mg/L and AGP >1.2 g/L (15,18–24).

et al. (42) found no correlation between hemoglobin and serum ferritin. Because the impact of the APR on hemoglobin is unclear, more work is needed to elucidate this relation.

CRP cutoffs of 5 or 10 mg/L are typically used to detect clinically relevant infections for a wide variety of micronutrients and populations (15,17–23); however, cutoffs relevant to shifts in micronutrient status indicators may be nutrient and age specific. Duncan et al. (39) found CRP cutoffs between 5 and 20 mg/L to be necessary to detect changes in blood concentration for various vitamins and minerals in adults, whereas Abraham et al. (43) found significant changes in blood nutrients, including serum ferritin and retinol, occurring at CRP concentrations of merely 0.26 mg/L in infants. CRP concentrations increase with age and tend to be higher in women than men (44). This increase

TABLE 4 Initial and final prevalence of micronutrient deficiencies by infection state as determined by acute phase proteins in Zambian children during a 70-d randomized, controlled trial[1]

	CRP >5 mg/L[2]					CRP >10 mg/L[2]				
Markers of infection	*n*	Anemia	Iron deficiency anemia	Vitamin A deficiency	Insufficient vitamin A liver stores	*n*	Anemia	Iron deficiency anemia	Vitamin A deficiency	Insufficient vitamin A liver stores
		%	%	%	%		%	%	%	%
Initial										
Total	178	70.2	1.1	21.9	11.8	178	70.2	1.1	21.9	11.8
C−A−	61	59.0	1.6	9.8	21.3	62	59.7	1.6	9.7	26.5
C+A−	1	0	100	100	100	0	—	—	—	—
C+A+[3]	27	88.9*	3.7	40.7*	7.4	12	91.7*	0	66.7**	16.7
C−A+[3]	89	71.9	0	24.7*	5.9*	104	74.0	1.0	24.0*	4.8*
Final										
Total	178	60.1	2.8	21.9	19.1	178	60.1	2.8	21.9	19.1
C−A−	106	46.2	2.8	14.2	13.2	109	45.9	2.8	14.7	12.8
C+A−	4	50.0	0	25.0	25.0	1	0	0	100	0
C+A+[3]	19	94.7**	0	57.9†	36.8*	10	90.0*	0	70.0†	40.0*
C−A+[3]	49	77.6†	4.1	24.5	24.5	58	81.0**	3.5	27.6	25.9

[1] Fisher's exact test was used to compare groups with elevated acute phase proteins with the group with no raised proteins: *$P \leq 0.05$, **$P < 0.0001$, and $^{\dagger}P < 0.001$. AGP, α_1–acid glycoprotein; C−A−, neither CRP nor AGP raised; C−A+, AGP raised only; C+A−, CRP raised only; C+A+, both CRP and AGP raised; CRP, C-reactive protein; MRDR, modified relative dose response.
[2] Micronutrient deficiencies were defined as follows: hemoglobin concentration of <110 g/L for anemia; anemia combined with serum ferritin concentration of <12 μg/L for iron deficiency anemia; serum retinol concentration of <0.70 μmol/L for vitamin A deficiency; and MRDR ratio of ≥0.060 for insufficient vitamin A liver stores (23,29,30,32–34).
[3] Raised serum concentrations of acute phase proteins were defined as CRP >5 or >10 mg/L and AGP >1.2 g/L (15,18–24).

is in part related to chronic inflammatory conditions (15,16), suggesting that a higher CRP cutoff may be necessary to assess acute infections in adults. In the current study, no differences were discovered in sensitivity between 5- and 10-mg/L CRP cutoffs, supporting the usefulness of the 5-mg/L cutoff for vitamin A and iron status assessments in children when AGP is also elevated. More research aimed at investigating CRP cutoffs of ≤5 mg/L on micronutrient status indicators in children is needed and should include a variety of kits and tests available. Additionally, AGP identified more cases of infection than CRP (74.7–91.3% vs. 8.7–25.3%) in this study because AGP typically remains elevated for a longer period of time, and children have recurrent infections in many developing countries. The decrease in the prevalence of elevated AGP concentration between baseline and final time points is likely reflective of the decrease in malaria prevalence caused by seasonal effects (26). Assessment of both early- and late-stage acute phase proteins is essential to gain a true understanding of infection within a population when using traditional measures of status.

The MRDR test relies on the principle that retinol binding protein accumulates when vitamin A liver reserves are low and is measured by the ratio of 3,4-didehydroretinol:retinol in serum (29,30,32). The present finding that the MRDR value is unaffected by the APR is supported by Wieringa et al. (21), who similarly reported no change through APR time stages measured by CRP and AGP in Indonesian children. The lack of difference in MRDR value across infection stage implies that retinol binding protein continues to accumulate during deficiency despite infection stage and is present in the liver to bind with 3,4-didehydroretinol during a short-term challenge test. Furthermore, this finding suggests that 3,4-didehydroretinol release is somewhat suppressed proportionally to retinol, such that the ratio between the two remains the same.

Serum retinol concentration is a widely used indicator for vitamin A status and is recommended for population assessment by the WHO (33). However, serum retinol provides an inferior status assessment when compared with the MRDR test because serum retinol is homeostatically regulated over a wide range of liver reserves (32) and suppressed during infection by ~25%, according to the current study and previous reports (17,45). No effect of time was observed for hemoglobin, serum ferritin, or serum retinol; however, MRDR values were significantly affected, reflecting a decrease in liver vitamin A reserves between the baseline and final assessment points of the feeding trial. Demonstrating the difference in sensitivity between these 2 indicators, the MRDR test, but not serum retinol concentrations, was able to detect a decrease in vitamin A status from the metabolism of a vitamin A supplement that was administered during Child Health Week in Zambia before the trial began. The MRDR test offers more information than serum retinol concentrations alone for vitamin A status assessments. The finding of a decrease in liver stores after consuming a high-dose supplement is in agreement with studies in Indonesian children where MRDR values were low after consuming high-dose supplements but became elevated before the next scheduled supplement (46–49). Biofortified maize with modest concentrations of provitamin A carotenoids did not maintain the high liver stores after supplement intake and no difference was found between treatment groups. Biofortified maize is now available with 3 times the amount of provitamin A carotenoids (50). Future trials with biofortified maize should consider isotope dilution methods, which are more sensitive to changes in total body stores than dose response tests. Considering that the mean MRDR value in this group of children indicated adequate liver stores and that provitamin A carotenoids from staple crops will slowly increase total body retinol stores over time, isotope dilution techniques may be the only method to measure the influence of biofortified crops on vitamin A status under controlled conditions (51).

In conclusion, this study's findings demonstrate that the APR significantly alters blood-based indicators of population-based iron and vitamin A status indicators in children, resulting in over- and possibly underestimations of nutrient deficiency prevalence. Early- and late-stage acute phase proteins, such as CRP and AGP, can be effectively employed to assess infection stages; a CRP elevation cutoff of 5 mg/L was sufficient in this population to affect serum-based indicators. Incorporating blood measurements of acute phase proteins will improve micronutrient status assessment.

Acknowledgments

The authors thank Peter Crump (University of Wisconsin-Madison College of Agricultural and Life Sciences Statistical Consulting Service) for statistical analysis consultation; Kevin Pixley and Natalia Palacios Rojas for breeding, producing, and sending sufficient high provitamin A seed for grain production in Zambia; Natalia Palacios Rojas for assisting with fieldwork; Bhola Verma for securing land for grain production; Raphael Mutale and Eliab Simpungwe for coordination of maize grain production in Zambia; and Bryan Gannon for proofreading the manuscript. K.A.B. analyzed data and wrote the manuscript. K.A.B., S.A., E.N., and R.S. conducted the fieldwork; J.C. coordinated the blood sample collection and storage as well as analysis of blood samples for acute phase proteins and micronutrient status indicators; R.S. standardized the Tropical Diseases Research Center laboratory; and C.M. oversaw the staff at the National Food and Nutrition Commission and worked with district officers for conduct of the trial. As co-principal investigator, E.K. (deceased 17 May 2010) was essential in setting up this study in Zambia. S.A.T. designed the research, provided oversight at the field stations during the trial, provided input for the statistical analysis, and revised the manuscript. All authors read and approved the final manuscript.

Literature Cited

1. Gruys E, Toussaint MJM, Niewold TA, Koopmans SJ. Acute phase reaction and acute phase proteins. J Zhejiang Univ Sci B. 2005;6:1045–56.
2. Thurnham DI, McCabe GP. Influence of infection and inflammation on biomarkers of nutritional status with an emphasis on vitamin A and iron. In: WHO. Report: priorities in the assessment of vitamin A and iron status in populations, Panama City, Panama, 15–17 September 2010. Geneva, Switzerland: WHO; 2012.
3. Shankar AH. Nutritional modulation of malaria morbidity and mortality. J Infect Dis. 2000;182:S37–53.
4. Bhaskaram P. Micronutrient malnutrition, infection, and immunity: an overview. Nutr Rev. 2002;60:S40–5.
5. Solomons NW. Malnutrition and infection: an update. Br J Nutr. 2007;98:S5–10.
6. Katona P, Katona-Apte J. The interaction between nutrition and infection. Clin Infect Dis. 2008;46:1582–8.
7. Reddy V, Bhaskaram P, Raghuramulu N, Milton RC, Rao V, Madhusudan J, Krishna KV. Relationship between measles, malnutrition, and blindness: a prospective study in Indian children. Am J Clin Nutr. 1986;44:924–30.
8. Louw JA, Werbeck A, Louw ME, Kotze TJ, Cooper R, Labadarios D. Blood vitamin concentrations during the acute-phase response. Crit Care Med. 1992;20:934–41.
9. Feelders RA, Vreugdenhil G, Eggermont AMM, Kuiper-Kramer PA, van Eijk HG, Swaak AJG. Regulation of iron metabolism in the acute-phase

response: interferon γ and tumor necrosis factor α induce hypoferraemia, ferritin production and a decrease in circulating transferrin receptors in cancer patients. Eur J Clin Invest. 1998;28:520–7.
10. Mitra AK, Alvarez JO, Wahed MA, Fuchs GJ, Stephensen CB. Predictors of serum retinol in children with shigellosis. Am J Clin Nutr. 1998;68:1088–94.
11. Raynes JG. The acute phase response. In: Kaufmann SHK, Steward MW, editors. Tropley and Wilson's microbiology and microbial infection. 10th ed. London: Hodder Arnold Ltd; 2014.
12. Heinrich PC, Castell JV, Andus T. Interleukin-6 and the acute phase response. Biochem J. 1990;265:621–36.
13. Clyne B, Olshaker JS. The C-reactive protein. J Emerg Med. 1999;17:1019–25.
14. Thompson D, Milford-Ward A, Whicher JT. The value of the acute phase protein measurement in clinical practice. Ann Clin Biochem. 1992;29: 123–31.
15. Stephensen CB, Gildengorin G. Serum retinol, the acute phase response, and the apparent misclassification of vitamin A status in the third National Health and Nutrition Examination Survey. Am J Clin Nutr. 2000;72:1170–8.
16. Ferrucci L, Corsi A, Lauretani F, Bandinelli S, Bartali B, Taub DD, Guralnik JM, Longo DL. The origins of age-related proinflammatory state. Blood. 2005;105:2294–9.
17. Thurnham DI, McCabe GP, Northrop-Clewes CA, Nestel P. Effects of subclinical infection on plasma retinol concentrations and assessment of prevalence of vitamin A deficiency: meta-analysis. Lancet. 2003;362: 2052–8.
18. Rosales FJ, Topping JD, Smith JE, Shankar AH, Ross AC. Relation of serum retinol to acute phase proteins in Papua New Guinea children. Am J Clin Nutr. 2000;71:1582–8.
19. Dijkhuizen MA, Wieringa FT, West CE, Martuti S, Muhilal. Effects of iron and zinc supplementation in Indonesian infants on micronutrient status and growth. J Nutr. 2001;131:2860–5.
20. Haskell MJ, Lembcke JL, Salazar M, Green MH, Peerson JM, Brown KH. Population-based plasma kinetics of an oral dose of [$^2H_4$]retinyl acetate among preschool-aged, Peruvian children. Am J Clin Nutr. 2003;77:681–6.
21. Wieringa FT, Dijkhuizen MA, West CE, Northrop-Clewes CA, Muhilal. Estimation of the effect of the acute phase response on indicators of micronutrient status in Indonesian infants. J Nutr. 2002;132:3061–6.
22. Northrop-Clewes CA. Interpreting indicators of iron status during an acute phase response – lessons from malaria and human immunodeficiency virus. Ann Clin Biochem. 2008;45:18–32.
23. Cogswell ME, Looker AC, Pfeiffer CM, Cook JD, Lacher DA, Beard JL, Lynch SR, Grummer-Strawn LM. Assessment of iron deficiency in US preschool children and nonpregnant females of childbearing age: National Health and Nutrition Examination Survey 2003–2006. Am J Clin Nutr. 2009;89:1334–42.
24. Paracha PI, Jamil A, Northrop-Clewes CA, Thurnham DI. Interpretation of vitamin A status in apparently healthy Pakistani children by using markers of subclinical infection. Am J Clin Nutr. 2000;72:1164–9.
25. Nuss ET, Arscott SA, Bresnahan K, Pixley KV, Rocheford T, Hotz C, Siamusantu W, Chileshe J, Tanumihardjo SA. Comparative intake of white- versus orange-colored maize by Zambian children in the context of promotion of biofortified maize. Food Nutr Bull. 2012;33:63–71.
26. Bresnahan KA, Chileshe J, Tanumihardjo SA. Quantification of food and nutrient intakes in Zambian children with and without malaria under controlled feeding conditions. Exp Biol Med (Maywood). 2014;239:45–51.
27. de Onis M, Blössner M. WHO global database on child growth and malnutrition [cited 2013 December 28]. Geneva, Switzerland: WHO; 1997. Available from: http://whqlibdoc.who.int/hq/1997/WHO_NUT_97.4.pdf
28. WHO. Guideline: vitamin A supplementation for infants 6–59 months of age [cited 2014 January 11]. Geneva, Switzerland: WHO; 2011. Available from: http://www.who.int/nutrition/ publications/micronutrients/guidelines/vas_6to59_months/en/
29. Valentine AR, Tanumihardjo SA. Adjustments to the modified relative dose response (MRDR) test for assessment of vitamin A status minimize the blood volume used in piglets. J Nutr. 2004;134:1186–92.
30. van Jaarsveld PJ, Faber M, Tanumihardjo SA, Nestel P, Lombard CJ, Benadé AJS. β-Carotene-rich orange-fleshed sweet potato improves the vitamin A status of primary school children assessed with the modified-relative-dose-response test. Am J Clin Nutr. 2005;81:1080–7.
31. WHO. Child growth standards: weight-for-height for boys and girls ages 2–5 yr [cited 2013 November 1]. Geneva, Switzerland: WHO. Available from: http://www.who.int/childgrowth/standards/ weight_for_height/en/index.html
32. Tanumihardjo SA. Vitamin A: biomarkers of nutrition for development. Am J Clin Nutr. 2011;94:658S–65S.
33. WHO. Serum retinol concentrations for determining the prevalence of vitamin A deficiency in populations [cited 2014 January 5]. Geneva, Switzerland: WHO; 2011. Available from: http://apps.who.int/iris/bitstream/10665/85859/4/ WHO_NMH_NHD_MNM_11.3_eng.pdf.
34. WHO. Haemoglobin concentrations for the diagnosis of anaemia and assessment of severity [cited 2014 January 5]. Geneva, Switzerland: WHO; 2011. Available from: http://www.who.int/vmnis/indicators/haemoglobin.pdf.
35. Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. N Engl J Med. 1999;340:448–54.
36. Wessling-Resnick M. Iron homeostasis and the inflammatory response. Annu Rev Nutr. 2010;30:105–22.
37. Beard JL, Murray-Kolb LE, Rosales FJ, Solomons NW, Angelilli ML. Interpretation of serum ferritin concentrations as indicators of total-body iron stores in survey populations: the role of biomarkers for the acute phase response. Am J Clin Nutr. 2006;84:1498–505.
38. Kung'u JK, Wright VJ, Haji HJ, Ramsan M, Goodman D, Tielsch JM, Bickle QD, Raynes JG, Stoltzfus RJ. Adjusting for the acute phase response is essential to interpret iron status indicators among young Zanzibari children prone to chronic malaria and helminth infection. J Nutr. 2009;139:2124–31.
39. Duncan A, Talwar D, McMillan DC, Stefanowicz F, O'Reilly DS. Quantitative data on the magnitude of the systematic inflammatory response and its effect on micronutrient status based on plasma measurements. Am J Clin Nutr. 2012;95:64–71.
40. Thurnham DI, Mburu ASW, Mwaniki DL, Wagt AD. Micronutrients in childhood and the influence of subclinical inflammation. Proc Nutr Soc. 2005;64:502–9.
41. Ramdath DD, Simeon DT, Wong MS, Grantham-McGregor SM. Iron status of schoolchildren with varying intensities of *Trichuris trichiura* infection. Parasitology. 1995;110:347–51.
42. Das BS, Thurnham DI, Das DB. Influence of malaria on markers of iron status in children: implications for interpreting iron status in malaria-endemic communities. Br J Nutr. 1997;78:751–60.
43. Abraham K, Müller C, Grüters A, Whan U, Schweigert FJ. Minimal inflammation, acute phase response and avoidance of misclassification of vitamin A and iron status in infants – importance of a high-sensitivity C-reactive protein (CRP) assay. Int J Vitam Nutr Res. 2003;73:423–30.
44. Woloshin S, Schwartz LM. Distribution of C-reactive protein values in the United States. N Engl J Med. 2005;352:1611–3.
45. Filteau SM, Morris SS, Abbott RA, Tomkins AM, Kirkwood BR, Arthur P, Ross DA, Gyapong JO, Raynes JG. Influence of morbidity on serum retinol of children in a community-based study in northern Ghana. Am J Clin Nutr. 1993;58:192–7.
46. Tanumihardjo SA, Permaesih D, Muherdiyantiningsih, Rustan E, Rusmil K, Fatah AC, Wilbur S, Muhilal, Karyadi D, Olson JA. Vitamin A status of Indonesian children infected with *Ascaris lumbricoides* after dosing with vitamin A supplements and albendazole. J Nutr. 1996;126: 451–7.
47. Allen LH, Haskell M. Estimating the potential for vitamin A toxicity in women and young children. J Nutr. 2002;132:2907S–19S.
48. Tanumihardjo SA, Permaesih D, Muhilal. Vitamin A status and hemoglobin concentrations are improved in Indonesian children with vitamin A and deworming interventions. Eur J Clin Nutr. 2004;58: 1223–30.
49. Tanumihardjo SA. Food-based approaches for ensuring adequate vitamin A nutrition. Compr Rev Food Sci Food Safety. 2008;7:373–81.
50. Schmaelzle S, Kaliwile C, Arscott SA, Gannon BM, Masi C, Tanumihardjo SA. Nutrient and nontraditional food intakes by Zambian children in a controlled feeding trial. Food Nutr Bull. 2014; 35:60–7.
51. Bouis H, Low J, McEwan M, Tanumihardjo S. Biofortification: evidence and lessons learned linking agriculture and nutrition [cited 2014 February 10]. Rome, Italy and Geneva, Switzerland: FAO and WHO; 2013. Available from: http://www.fao.org/food/nutritional-policies-strategies/icn2/expert-papers/en/.

CHAPTER 6

Conclusions and future directions

Kara Bresnahan

Interdepartmental Graduate Program in Nutritional Sciences

University of Wisconsin, Madison, WI 53706, USA

Vitamin A deficiency is a major global health problem with high prevalence in sub-Saharan Africa, South Asia, and regions of Latin America. The introduction of provitamin A biofortified staple crops may provide a cost-effective and sustainable method to alleviate vitamin A deficiency that prevents the cyclic liver reserves and toxicity risk associated with preformed vitamin A supplementation and fortification. However, the effects of a variety of factors can impact provitamin A carotenoid bioefficacy from plant foods. The research presented in this dissertation investigated several of these factors with a concentration on species of carotenoid, plant matrix effects, and host infection. Of paramount importance to nutrition-focused public health interventions, the impact of the acute phase response to infection on indicators of micronutrient status and estimates of deficiency prevalence is also examined in Zambian children.

The primary focus of Chapter 2 was the relative vitamin A values of 9-*cis*- and 13-*cis*-β-carotene compared with the all-*trans*-isomer. The bioefficacies of these *cis*-isomers is of interest because thermal processing of plant foods can considerably increase their concentration. Using Mongolian gerbils and moderate (30 nmol) oil doses, the 9-*cis*- and 13-*cis*-isomers showed relative vitamin A values of 63 and 70%, respectively, when compared with all-*trans*-β-carotene, which are greater than the value currently accepted by the Institute of Medicine. This work is of merit because it is the only investigation of *cis*-isomer bioefficacy using doses reflective of dietary intake in an animal model that metabolizes carotenoids similarly to humans. Moreover, this work suggests that *cis*-isomer generation due to thermal processing may not significantly decrease the vitamin A value of plant foods. A previous study, implementing much

larger oil doses, reported lower bioefficacies of the *cis*-isomers, suggesting more work is needed to determine the effect of dose size and vitamin A status on the bioavailability of β-carotene supplements. *In vivo* isomerization of all isomers was apparent, with *9-cis*-β-carotene appearing to preferentially accumulate in the liver and all-*trans*-retinol in the serum. The mechanisms of isomerization remain to be fully elucidated.

Chapter 3 examined the impact of matrix effects and thermal processing on the bioavailability of provitamin A carotenoids in various cultivars of staple crop banana in Mongolian gerbils. The results presented in this manuscript assisted plant breeders in the selection of banana cultivars for continued advancement in biofortification initiatives in Uganda and Australia. Thermal processing was found to decrease resistant starch concentration in banana diets and increase provitamin A carotenoid bioavailability. No differences were observed in resistance starch concentration or provitamin A carotenoid bioavailability of ripe versus unripe banana diets. Interestingly, resistant starch concentration corresponded to changes in dietary intake, but final gerbil body weights did not differ, suggesting a greater indigestible component in uncooked banana diets. More work is needed to establish a causal relationship between matrix effects in plant foods and provitamin A carotenoid bioavailability. Studies examining the influence of thermal processing on this relationship would also be beneficial.

Chapters 4 and 5 consider the impact of infection on dietary intake and blood-based indicators of micronutrient status in preschool-age, rural Zambian children during a 70-day, controlled feeding trial with high provitamin A carotenoid biofortified (orange) maize. The feeding trial was a collaborative initiative between the Tanumihardjo Laboratory, HarvestPlus, and Zambian governmental organizations. Dietary intakes were

similar overall between orange and white maize groups and no differences were found between groups in regards to malaria prevalence or decreases in intake during infection. A common 3-day malaria episode was associated with a decreased intake of 11% for food and calories, 10% for macronutrients, 9% for vitamin A, 13% for iron, and 14% for zinc. The loss in vitamin A intake is equivalent to 5-6% of the RDA. Given the deficits due to appetite suppression and increased urinary retinol excretion during infection, vitamin A requirements are likely higher in regions with endemic infection. Provitamin A carotenoid biofortification targets, which are currently set to meet 50% of the EAR based on food intake, may need to be increased to meet the needs of target populations.

The acute phase response to infection alters plasma protein concentrations, thus impacting micronutrient status indicators. Using biochemical data from baseline and final assessment points, common markers of vitamin A (serum retinol and the modified relative dose response test [MRDR]) and iron (hemoglobin and serum ferritin) status were assessed across infection stages identified by early and late stage acute phase proteins. Serum retinol and hemoglobin concentration were decreased during infection, leading to inflated estimations of vitamin A deficiency and anemia, respectively. Serum ferritin increased drastically by 67-132%, likely decreasing estimations of iron deficiency anemia. The MRDR was unaffected by infection, but increased by timepoint, demonstrating a decrease in vitamin A liver reserves with orange maize on the background of semiannual vitamin A supplementation. These results support MRDR as a superior and more sensitive vitamin A status indicator than serum retinol concentrations. More research is needed to determine the impact of the acute phase response to infection on the vitamin A stable isotope dilution test, which is considered the gold standard for

status assessment next to direct liver measurement. Currently, a standardized method for adjusting micronutrient status indicators during infection does not exist. It is essential to establish universal cutoff points for acute phase proteins as indicators of infection, which may need to be nutrient and population specific. The acute phase response should be assessed and micronutrient status indicators adjusted when using micronutrients that are sensitive to it in populations with high or unknown prevalence of infection.

The chapters presented in this thesis have important implications for provitamin A carotenoid biofortification efforts and emphasize the value of multidisciplinary collaboration in the successful application of scientific findings to human problems. In order to be successful, biofortified crops must possess desirable agronomic characteristics so as to be economically viable, demonstrate efficacy as a vehicle for the delivery of vitamin A, and be partnered with ongoing educational initiatives to promote acceptance by the target population. The role of nutritionists in this process is crucial to the development of superior provitamin A biofortified staple crops. The results presented here are valuable to biofortification initiatives because they expose factors impacting the vitamin A value of staple crops as well as the assessment of nutritional status of target populations.

APPENDIX

Comparative intake of white- versus orange-colored maize by Zambian children in the context of promotion of biofortified maize

Emily T. Nuss, Sara A. Arscott, Kara Bresnahan, Kevin V. Pixley, Torbert Rocheford, Christine Hotz, Ward Siamusantu, Justin Chileshe, and Sherry A. Tanumihardjo

Abstract

Background. *Vitamin A deficiency is associated with poor health outcomes related to reproduction, growth, vision, and immunity. Biofortification of staple crops is a novel strategy for combating vitamin A deficiency in high-risk populations where staple food intakes are high. African populations are proposed beneficiaries of maize (*Zea mays*) biofortified with provitamin A carotenoids, often called "orange maize" because of its distinctive deep yellow-orange kernels. The color facilitates ready recognition but presents a cultural challenge to maize-consuming populations, including those in much of Africa, who traditionally eat white varieties.*

Objective. *This study explores the intake patterns of, as well as adaptation to, traditional foods made with provitamin A–biofortified maize compared with white maize in rural Zambian children 3 to 5 years of age (*n = *189) during a 3-month feeding trial.*

Methods. *The subjects were fed a breakfast of maize porridge (sweet mush), a lunch of maize* nshima *(stiff mush) with various side dishes, and an afternoon snack based on a 6-day rotating menu. The trial was conducted in 2010. The orange maize used in the trial came from three different sources. O1 maize was from the 2009 harvest and was stored in a freezer until use in 2010. O2 maize was also from the 2009 harvest and was stored in a cold room until 2010. O3 ("fresh") maize was from the 2010 harvest and was fed immediately after harvest in week 9 of the study and then stored in a freezer until milling for the final four weeks.*

Results. *Consumption of menu items, except snacks, was influenced by week ($p < .0084$). The intakes of porridge and nshima made with orange maize equaled those of porridge and nshima made with white maize from week 2 onward. The intakes of porridge and nshima prepared from O1 and O2 did not differ, but intakes became significantly higher when meals made from O3 were introduced ($p < .014$ for porridge and $p \leq .013$ for nshima).*

Conclusions. *These results demonstrate quick adaptation to orange maize, a preference for recently harvested maize, and an optimistic outlook for similar adaptation patterns in other biofortified-maize target countries.*

Key words: Africa, β-carotene, food intake, maize (*Zea mays*)

Introduction

More than 2 billion people are affected by deficiencies of micronutrients, particularly iron, zinc, and vitamin A, with the highest rates in regions of sub-Saharan Africa, parts of Asia, and Latin America [1]. Chronic undernutrition related to micronutrient deficiencies not only has a negative impact on human health but also can hinder community mobilization through reduced physical productivity, increased morbidity and mortality, and impaired cognitive development [2]. Vitamin A is needed for reproduction, growth, vision, and immunity. Vitamin A deficiency is the leading preventable cause of blindness and continues to be a major problem in more than half of the world's countries. An estimated 250,000 to 500,000 children become blind each year due to vitamin A deficiency, and half of them die within 12 months after losing their sight [3].

Emily T. Nuss, Sara A. Arscott, Kara Bresnahan, Sherry A. Tanumihardjo, and Kevin V. Pixley are affiliated with the University of Wisconsin, Madison, Wisconsin, USA; Kevin V. Pixley is also affiliated with the International Maize and Wheat Improvement Center (CIMMYT), Texcoco, Mexico; Torbert Rocheford is affiliated with Purdue University, West Lafayette, Indiana, USA; Christine Hotz was affiliated with HarvestPlus, Washington, DC; Ward Siamusantu is affiliated with the National Food and Nutrition Commission, Lusaka, Zambia; Justin Chileshe is affiliated with the Tropical Diseases Research Centre, Biomedical Sciences Department, Ndola, Zambia.

Please direct queries to the corresponding author: Sherry A. Tanumihardjo, University of Wisconsin-Madison, Department of Nutritional Sciences, 1415 Linden Dr., Madison, WI, 53706, USA; e-mail: sherry@nutrisci.wisc.edu.

Current strategies for alleviating vitamin A deficiency are expansive and progressive. For decades, supplementation programs have been distributing high-dose vitamin A capsules to high-risk women and children [4]. In addition, extrinsic vitamin A fortification of commonly consumed foods, such as flour, oil, and sugar, has been widely encouraged [5, 6]. Supplementation and fortification, nonetheless, have limitations related to sustainability and coverage. "Biofortification," an umbrella term describing intrinsic enhancement of nutrient synthesis, accumulation in staple crops, or both, is receiving increased global attention [7, 8]. Biofortification has been achieved through the use of fertilizer, conventional breeding techniques, mutagenesis, and transgenic methods [9, 10]. A major difference among nutrition strategies is that supplementation and extrinsic fortification supply vitamin A as preformed retinol, whereas biofortification provides provitamin A carotenoids. Biofortification of staple crops to alleviate vitamin A deficiency would provide consistent dietary intakes of small amounts of provitamin A carotenoids, which over time can facilitate vitamin A adequacy [11, 12]. Biofortification programs may also provide an alternative to high-dose vitamin A supplements, in consideration of recent concern about their effectiveness [13]. Biofortified crops may also decrease dependence on food aid by creating sustainable opportunities for domestic farmers to expand their agricultural capacities [14].

Maize is a staple food for more than 200 million people. With nearly 750 million MT harvested annually [15], maize provides the world with an estimated 15% of total protein and 20% of total calories [16]. Maize kernels supply many macronutrients and micronutrients; however, concentrations of provitamin A carotenoids are extremely low in white cultivars [17]. The importance of maize as a traditional staple food in Zambia is paramount. Maize was introduced to African agriculture in the 16th century, and today Zambians derive more than half of their calories from this source [18]. Maize is eaten as milled flour and whole kernels, which are cooked into sweet or savory dishes. Fresh, "green" maize is eaten roasted or boiled. Most maize is consumed in either of two forms: as flour, locally called "mealie meal," which is most often cooked with water into a thick sweet porridge; or as a stiff mush called *nshima*, which is consumed in large quantities in at least two meals a day by most Zambians. Maize meal is also used for breakfast porridges, fermented drinks, and sweet breads [19].

Most people in Africa consume white maize; therefore, replacement of white maize with provitamin A–biofortified varieties might have a significant and positive impact on vitamin A status for populations who subsist on maize. Biofortified maize, which is characterized by a deep yellow to orange phenotype, has been tested using *in vitro* and animal models [20–23] and in small studies in humans [24, 25]. The target level for biofortification of maize grain is 15 μg of provitamin A per gram dry weight [8], which would provide the estimated average requirement of vitamin A to children who consume 200 g of dry maize a day. Unfortunately, although recent studies in Zambia, Zimbabwe, Kenya, and Mozambique suggest that acceptance is likely [26–28], the obvious yellow-orange color of the biofortified kernels represents an obstacle to acceptance by consumers accustomed to eating almost exclusively white maize. This study tested the intake patterns of, and adaptation to, biofortified maize in Zambian children during a 3-month intervention by comprehensively assessing food intake patterns, between and within feeding groups, as a function of time.

Materials and methods

Subjects and maize

All procedures involving human subjects were approved by the Tropical Diseases Research Centre Ethics Review Committee in Zambia and the University of Wisconsin's Health Sciences Human Subjects Institutional Review Board. Written informed consent was obtained from the parents or caregivers. The trial was conducted in 2010 in Nyimba District of the Eastern Province of Zambia on children 3 to 5 years of age (n = 189 initial enrollment). The anticipated dropout rate was 10% of initial enrollment, and the target number was set higher than what was actually needed to accommodate dropouts. The number of observations during the feeding gave us greater than 90% power to detect a difference between groups during the transition to orange maize. Nyimba District was selected because local communities had expressed high interest in participating in the study following community sensitization programs related to orange maize performed by the National Food and Nutrition Commission (Lusaka, Zambia) in 2009. Using white and orange dots placed in opaque envelopes, we randomly assigned 27 to 35 children in each of six rural villages to receive white maize or biofortified orange maize. The subjects were screened for absence of severe anemia (hemoglobin < 7.0 g/dL) and malnutrition (< –3 SD weight-for-age or height-for-age) [30]. All subjects and their families were educated and encouraged to participate in open discussions about the health benefits of orange maize in their local languages before recruitment, during enrollment, and at various community meetings throughout the trial to extinguish rumors and facilitate cooperation. Field notebooks were kept by researchers to record successes and problems.

The children were fed three times per day (breakfast,

lunch, and snack), 6 days per week, for a total of 70 days of feeding, excluding Sundays. All biofortified maize used in this study was from conventional breeding programs that select for increased provitamin A kernel content. Because of the length of the feeding trial and the limited scale of biofortified maize production, orange maize from two harvest cycles was used over the course of the trial. O1 maize was from the 2009 harvest and was stored in a freezer until use in 2010. O2 maize was also from the 2009 harvest and was stored in a cold room until 2010. O3 ("fresh") maize was harvested in 2010 and was fed immediately after harvest in week 9 of the study and then stored in a freezer and milled weekly for the final four weeks (**fig. 1**). O1 and O2 were the same genotype (CI7XDexp3), and O3 was a bulk mixture of grain from yield trials of advanced experimental provitamin A–biofortified hybrids. White maize was typical maize purchased from a local mill. The progression of orange maize feeding occurred due to limited freezer capacity to store all of the 2009 harvested orange maize. Within the orange-maize group, we had greater than 90% power to detect a difference in intake between dishes made from O1 and O3 and a 70% power to detect a difference in intake between dishes made from O2 and O3.

Project menu

The project menu was established based on commonly consumed foods and cooking methods outlined by a recent national food intake survey (U. Palaniappan and C. Hotz, personal communication). A 6-day rotation of traditional recipes provided dietary variety (**fig. 2**). Before the start of the trial, all food recipes were tested for general acceptance by a team of Zambian nutritionists who were also responsible for overseeing the study's six feeding sites. Whole-grain roller meal flour was chosen for use in the porridge and nshima. Although side dishes ("relishes") at lunch and a snack were provided each day, maize consumption was of primary interest. Serving sizes of maize dishes, based on the national food intake survey and discussion with Zambian nutritionists, provided children with a total of 158 g of dry maize per day (41 g in breakfast porridge and 117 g in lunch nshima). An evening meal was not provided, to reduce the burden on families and caregivers and based on the assumption that the subjects would not consume much at home because they were fed well while at the feeding centers. Consumption of any foods other than those provided by the project was strongly discouraged while the subjects were at the

Orange maize 1 (2009) 33–35 days	Orange maize 2 (2009) 11–13 days	Orange maize 3 (2010) 24 days
White maize (from local mill) 70 days		

FIG. 1. Maize progression timeline. The commencement of feeding occurred over 3 days, with two feeding sites beginning at a time. This "rollout" start caused the range of feeding days for orange maize feeding for O1 and O2 seen above

	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
Breakfast	Porridge with groundnuts (270 g)	Porridge with milk (270 g)	Porridge with groundnuts (270 g)	Porridge with milk (270 g)	Porridge with groundnuts (270 g)	Porridge with milk (270 g)
Lunch	Nshima (350 g)	Nshima (350 g)	Nshima (350 g)	Nshima (350 g)	Nshima (350 g)	Nshima (350 g)
	Kapenta + cowpea relish (150 g)	Chicken + cabbage with groundnuts relish (200 g)	Beans + cabbage relish (200 g)	Kapenta + pumpkin leaf relish (150 g)	Chicken + pounded cowpea relish (200 g)	Pumpkin leaf with groundnuts relish (175 g)
Snack	Tea + bread (250 g)	Guavas or bananas (100 g)	Bananas + roasted groundnuts (125 g)	Tea + biscuits (230 g)	Bananas (100 g)	Guavas or bananas + roasted groundnuts (125 g)

FIG. 2. Weekly rotating menu items and serving sizes for a 3-month intervention in rural Zambian children. Kapenta are small dried fish, and groundnuts are Zambian peanuts. Values listed in parentheses are the standardized serving sizes measured for each subject at that given feeding time. The serving size for porridge refers to a sweet maize meal mush mixed well with either milk or groundnut flour

feeding centers, and taking any project food from the feeding center was strictly prohibited.

Food waste records and equipment

Menu items were served to the children in standardized serving quantities (**fig. 2**) with tared dishes. The subjects were monitored by nutritionists for uneaten food, termed "food waste," which was quantified in grams as the difference between the weight of food waste in the serving dish and the weight of the dish. Menu items (porridge, nshima, relishes, and snack) were served in separate dishes so that records reflected food-specific intakes. Meal attendance was also recorded so that zero food waste resulting from absence of the child would not be mistaken for zero food waste resulting from complete meal consumption. Food waste records were facilitated by writing subject identification numbers on the children's bracelets or name tags and eating placemats. Food waste values were translated into "food consumed" by taking the difference between the standard serving size and food waste.

Digital kitchen scales (My-weigh KD7000, China) with a maximum capacity of 7,000 g were used to measure recipe ingredients, food portions, and food waste. The scales were automatically calibrated at the beginning of the study. The field staff checked that all scales were accurate within 2 g before and during the study.

Feeding environment

Feeding activities were standardized across all six villages in designated areas on site. The children sat at their placemats separated by treatment group (orange vs. white maize eaters) at each feeding time. Because children tend to want to be like their peers, the different color groups were separated so that they were not influenced by the children eating the other color of maize. Acceptability of the orange maize therefore was based more on taste than on peer influence. All food ingredients were measured by on-site Zambian nutritionists and prepared by locally recruited women using standardized recipes. Supervisors monitored the mealtimes to prevent food sharing and to ensure that each child remained at his or her placemat until feeding was completed. All subjects were equally encouraged to finish their food but were never forced, and most ate their food voluntarily. Family members and friends ate meals separately from the subjects but were allowed to assist their children if they desired.

Weekly intake patterns

For weekly intake patterns of menu items, days of feeding were translated into weeks of feeding to account for the 6-day rotating snack and relish combinations. A "week" in this analysis includes 6 days of project feeding, excluding the "rest day," translating to a complete cycle of the rotating project menu. Food intakes were calculated for each maize feeding group (orange and white) and categorized according to menu item (porridge, nshima, relish, and snack) for each week of feeding (weeks 1 to 12). The beginning and ending of feeding were staggered so that two sites began or ended per day during weeks 1 and 12 of the study (**fig. 1**). Since the rotating menu for all sites was identical by "weekday" and not "study day", comparison of "weeks" of feeding was done by aligning the weekdays instead of the study days.

Statistical analysis

The data were analyzed by ANOVA followed by least squares difference (LSD) of variance testing and linear regression of slopes using the SAS statistical package (version 9.2). Power analysis was done using the degrees of freedom and F values from our observations at $\alpha < .05$. For all comparisons, individual intakes were averaged per menu item according to the time period being considered (weekly intakes or orange maize feeding period). Individual means were then used to calculate feeding group means for the time period being considered. Two-way ANOVA was used to calculate significant differences between group means for each time period considered. All tests were two-sided, and $p < .05$ was considered to indicate a significant difference. Meal absences were excluded from the data analysis and were not related to treatment group. Values were rounded to the nearest gram to reflect the precision of the field scales.

Results

Subject data

Anthropometric data are given in **table 1**. Eight children (4%) dropped out, including two who left before the first day of feeding. The reasons for discontinuing participation in the study included persistent, severe illnesses and noncompliance with study requirements.

Between-group comparison of weekly intakes

The unique daily recording of each subject's food consumption allowed comparisons of maize and nonmaize item intakes over time, between feeding groups, and within the orange-maize group during the progression of meals made with O1, O2, and O3 maize. The mean weekly intakes of menu items generally did not differ between groups (**fig. 3**), with decreasing variance in intake over time. However, at weeks 1 and 4, the intake of porridge was greater in the white-maize group than

TABLE 1. Baseline anthropometric measurements by treatment group (mean ± SD)

Measurement	Orange maize (n = 96	White maize (n = 93)	All subjects (n = 189)
Weight (kg)	14.8 + 1.9	14.7 + 2.1	14.8 + 2.0
Height (cm)	98.3 + 6.9	98.5 + 7.2	98.4 + 7.0
Age (mo)	53.0 + 10.0	55.1 + 11.3	54.0 + 10.7
Weight-for-height z score[a]	−0.22 + 0.87	−0.18 + 0.92	−0.20 + 0.90

a. World Health Organization Child Growth Standards, ages 2–5 years [29].

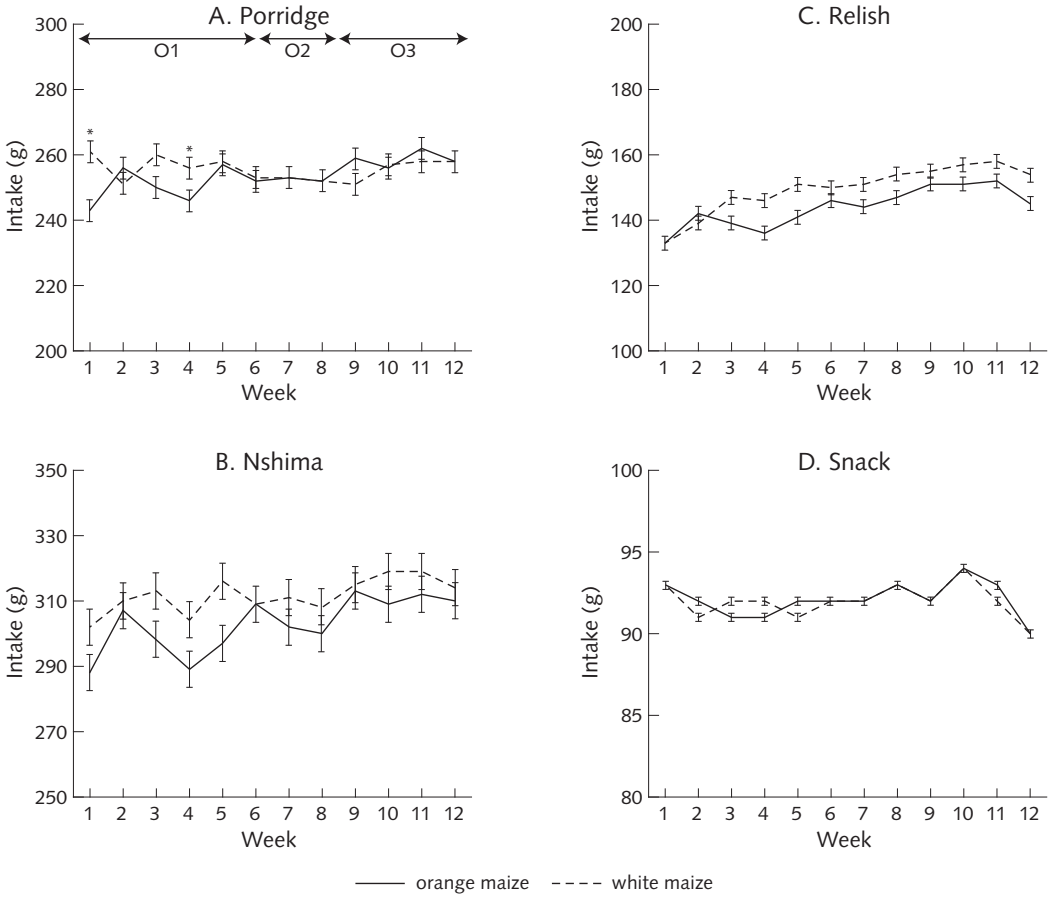

FIG. 3. Weekly intakes (mean ± SE) of maize-based (A and B), relish (C), and snack (D) items by treatment group in rural Zambian children. Arrows represent the periods when different types of orange maize (O1, O2, O3) were served. Overall, maize intake increased with time ($p < .0084$), and an interaction between group and week occurred for porridge intake ($p < .0001$). Differences between groups that were influenced by both group and week are denoted by an asterisk, where white porridge intakes were slightly higher than orange porridge intakes ($p < .05$). Relish intake consistently increased over time in both groups ($p < .0001$), and snack intake varied significantly by week (± 2 g, $p < .0001$)

in the orange-maize group by 17 and 10 g, respectively ($p < .05$). By week 2, the intake of porridge was equal in both groups, suggesting that the initial aversion to orange maize was only temporary. The intakes of nshima, relishes, and snacks did not differ between groups.

The total changes in intake between the first and final weeks were highly significant for all dishes made from orange or white maize except for porridge made from white maize. Consumption of porridge made from orange maize, nshima made from orange maize, and nshima made from white maize increased by 6.2%, 8.3%, and 5.4%, respectively (**fig. 3**). The intake of relishes increased by 12.2% in the orange-maize group and 16.0% in the white-maize group. These changes indicate that eating capacity increased during the trial.

Within-group changes in weekly intakes

In the orange-maize group, the mean porridge intake

was 251 g/day in the first 8 weeks of feeding and increased significantly by 3.0% at week 9, where it remained higher at 259 g/day for the final weeks. Interestingly, orange nshima intakes followed a very similar trend, i.e., a sharp, significant increase of 20 g (6.5%) from weeks 1 to 2, followed by another increase of 12 g (3.8%) at week 9 (**fig. 3**).

In the orange-maize group, the intakes of both porridge and nshima decreased nonsignificantly in week 4 by 1.6% and 3.0%, respectively, followed by significant increases of 4.3% and 2.3%, respectively, in week 5 (**fig. 3**). The intakes of both porridge and nshima also decreased in week 4 in the white-maize group. Despite these temporary decreases in intake, a consistent increasing trend was restored for the remainder of the feeding trial.

The pattern of relish intake was similar to that of maize-based dishes. Intake rapidly increased between weeks 1 and 2 in both feeding groups, by 8.5% in the orange maize group and 5.8% in the white-maize group. The overall intake of relishes increased through week 8, whereas the intake of snacks did not appreciably change. Snacks were well received, and intakes for both groups were 92 g/day (range, 89 to 94 g/day).

Intake patterns related to orange maize progression

The provitamin A contents of the maize varieties used in this trial are shown in **table 2**. Mean intakes of orange-maize meals prepared from O1, O2, and O3 were compared by LSD. The mean intakes were calculated for each individual subject and then averaged according to each feeding period for O1, O2, and O3 (**fig. 1**). The intakes of porridge and *nshima* prepared from O1 and O2 did not differ, but intakes became significantly higher when meals made from O3 were introduced ($p < .014$ for porridge and $p \leq .013$ for *nshima*) (**table 3**). This pattern complements the findings related to the increase by time analysis which found an increase at week 9 when recently harvested maize (O3) was introduced and fed until the end of the study.

TABLE 2. Characteristics of orange maize meal types (mean ± SD)

Type	Color	β-Carotene (nmol/g)	Theoretical vitamin A[a] (μg/g)
O1	Orange	10.4 ± 0.14	6.21 ± 0.09
O2	Orange-yellow	4.90 ± 0.85	2.95 ± 0.51
O3	Bright orange	7.54 ± 0.49	6.29 ± 0.39

a. Theoretical vitamin A includes all provitamin A carotenoids and not just β-carotene.

TABLE 3. Differences in intakes of Zambian dishes made from different types of orange maize[a]

	Intake (g)	
Types	Porridge	Nshima
O1 vs. O2	1.0 ($p = .739$)	4.2 ($p = .355$)
O2 vs. O3	7.0 ($p = .014$)	8.9 ($p = .013$)
O1 vs. O3	7.8 ($p = .001$)	14 ($p = .001$)

a. Differences are considered significant if $p < .05$.

Discussion

Maize has a distinct imprint across African landscapes, and 90% of cereal produced for human consumption in Africa is white maize [18]. The current preference for white maize among Africans is largely shaped by historical, political, and social influences. The aversion to yellow maize stems from prejudice and negative associations, such as associations with food aid and animal feed, rather than from its consumer characteristics [31]. Historically, production of yellow maize has been limited to large-scale commercial farmers for stock feed, and yellow maize has been considered for human consumption only when domestic production of white maize has been inadequate. For example, the drought of 1991/92 in Zambia required the government to import large quantities of maize for human consumption; this maize was mostly yellow and was sold at prices 10% to 35% lower than that of white maize [32]. In addition, greater allocation of research funds for white maize breeding programs has increased the yield of white maize more than that of yellow varieties [18, 31].

Despite any pre-existing cultural aversions toward yellow maize, the children in this feeding trial adapted to the biofortified orange maize dishes within the first week. After week 1, dietary intakes of maize-based dishes in the orange-maize group were essentially not different from those in the white maize group across the 70-day intervention. The field notes state that the children were reluctant to come fasted for the first few days of the feeding study, so the lower intake of porridge may have been caused, in part, by poor appetite resulting from off-site morning snacking. Although the study included a "pilot day," a run-in feeding period may be considered, depending on the objectives of future studies, if the subjects are unfamiliar with biofortified maize. The intake of orange maize was significantly lower than that of white maize at week 4 for both porridge and nshima; the difference may have been due to appetite changes related to seasonal morbidity or may have been a random effect. This observation is further supported by mean relish intakes, which also decreased that week. Changes in nutritional status may also have occurred during the intervention. The field notes record conversations with caretakers indicating that the children typically received one meal and

various small snacks over the course of the day instead of two full meals and a snack. Hunger does not always equate to food intake, because stomach capacity can be limited [33]. Perhaps underlying improvements in overall health, in addition to an expected increase in body size, in part supported by the nutritious feeding regimen that the rotating menu provided, resulted in increased food intake over the 3-month trial in all groups.

The field notes provide another perspective for intake patterns of relishes. For example, children were previously educated by society not to overindulge in relishes because their contents are "expensive" and "precious," and they are often shared among family members. The intake of relish increased more than that of any other menu item, perhaps because the children became accustomed to eating from a single, nonshared bowl. Similar trends in psychobehavioral feeding studies in children suggest that children require multiple encounters with a new food before they will taste or eat it [34–37]. Additional field notes suggest that children were particularly fond of the snack foods provided by the feeding study, especially tea and bread or biscuits.

Future efforts will target increased acceptance of orange maize in the developing world. Many factors must be considered for maize-eating populations, such as Zambians, to shift their preferences from white to orange provitamin A–biofortified maize varieties. Socioeconomic factors, such as education, income, and family and community traditions and customs, influence food choices, which include maize color preference. For example, consumer preference for white over yellow maize meal in Kenya is strongest among wealthier and more highly educated consumers [26], perhaps reflecting a societal perception of status because yellow maize has been associated with food aid. The cost of a maize meal greatly influences consumer acceptability or willingness to procure a certain type; experimental auctions in Kenya, for example, confirmed that consumers generally prefer white maize but would buy yellow maize at a price discount of 11% [38]. A similar observation was made using a simulated multimarket model based on supply and demand in Mozambique [39]. Aside from purchasing prices, consumer aversion to yellow maize decreases when people have had opportunities to become familiar with it, taste cooked products, or become educated about vitamin A [38]. Ninety-four percent of surveyed households in Zimbabwe reported that they would consume yellow maize meal if they were educated about its superior nutritional qualities compared with white maize [27]. A study in Mozambique concluded that existing preferences for white maize did not preclude the acceptance of biofortified varieties [28], demonstrating that maize meal preferences are malleable beyond preexisting biases.

The preference for O3 by the children in the orange maize group suggests that maize meal characteristics have a significant impact on acceptance of biofortified maize products. O3 maize was fed shortly after harvest in 2010 and did not go through the same long storage period as O1 and O2. Although the significant, positive intake trend at week 9 might have been influenced by an underlying appetite change in the later stages of the feeding trial, the mothers and field staff also preferred the newer orange maize to the older maize when sampling the leftovers. The patterns of intake most likely reflect rapid adaptation to nontraditional orange maize products, as shown by the initial increase between week 1 and 2, as well as a possible preference for O3 maize meal, which was introduced in week 9 and did not go through the long storage times like O1 and O2. This finding was strengthened by the significant 10-g (3.8%) decrease in consumption of white-maize porridge and the nonsignificant 9-g (2.9%) increase in consumption of white-maize nshima between weeks 1 and 2. Furthermore, the intake of relishes did not increase at week 9, suggesting that increases in intake of orange maize at and after that time were related to the favored O3 maize.

As mentioned above, the target level for biofortification of maize grain is 15 μg of β-carotene equivalents per gram dry weight, which would provide the estimated average requirement of vitamin A to children who consumed 200 g of dry maize per day [8]. The study menu provided a daily quantity that was 42 g below that estimated intake, which was based on survey data collected prior to study commencement in the same age group. A third daily meal or snack could increase this amount to 200 g. Orange maize used in this study had not reached the target provitamin A carotenoid concentrations. A few prospective cultivars included in ongoing field testing have met this target concentration.

Agricultural aspects must also be considered. A survey in Zimbabwe found that although only 6% of farmers grow yellow maize varieties, more than 90% of them would be willing to grow it if it had similar qualities to traditional white varieties (e.g., drought tolerance, early maturation, and pest resistance) [27]. Much of Africa is still skeptical of genetically modified organisms (GMOs), such as transgenic high-provitamin A maize [40, 41]. For this reason, selective plant husbandry programs, such as those used in this study, may be better received, because conventional breeding techniques are believed to maintain functionality of the plant in a more "natural" way than the techniques used with GMOs [42]. Furthermore, biofortified orange maize is likely to be embraced in regions where yellow maize is currently grown if the orange maize has similar agronomic, culinary, and sensory characteristics [38]. The Zambian Agricultural Research Institute, the

International Institute of Tropical Agriculture, and the International Maize and Wheat Improvement Center have specific research programs for breeding and testing provitamin A–biofortified maize in Zambia and elsewhere. Within the next decade, Zambian-grown orange maize is projected to be agronomically competitive with most popular white varieties. The results of this study, in coordination with plans to initiate the release of this orange maize to rural farmers, show promise of acceptance and sustained use of biofortified orange maize in communities most at risk for vitamin A deficiency.

Food intake can be influenced by many factors, including morbidity, climate, and social pressures. The findings from this intervention demonstrate modifiable preferences for maize meal color among individuals at young ages, which is of great importance for those interested in the future use of biofortified maize. In summary, preschool-aged children in rural Zambia were highly receptive to biofortified orange maize meals substituted for traditional white maize dishes. Future investigations may provide deeper insight into appetite and consumption patterns related to acceptance of orange maize meals.

Acknowledgments

Financial support for this study was provided by HarvestPlus (www.HarvestPlus.org), contract number 8217, a global alliance of agricultural and nutritional research institutions working to increase the micronutrient density of staple food crops through biofortification. The views expressed do not necessarily reflect those of HarvestPlus. This paper is dedicated to the memory of Dr. Emmanuel Kafwembe (deceased May 17, 2010) who was essential in setting up this project in Zambia. We thank Peter Crump, Senior Information Processing Consultant of the University of Wisconsin-Madison College of Agriculture and Life Sciences Statistical Consulting Service, for statistical assistance. We thank Uma Palaniappan, nutrition consultant, who assisted in finalizing the recipes to be used and provided logistic guidance while in Zambia. We give special thanks to Natalia Palacios, CIMMYT/HarvestPlus, for developing hybrids and planning trials, and Bhola Nath Verma, Kabamba Mwansa, Raphael Mutale, Herbert Masole, and Eliab Simpungwe, who assisted in procuring the maize for this trial in Zambia.

References

1. Micronutrient Initiative/United Nations International Children's Emergency Fund. Vitamin and mineral deficiency: A global progress report. 2004. Available at: http://www.micronutrient.org/CMFiles/PubLib/VMd-GPR-English1KWW-3242008-4681.pdf. Accessed 16 November 2011.
2. Allen L, de Benoist B, Dary O, Hurrell R, eds. Guidelines on food fortification with micronutrients. World Health Organization/Food and Agriculture Organization, 2006. Available at: http://www.who.int/nutrition/publications/micronutrients/guide_food_fortification_micronutrients.pdf. Accessed 16 November 2011.
3. World Health Organization. Micronutrient deficiencies: Vitamin A deficiency. 2010. Available at: http://www.who.int/nutrition/topics/vad/en/. Accessed 16 November 2011.
4. World Health Organization/United Nations International Children's Emergency Fund/ International Vitamin A Consultative Group Task Force. Vitamin A supplements: A guide to their use in the treatment of vitamin A deficiency and xerophthalmia, 2nd ed. Geneva: WHO, 1997. Available at: http://whqlibdoc.who.int/publications/1997/9241545062.pdf. Accessed 16 November 2011.
5. Micronutrient Initiative. Investing in the future: A united call to action on vitamin and mineral deficiencies. In: Global report summary 2009. Available at: http://www.unitedcalltoaction.org/documents/Investing_in_the_future_Summary.pdf. Accessed 16 November 2011.
6. Fortifying Africa's future. 2010. Available at: http://www.fortaf.org/index.htm. Accessed 16 November 2011.
7. Meenakshi JV. Cost-effectiveness of biofortification; Copenhagen Consensus Center Best Practice Paper: New Advice from cco8. 2008. Available at: http://www.copenhagenconsensus.com/Files/Filer/CCC/Best%20Practice%20Papers/bio.pdf. Accessed 16 November 2011.
8. Tanumihardjo SA, Bouis H, Hotz C, Meenakshi JV, McClafferty B. Biofortification of staple crops: An emerging strategy to combat hidden hunger. Compr Rev Food Sci Food Saf 2008;7:329–34.
9. Glenn KC. Nutritional and safety assessments of foods and feeds nutritionally improved through biotechnology: Lysine maize as a case study. J AOAC Int 2007;90:1470–9.
10. Zhu C, Naqvi S, Breitenbach J, Sandmann G, Cristou P, Capell T. Combinatorial genetic transformation generates a library of metabolic phenotypes for the carotenoid pathways in maize. Proc Natl Acad Sci USA 2008;105:18232–7.
11. Low JW, Arimond M, Osman N, Cunguara B, Zano F, Tschirley D. A food-based approach introducing orange-fleshed sweet potatoes increased vitamin A intake and serum retinol concentrations in young children in rural Mozambique. J Nutr 2007;137:1320–7.
12. Tanumihardjo SA. Food-based approaches for ensuring adequate vitamin A nutrition. Compr Rev Food Sci Food Saf 2008;7:373–81.
13. Latham M. The great vitamin A fiasco. World Nutr 2010;1:12–45.
14. International Maize and Wheat Improvement Center.

Maize. Global alliance for improving food security and the livelihoods of the resource-poor in the developing world. 2011. Available at: http://www.cimmyt.org/index.php/en/what-we-do/maize-and-wheat-cgiar-programs. Accessed 16 November 2011.
15. US Department of Agriculture and Food Agriculture Service. Corn 2008: Production, supply, demand database. 2008. Available at: http://www.pecad.fas.usda.gov/cropexplorer/. Accessed 16 November 2011.
16. Brown WL, Bressani R, Glover DV, Hallauer AR, Johnson VA, Qualset CO. Malnutrition and protein quality. In: Board on Science and Technology for International Development, National Research Council, in cooperation with the Board on Agriculture, National Research Council. Quality-protein maize: Report of an ad hoc panel of the advisory committee on technology innovation. Washington, DC: National Academy Press, 1988:1-7.
17. Nuss ET, Tanumihardjo SA. Maize: A paramount staple crop in the context of global nutrition. Compr Rev Food Sci Food Saf 2010;9:417–36.
18. McCann JC. Africa and the world ecology of maize. In: McCann JC, ed. Maize and grace: Africa's encounter with a New World crop, 1500-2000. Cambridge, Mass, USA: Harvard University Press, 2005:1–22.
19. Taylor SD. Cuisine and traditional dress. In: Toyin Falola S, ed. Culture and customs of Zambia. Westport, Conn, USA, and London: Greenwood Press, 2006:75–90.
20. Thakkar SK, Failla ML. Bioaccessibility of pro-vitamin A carotenoids is minimally affected by non pro-vitamin A xanthophylls in maize (*Zea mays* sp.). J Agric Food Chem 2008;56:11441–6.
21. Howe JA, Tanumihardjo SA. Carotenoid-biofortified maize maintains adequate vitamin A status in Mongolian gerbils. J Nutr 2006;136:2562–7.
22. Davis CR, Howe JA, Rocheford T, Tanumihardjo SA. The xanthophyll composition of biofortified maize (*Zea mays* sp.) does not influence the bioefficacy of provitamin A carotenoids in Mongolian gerbils (*Meriones unguiculatus*). J Agric Food Chem 2008;56:6745–50.
23. Davis CR, Jing H, Howe JA, Rocheford T, Tanumihardjo S. β-Cryptoxanthin from supplements or carotenoid enhanced maize maintains liver vitamin A in Mongolian gerbils (*Meriones unguiculatus*) better than or equal to β-carotene supplements. Br J Nutr 2008;100:786–93.
24. Li S, Nugroho A, Rocheford T, White WS. Vitamin A equivalence of the β-carotene in β-carotene biofortified maize porridge consumed by women. Am J Clin Nutr 2010;92:1105–12.
25. Muzhingi T, Gadaga TH, Siwela AH, Grusak MA, Russell RM, Tang G. Yellow maize with high β-carotene is an effective source of vitamin A in healthy Zimbabwean men. Am J Clin Nutr 2011;94;510-9.
26. De Groote H, Chege Kimenju S. Comparing consumer preferences for color and nutritional quality in maize: Application of a semi-double-bound logistic model on urban consumers in Kenya. Food Policy 2008;33:362–70.
27. Muzhingi T, Langyintuo AS, Malaba LC, Banziger M. Consumer acceptability of yellow maize products in Zimbabwe. Food Policy 2008;33:352–61.
28. Stevens R, Winter-Nelson A. Consumer acceptance of provitamin A-biofortified maize in Maputo, Mozambique. Food Policy 2008;33:341–51.
29. World Health Organization. Child growth standards: Weight-for-height for boys and girls ages 2–5 yr. 2011. Available at: http://www.who.int/childgrowth/standards/weight_for_height/en/index.html. Accessed 16 November 2011.
30. de Onis M, Blössner M. WHO global database on child growth and malnutrition. Geneva: World Health Organization, 1997. Available at: http://whqlibdoc.who.int/hq/1997/WHO_NUT_97.4.pdf. Accessed 16 November 2011.
31. Food and Agriculture Organization/International Maize and Wheat Improvement Center. White maize: A traditional food grain in developing countries. 1997. Available at: http://www.fao.org/docrep/w2698e/w2698e00.htm. Accessed 16 November 2011.
32. Rubey L, Ward RW, Tschirley D. Maize research priorities: The role of consumer preferences. In: Byerlee D, Eicher CK, eds. Africa's emerging maize revolution. London: Lynne Publishing, 1997:145–55.
33. Patel D, Gupta P, Shah D, Sethi K. Home-based rehabilitation of severely malnourished children in resource poor setting. Indian Pediatr 2010;8:694–701.
34. Addessa E, Galloway AT, Birch L, Visalberghi E. Taste perception and food choices in capuchin monkeys and human children. Primatologie 2004;6:101–28.
35. Birch LL, Marlin DW. I don't like it; I never tried it: Effects of exposure on two-year-old children's food preferences. Appetite 1982;4:353–60.
36. Sullivan SA, Birch LL. Infant dietary experience and acceptance of solid foods. Pediatrics 1994;2:271–7.
37. Birch LL, McPhee L, Shoba BC, Pirok E, Steinberg L. What kind of exposure reduces children's food neophobia? Looking vs. tasting. Appetite 1987;9:171–8.
38. De Groote H, Chege Kimenju S, Morawetz UB. Estimating consumer willingness to pay for food quality with experimental auctions: The case of yellow versus fortified maize meal in Kenya. Agric Econ 2010;1–16.
39. Dorosh P, del Ninno C, Sahn DE. Poverty alleviation in Mozambique: A multi-market analysis of the role of food aid. Agric Econ 1995;13:89–99.
40. Chege Kimenju S, De Groote H. Consumer willingness to pay for genetically modified food in Kenya. Agric Econ 2008;38:35–46.
41. Eicher CK, Maredia K, Sithole-Niang I. Crop biotechnology and the African farmer. Food Policy 2006;31:504–27.
42. Manshardt R. Crop improvement by conventional breeding or genetic engineering: How different are they? Biotechnology 2004;5:1–3.