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Linoleic Acid:Dihomo-γ-Linolenic Acid Ratio Predicts the Efficacy of Zn-Biofortified Wheat in Chicken (*Gallus gallus*)

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Supporting Information

ABSTRACT: The amount of Zn absorbed from Zn-biofortified wheat material has been determined using an *in vivo* model of Zn absorption. The erythrocyte linoleic:dihomo -γ-linolenic acid (LA:DGLA) ratio was used as a biomarker of Zn status. Two groups of chickens (n = 15) were fed different diets: a high-Zn (46.5 μg Zn g^{-1}) and a low-Zn wheat-based diet (32.8 μg Zn g^{-1}). Dietary Zn intakes, body weight, serum Zn, and the erythrocyte fatty acid profile were measured, and tissues were taken for gene expression analysis. Serum Zn concentrations were greater in the high Zn group ($p < 0.05$). Duodenal mRNA expression of various Zn transporters demonstrated expression upregulation in the birds fed a low Zn diet (n = 15, $p < 0.05$). The LA:DGLA ratio was higher in the birds fed the low Zn diet ($p < 0.05$). The higher amount of Zn in the biofortified wheat resulted in a greater Zn uptake.

KEYWORDS: *zinc, Zn biofortification, wheat, Zn transporters, LA:DGLA, Zn biomarker*

INTRODUCTION

Zinc (Zn) deficiency is a worldwide health problem that is projected to affect 17% of the total world's population.[1] Zn inadequacy has been related to poor growth, reduced immune function, increased susceptibility to and severity of infection, neurobehavioral abnormalities, and adverse outcomes of pregnancy.[2−5] Zn deficiency is a major cause of early childhood morbidity and mortality.[6] In many developing countries, Zn deficiency is attributable to the low consumption of animal products, and an increased intake of cereals that contain substantial amounts of phytate, which is a compound known to inhibit Zn absorption.[7] Wheat is one of those cereals with a high phytate content, yet it is a major food staple for almost a half of the worlds' population.[8] In many developing countries (i.e., North Africa and the eastern Mediterranean), nearly 50% of the daily food energy originate from wheat.[2] However, after significant extraction, the remaining white flour of modern wheat cultivars is inherently poor in Zn.[9]

The wide-ranging occurrence of Zn deficiency in developing countries and a large number of people dependent on wheat as a major food source, encouraged the development of biofortified wheat varieties with enhanced Zn concentration. Biofortification, the delivery of Zn via staple food crops, has been proposed to facilitate current efforts for the alleviation of Zn deficiency.[10] The trait for high Zn concentration in grain can be backcrossed into local varieties that are suited to particular regional conditions.[11−13]

While these conventional plant breeding initiatives are particularly beneficial to poor rural populations that are affected by dietary Zn deficiency,[11,13,14] quantitative data on efficacy of the biofortified product is still limited. Welch et al.[13] and Rosado et al.[14] provided limited evidence that newly developed Zn-biofortified varieties of wheat may be useful in improving the Zn status of consumers. Nevertheless, we still require a more precise measurement of Zn bioavailability.

Additionally, is the extra Zn in biofortified wheat seed at least similarly absorbable as Zn in seed that has not been biofortified, and can the Zn-enriched wheat varieties be used to successfully improve the Zn status of Zn deficient people in developing countries? In recent years, the chicken (*Gallus gallus*) model has been used for assessing the efficacy of biofortified food crops.[15−17] Recently, a strong correlation between the results obtained via this animal model and through human efficacy trials was confirmed, additionally proving the suitability of the model in examining mineral bioavailability.[16] The *Gallus gallus* model has also been shown to be highly sensitive for Zn related studies.[18−20] The cost effectiveness, faster output and the ability to assess a wide range of physiological and molecular parameters in great detail are characteristics that make the use of the model appealing for testing dietary Zn bioavailability of wheat crops. The LA:DGLA ratio, a potentially new biomarker of Zn status, has been previously tested, demonstrating the efficacy of the biomarker in predicting Zn status in both animals and humans, as the LA:DGLA ratio responded to the changes in supplemental Zn intake within *Gallus gallus*[15] and closely tracked dietary Zn intakes within humans.[21] The major aim of this study was to determine the amount of Zn absorbed from Zn-biofortified wheat material using an in vivo (*Gallus gallus*) model of Zn absorption. In addition, this study assesses the usefulness of the LA:DGLA ratio to predict the Zn status of Zn deficient subjects consuming wheat based diets.

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MATERIALS AND METHODS

Development of Zn-Biofortified Wheat. The commercial wheat variety, Correll (*Triticum aestivum*) was grown by Jordan Farms at Murtoa in the West Wimmera Shire of Victoria, Australia. Seeds were sown in the last week of May 2015 and harvested late November to early December.

The high Zn grain was obtained by a foliar application of 1.5 L ha^{-1} $ZnSO_4$ during the mid-vegetative growth stage (mid-August), followed by 2 L ha^{-1} $ZnSO_4$ two weeks after flowering (mid-October). The lower Zn grain was grown in the same paddock and was treated with one foliar application of $ZnSO_4$ at the rate of 2 L ha^{-1} one week after flowering, and this was a common practice in the region to mitigate any effects of Zn deficiency in the soil. Wheat samples were prepared under trace element-free conditions to eliminate contaminant sources of Zn. The samples were dried at 80 °C in a conventional oven, and subsequently, they were milled using a trace-element-free mill. The wheat flour was transported to Cornell University, Ithaca, New York in sealed containers for testing in the *Gallus gallus* model. Zn, Fe, phytate, calcium, fatty acid, and protein concentrations were measured in the original grain and in experimental wheat-based diets (Table 1).

Table 1. Zn, Fe, Phytate, Ca Content, and Fatty Acid Concentration of Wheat Flours

components	high Zn flour	low Zn flour
Zn concentration[a] (μg/g)	47.2	33.6
Fe concentration (μg/g)	58	53
phytate[b] (mg/g)	11.1	9.9
calcium (mg/kg)	340	330
fatty acids		
total saturated	338.8	330.3
total transaturated	0.4	0.3
total monounsaturated	286.1	274.4
total omega 3	55.2	55.8
total omega 7	19.0	19.1
total omega 9	265.8	254.1
total omega 6	975.8	981.0
LA (18:2n-6)	973.1	978.4
DGLA (20:3n-6)	0.6	0.5

[a,b]The methods employed for mineral and phytate analyses and determination of fatty acids are described in the Materials and Methods section. LA: linoleic acid; DGLA: dihomo-γ-linolenic acid. Fatty acids mg/100g.

Assessment of Micronutrient and Protein Content of Wheat Flour. Micronutrient content was measured by inductively coupled-mass spectrometry (ICPMS 7500cx, Agilent Technologies, U.S.A.) following closed-tube digestion of 0.2 g of flour with nitric acid and hydrogen peroxide.

Digest solutions were further diluted 10 times with the addition of a mixture of internal standards. The Agilent 7500cx is equipped with a CETAC ASX560 autosampler and the ASXpress Plus Rapid Sample Introduction System, a Glass Expansion OpalMist nebulizer (0.4 mL/min) and nickel cones. It was run in the helium (He) collision mode using two He tune methods to allow full analysis of all elements. NIST reference material was analyzed concurrently for quality control.

Nitrogen analysis was done using a Vario EL Cube (Elementar, Germany) using a modified Dumas combustion as outlined in Muñoz-Huerta et al.[22] and Watson and Galliher.[23] Sample (19 ± 2 mg) was weighed into a 4 × 4 × 11 mm tin boat and sealed tightly. Samples were analyzed using the default settings of the instrumentation, the method used for analysis had a 120 s oxygen dose. Sulfanilamide (Elementar, 15.00-0062) was used to adjust the calibration and a NIST reference material (Durum Wheat, 8436) was run at the start of each batch.

Phytate Content of Wheat Flour. Phytate content, in the form of myo-inositol hexaphosphate (IP-6) was measured by Dionex liquid chromatography (Dionex Corporation, Sunnyvale, CA, U.S.A.) using a method developed by Dionex[24] and Lehrfeld.[25,26] Please refer to Supporting Information for more details.

Briefly, 250 mg of dry, lyophilized diet sample was diluted in 10 mL of a 1.25% H_2SO_4 solution in order to extract phytate. The extraction process was carried out for 2 h and samples were centrifuged at 3660*g* for 10 min. Subsamples were then diluted with deionized water (1:10), and 10 μL was inserted and analyzed (n = 3).

Animals, Diets, and Study Design. Thirty Cornish cross fertile broiler eggs were taken from a commercial hatchery (Moyer's chicks, Quakertown, PA, U.S.A.). The eggs were incubated in optimal conditions at the Cornell University Animal Science poultry farm incubator. The procedure was described in detail elsewhere.[15−17] Upon hatching (hatchability rate was 94%), chicks were assigned to two treatment groups based on gender and body weight to make an equal dissemination between groups (n = 15): 1. "High Zn": 75% Zn wheat diet (46.5 μg Zn g^{-1}); 2. "Low Zn" = 75% wheat diet (32.8 μg Zn g^{-1}).

The NRC recommendations and requirements for poultry[27] were consulted to formulate wheat based diets that meet the nutrient supplies for the broiler, excluding Zn (Table 2). For further details, please see Supporting Information.

Table 2. Composition of the Experimental Diets

ingredient	high-Zn (biofortified)	low-Zn (standard)
	g/kg (by formulation)	
high-Zn wheat	750	–
low-Zn wheat	–	750
skim milk, dry	100	100
DL-methionine	2.5	2.5
corn starch	46.75	46.75
corn oil	30	30
choline chloride	0.75	0.75
vitamin/mineral premix[a] (no Zn)	70	70
total (g)	1000	1000
selected components	mean ± SEM, n = 5 (by analysis)	
dietary Zn concentration[b] (μg/g)	46.5 ± 0.99[c]	32.8 ± 0.17[d]

[a]Vitamin and mineral premix provided/kg diet (330002 Chick vitamin mixture; 235001 Salt mix for chick diet; Dyets Inc. Bethlehem, PA). [b]Zn concentrations in the diets were determined by an inductively coupled argon-plasma/atomic emission spectrophotometer (ICAP 61E Thermal Jarrell Ash Trace Analyzer, Jarrell Ash Co. Franklin, MA) following wet ashing. [c,d]Within a row, means without a common letter are significantly different (P < 0.05).

Blood Collection and Serum, Nail, and Feather Zn and Fe Content Measurements. On a weekly basis, approximately 100 μL of blood was collected from the wing vein (n = 15) using a microhematocrit heparinized capillary tubes (Fisher Scientific, Pittsburgh, PA, U.S.A.). Samples were taken in the morning after an 8 h overnight fast. Nail and feather samples (1−2 g) were gathered on the last day of the experiment. Serum, nail, and feather Zn and Fe concentrations were measured by an inductively coupled argon-plasma/atomic emission spectrophotometer (ICAP 61E Thermal Jarrell Ash Trace Analyzer, Jarrell Ash Co., Franklin, MA, U.S.A.) following wet ashing.

Fatty Acids Analysis of Erythrocytes and Experimental Wheat-Based Diets. The method for fatty acid analysis was previously described in detail by Reed et al.[15]

Fatty acid levels were expressed as weight % of total FA (% w/w). Fatty acid analysis of the wheat based diets was performed at the Waite Lipid Analysis Services according to their standard fatty acid analysis protocol.

Isolation of Total RNA. Total RNA was extracted as previously described by Tako et al.[16,28]

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Table 3. DNA Sequences of the Primers Used in This Study

analyte	organ	forward primer (5′−3′) (nucleotide position)	reverse primer (5′−3′)	base pairs length	GI identifier
ZnT1	int	GGTAACAGAGCTGCCTTAACT	GGTAACAGAGCTGCCTTAACT	105	54109718
ZnT5	int	TGGTTGGTATCTGTGCCTTTAG	GGCTGTGTCCATGGTAAGATT	99	56555150
ZnT7	int	GGAAGATGTCAGGATGGTTCA	CGAAGGACAAATTGAGGCAAAG	87	56555152
ZIP6	int	GCTACTGGGTAATGGTGAAGAA	GCTGTGCCAGAACTGTAGAA	99	66735072
ZIP9	int	CTAAGCAAGAGCAGCAAAGAAG	CATGAACTGTGGCAACGTAAAG	100	237874618
Zip4	int	TCTCCTTAGCAGACAATTGAG	GTGACAAACAAGTAGGCGAAAC	95	107050877
NF-κB	liv	CACAGCTGGAGGGAAGTAAAT	TTGAGTAAGGAAGTGAGGTTGAG	100	2130627
SI	int	CCAGCAATGCCAGCATATTG	CGGTTTCTCCTTACCACTTCTT	95	2246388
Na+ K+ ATPase	int	CCTTGGAGGTTTCTTCACCTATT	GGTCATCCCACTGAAGTCTAATC	92	14330321
SGLT-1	int	GCATCCTTACTCTGTGGTACTG	TATCCGCACATCACACATCC	106	8346783
Δ6 desaturase	liv	GGCGAAAGTCAGCCTATTGA	AGGTGGGAAGATGAGGAAGA	93	261865208
DMT-1	int	TTGATTCAGAGCCTCCCATTAG	GCGAGGAGTAGGCTTGTATTT	101	206597489
ferroportin	int	CTCAGCAATCACTGGCATCA	ACTGGGCAACTCCAGAAATAAG	98	61098365
DcytB	int	CATGTGCATTCTCTTCCAAAGTC	CTCCTTGGTGACCGCATTAT	103	20380692
18S rRNA	liv, int	GCAAGACGAACTAAAGCGAAAG	TCGGAACTACGACGGTATCT	100	7262899
actinB	liv, int	CCAAAGCCAACAGAGAGAAGA	ATCACCAGAGTCCATCACAATAC	137	NM 205518

Table 4. Body Weight, Feed Consumption, and Zn Intake of Chickens Fed a Low and a High Zn Diet from Day 0 to Day 42[§]

	day 0	day 14	day 28	day 42
body weight (g)				
low Zn group	41.1 ± 0.7[a]	125.4 ± 4.8[a]	301.5 ± 11.4[a]	598.6 ± 22.8[a]
high Zn group	41.4 ± 0.8[a]	122.5 ± 3.9[a]	298.7 ± 12.8[a]	585.8 ± 29.7[a]
feed consumption (kg/d)				
low Zn group		0.41 ± 0.2[a]	1.13 ± 0.5[a]	2.12 ± 0.8[a]
high Zn group		0.40 ± 0.3[a]	1.12 ± 0.6[a]	2.11 ± 0.7[a]
zinc intake (g)				
low Zn group		13.15 ± 0.6[b]	36.21 ± 4.8[b]	69.18 ± 7.5[b]
high Zn group		19.58 ± 0.8[a]	49.85 ± 5.1[a]	100.14 ± 9.8[a]

[§]Values are mean daily feed intakes for the 14 days preceding the day designated in the column heading. Values are cumulative weekly from day 0. [a,b]Within a column and for each parameter, means without a common letter are significantly different ($n = 15$, $p < 0.05$). Values are means ± SEM.

Table 5. Differences in Zn Status among the Groups as Assessed by Various Zn Biomarkers Serum, Nail, and Feather Zn Concentration)

	day 0	day 14	day 28	day 42
serum Zn (μg/g)				
low Zn group	1.07 ± 0.15[a]	0.48 ± 0.05[a]	0.35 ± 0.03[a]	0.43 ± 0.04[a]
high Zn group	1.07 ± 0.15[a]	0.66 ± 0.08[b]	0.69 ± 0.07[b]	0.55 ± 0.04[b]
feather Zn (μg/g)				
low Zn group				89.6 ± 7.7[a]
high Zn group				117.9 ± 10.2[b]
nail Zn (μg/g)				
low Zn group				67.9 ± 3.9[a]
high Zn group				85.8 ± 3.9[b]

[a,b]Within a column and for each parameter, means without a common letter are significantly different ($n = 15$, $p < 0.05$). Day 0−day 42. Values are means ± SEM.

Gene Expression Analysis, Primer Design, and Real-Time qPCR Design. These procedures were carried out as described previously.[29,30] For further details please refer to Supporting Information.

Real-time RT-PCR efficiency (E) values for the genes were as follows: NK-kB, 1.33; SGLT-1, 1.32; LepR, 1.205; ActinB, 1.09; DMT-1, 1.11; Ferroportin, 1.27; ZnT5, 1.43; Na+K+/ATPase, 1.33; ZnT7, 1.39; 18s rRNA, 1.28; Zip4, 1.11; Δ6-desaturase, 1.34; Zip9, 1.37; DcytB, 1.36; ZnT1, 1.31; Zip6, 1.33; SI, 1.43 (see Table 3).

Liver Fe and Ferritin Analysis. Liver samples were treated as described by Mete et al.[31] and Passaniti and Roth[32] with some modifications as in Tako et al.[17,28]

Statistical Analysis. Results were analyzed by ANOVA using SAS software (SAS Institute Inc. Cary, NC). Dissimilarities among treatments were computed by Tukey's test. $p < 0.05$ was considered statistically significant. Data are presented as means ± SEM.

RESULTS

Composition of the Wheat-Based Diets. Composition of the wheat based diets is presented in Table 2. High and low Zn wheat made 75% of the total diet. The concentrations of Zn in the high and the low Zn based diets were 46.5 ± 0.99 and 32.8 ± 0.17 mg kg^{-1}, respectively.

Except for the variation in Zn concentration there were no statistically significant variances in the fatty acid content or Fe concentrations between the low and high Zn diets (Table 1).

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The phytate concentration of the high Zn flour was higher; 11.1 compared to 9.9 mg g^{-1} in the low Zn wheat.

General Information (Body Weight, Feed Consumption, and Dietary Zn Intake). There were no statistically significant differences in feed consumption and body weights between groups of birds fed different diets. However, Zn intakes were consistently lower in the low Zn group versus high Zn group ($p < 0.05$, Table 4).

Differences in Zn Status among the Groups (Various Biomarkers). Serum Zn concentrations were lower in the low Zn group with statistically significant differences between the groups noted at each time point ($p < 0.05$). Similarly, the concentration of Zn in both tissues (feather and nail) was lower in the low Zn group of birds versus the birds fed high Zn diet (day 42, $n = 15$, $p < 0.05$, Table 5).

There were statistically significant differences in the LA:DGLA ratio among the groups, with the higher ratio measured in the group of birds fed the low Zn diet.

The LA:DGLA ratio was increasing as the study progressed from day 0 to day 42 and was dissimilar among the treatment groups at each time point ($p < 0.05$, Figure 1).

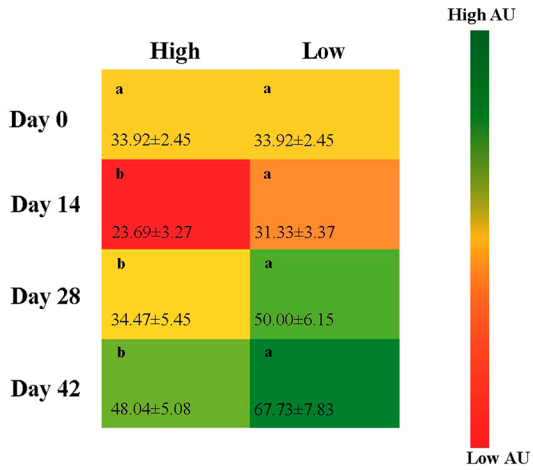

Figure 1. Changes in the LA:DGLA ratio among the groups from day 0 to day 42. AU – arbitrary units.

Changes in the Expression of Zn and Fe Transporters among the Groups. Duodenal mRNA expression of numerous Zn transporters (Figure 2) confirmed a higher mean arbitrary unit (AU) value in the tissues taken from the birds fed the low Zn diet ($n = 15$, $p < 0.05$). Similarly, the expression of iron (Fe) related transporters (DMT1, FPN1, and DcytB) was statistically different among the groups with the higher expression measured in the low Zn group ($n = 15$, $p < 0.05$).

Further, higher expression of functional genes (SGLT-1, SI and ATPase) was measured in the birds fed with the low Zn wheat based diet. Finally, the measurements of hepatic Δ6 desaturase revealed significant variations among the groups, with a lower mean value for birds in the low Zn group (Figure 2).

Liver Zn, Fe, and Ferritin Concentrations. No significant dissimilarities in the liver Zn, Fe and ferritin concentrations were noticed between the treatment groups (Table 6).

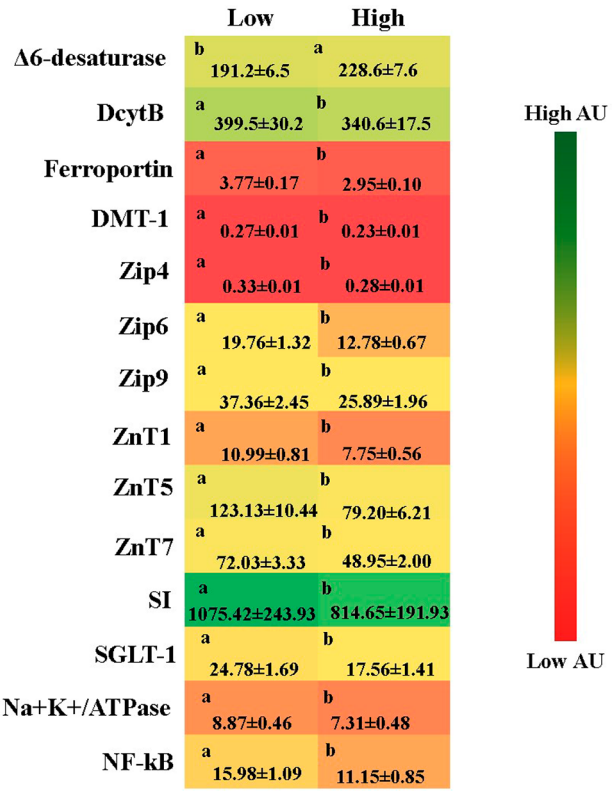

Figure 2. Gene expression of Zn and Fe transporters in duodenum and liver. Chicken mRNA expression of hepatic Δ6 desaturase and NF-κB1 and duodenal transporters: Cytochrome *b* (DcytB), ferroportin, divalent metal transporter 1 (DMT1), solute carrier family 39 member 4 (Zip4), solute carrier family 39 member 6 (Zip6), solute carrier family 39 member 9 (Zip9), Zn transporter 1 (ZnT1), Zn transporter 5 (ZnT5) and Zn transporter 7 (ZnT7), sucrose-isomaltase (SI), sodium/glucose cotransporter (SGLT1), ATPase Na+/K+ transporter (NaKATPase), nuclear factor kappa B subunit 1 (NF-kB) in birds given "high Zn wheat" diet and "low Zn wheat". Changes in mRNA expression are shown relative to expression to 18s rRNA in arbitrary units (AU). Values are means ± SEM $n = 15$, $p < 0.05$.

Table 6. Concentrations of Zn, Fe and Ferritin in the Liver of Birds on Low and High Zn Diets

	Zn concentration (μg/g)	Fe concentration (μg/g)	liver ferritin (μg/g wet weight)
low Zn group	16.72 ± 0.82[a]	144.59 ± 10.95[a]	254.5 ± 12.6[a]
high Zn group	16.76 ± 0.82[a]	145.31 ± 11.53[a]	272.5 ± 15.3[a]

[a]Within a column and for each parameter, means without a common letter are significantly different ($n = 15$, $p < 0.05$). Values are means ± SEM.

DISCUSSION

Nutritional deficiency of Zn in humans is prevalent throughout the world, particularly in areas where cereal grains are the primary staple in local diets.[6,33] Zinc-biofortified crops are a means of addressing nutritional deficiencies and knowledge of their efficacy in improving Zn status of subjects needs to be demonstrated. This study shows that the additional Zn present in the biofortified wheat is readily available for absorption; the higher amount of Zn in biofortified wheat contributed to a larger uptake of Zn by the intestinal enterocytes.

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A 14 μg Zn g^{-1} differential in dietary Zn intake was sufficient to discriminate between the Zn statuses of the birds used in the study.

Higher serum, feather and nail Zn concentrations were measured in the group of birds consuming a high-Zn wheat-based diet. The absorption of Zn was greater from the Zn-biofortified wheat dietary treatment than from the wheat with lower Zn concentration, when the same amounts of each type of wheat flour were consumed. Our results are consistent with previous studies, providing evidence that valuable increases in Zn absorption can be accomplished by a consumption of a Zn-biofortified wheat product.[13,14] Welch et al.[13] were the first to demonstrate the beneficial effect of Zn-biofortified wheat in improving the Zn status of rats. The wheat genotypes with enriched grain Zn concentrations had increased quantities of bioavailable Zn, supporting the idea that breeding for Zn enhanced wheat grain may lead to reducing the Zn deficiency problem in target populations.[13] Similar findings were provided by Rosado et al.[14] who confirmed greater net absorption of Zn in women eating Zn-biofortified wheat.

In that study, adult women were given 300 g of the high or low extraction flours as tortillas for two successive days using either biofortified (41 mg Zn g^{-1}) or control (24 mg Zn g^{-1}) wheat. The absorption of Zn from the Zn-biofortified wheat was significantly higher than that from the control wheat.

In addition, it was established that the amount of Zn absorbed from cereal products with high phytate content is greater from those fortified with Zn than when they are not fortified.[14]

Phytate is a major inhibitory compound present in plant foods and is known to inhibit dietary Zn bioavailability and absorption.[34,35] In this study, slightly higher levels of phytate were measured in the biofortified wheat variety (11 vs 9 mg g^{-1} in the low Zn wheat grain); however, this increase in phytate did not change Zn absorption negatively as the phytate to Zn molar ratio was not elevated.

Serum Zn responded to dietary Zn intake, as birds given the low Zn diet had a consistently lower level of Zn in serum. There were also significant differences in feather and nail Zn concentrations among the groups. These results are compatible with previous studies in humans that indicated serum, hair, and nail Zn concentration are contemplative of dietary Zn intake.[36−38] However, over the years, it has been shown that the sensitivity of these biomarkers is very often affected by factors not related to Zn intake/status (i.e., infection, inflammation, stress, sample handling).[38,39] Hence, the development of a sensitive Zn biomarker is still a high priority, as proposed by the World Health Organization.[1,40] The current study demonstrated the effectiveness of the LA:DGLA ratio to predict Zn status of subjects consuming a wheat based diet—a diet more representative of a diet in target Zn-deficient populations.

The erythrocyte LA:DGLA ratio reacted to dietary Zn manipulations rapidly (within 2 weeks). A difference of 14 μg Zn g^{-1} in Zn concentration among the wheat based diets was enough to show that the production of the DGLA was reduced and accordingly the LA:DGLA ratio was increased in subjects consuming wheat diets with lower Zn concentrations.

The Δ6 desaturase expression was increased in the group of birds fed the high Zn wheat based diet. Zn is an essential and vital cofactor for a proper activity of the Δ6 desaturase enzyme;[41,42] therefore, the higher concentrations of Zn lead to the higher expression of this enzyme. In contrast, Zn deficiency inhibits functioning and expression of the hepatic Δ6 desaturase.[41]

The LA:DGLA ratio was statistically different among the groups of birds, with a higher LA:DGLA ratio measured in the subjects that were fed the low Zn wheat based diet, at each time point (weeks 2, 4, and 6).

The ratio increased in both groups as the study progressed (from day 0 to day 42), which is due to the fact that both groups of birds were getting progressively more Zn deficient.

It is important to note that with only 14 μg Zn g^{-1} distinction in dietary Zn concentration, the LA:DGLA ratio still distinguished clearly between the groups, which demonstrates the sensitivity of the biomarker to change according to the dietary Zn intake. Besides, variations in the LA:DGLA ratio were evident within 7 days, indicating that this biomarker can display changes in the dietary Zn status reasonably quickly and it may be able to identify early stages of Zn deficiency that usually, due to the lack of obvious signs and symptoms, pass unrecognized. The results are in agreement with our former studies,[15,21] demonstrating that LA:DGLA is able to distinct Zn status between Zn adequate and Zn deficient subjects. When birds were fed either Zn adequate control diet (42.3 μg Zn g^{-1}) or a Zn deficient diet (2.5 μg Zn g^{-1}), the hepatic Δ6 desaturase expression was considerably higher in the control group.[15] Consequently, the LA:DGLA ratio was markedly reduced in the group of birds fed a high Zn diet.

The dietary Zn intake and plasma Zn status were similarly compared to the content of plasma phospholipid LA, DGLA, and variations in the LA:DGLA ratio in healthy human subjects.[21]

It was demonstrated that while the plasma Zn concentrations of participants stayed unchanged (presumably due to the good homeostatic regulation), there was a statistically significant discrepancy in DGLA production and the LA:DGLA ratio between the study participants.

The concentration of DGLA declined and the LA:DGLA ratio increased in people with lower dietary Zn intakes. The LA:DGLA biomarker needs additional testing in order to determine its full potential, but a number of studies up to date confirm that the LA:DGLA ratio may be a valuable additional indicator for assessing Zn status more precisely.[15,21]

In this study, the expression of various Zn transporters were also responsive to dietary Zn manipulations, with higher expression of all investigated transporters found in birds fed the lower Zn diet.

Comparable findings have been observed by others; a rapid upregulation of Zip4 expression in the small intestine was seen during Zn deficient conditions.[43−45] Zip4 is the most important import protein, so rapid Zip4 accumulation demonstrates the molecular basis of systemic Zn homeostatic regulation.[44,46] Similarly, the expression of other Zn import proteins, Zip6 and Zip9, was also significantly greater in subjects on lower Zn diets. The increased expression of the major Zn export protein, ZnT1, has been previously noted during Zn deficiency.[47,48] As was previously suggested, this initial increase in the expression most likely occurred in order to improve the transfer of Zn into systemic circulation in order to minimize the difference between the Zn demand and supply and to alleviate the undesirable consequences of extended Zn depletion.[47,49] The same applies for Fe uptake, which explains the increased activity of the ferroportin in the group of birds fed lower Zn diets. In addition, increased ferroportin expression may be explained by the point that Zn has a protecting role against Fe induced

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oxidative damage, so reduced amounts of Zn in the cells signify that the level of Fe in the cells should also be reduced.[49]

ZnT5 and ZnT7 are transporters ubiquitously expressed in the small intestine and are believed to perform partly overlapping functions in intestinal Zn homeostasis.[20] The expression patterns of ZnT5 and ZnT7 suggests a role in dietary Zn absorption, and in this study increased expression was measured in birds fed the low Zn diet. Both increased and reduced expression of ZnT5 and ZnT7 have been found in response to Zn.[45,50]

Expression of DcytB reductase and ferroportin transporter was different among the groups, which confirms that cellular concentrations of Zn may also affect the process of Fe absorption by modulating the activities of transporters responsible for the uptake of Fe into the cells. This proposition has already been confirmed by a number of studies.[47,49,51,52]

In conclusion, this study demonstrated that the increased amount of Zn in the biofortified wheat produced a higher dietary bioavailability of Zn and therefore enhanced uptake of Zn by the intestinal enterocytes.

This shows the potential of Zn-biofortified wheat varieties in improving the Zn status of consumers. The LA:DGLA ratio responded to dietary Zn manipulations and the consumption of the Zn-biofortified wheat lowered the LA:DGLA ratio. Hence, these observations suggest that the LA:DGLA ratio can be utilized as an additional physiological indicator of Zn status.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b04905.

Detailed description of Materials and Methods used in this study: development of Zn-biofortified wheat; assessment of micronutrient and protein content of wheat flour; phytate content of wheat flour; animals, diets, and study design; blood collection and serum, nail, and feather Zn and Fe content measurements; fatty acids analysis of erythrocytes and experimental wheat-based diets; isolation of total RNA; gene expression analysis; primer design; and real-time qPCR design liver Fe and ferritin analysis (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Wessells, K. R.; Brown, K. H. Estimating the global prevalence of zinc deficiency: Results based on zinc availability in national food supplies and the prevalence of stunting. *PLoS One* **2012**, *7* (11), e50568.

(2) International Zinc Nutrition Consultative Group; Brown, K. H.; Rivera, J. A.; Bhutta, Z.; Gibson, R. S.; King, J. C.; Lonnerdal, B.; Ruel, M. T.; Sandtrom, B.; Wasantwisut, E.; Hotz, C. International Zinc Nutrition Consultative, G., International Zinc Nutrition Consultative Group (IZiNCG) technical document #1. Assessment of the risk of zinc deficiency in populations and options for its control. *Food and nutrition bulletin* **2004**, *25* (1 Suppl 2), S99−203.

(3) Gibson, R. S. Zinc: the missing link in combating micronutrient malnutrition in developing countries. *Proc. Nutr. Soc.* **2006**, *65* (1), 51−60.

(4) Kaur, K.; Gupta, R.; Saraf, S. A.; Saraf, S. K. Zinc: The Metal of Life. *Compr. Rev. Food Sci. Food Saf.* **2014**, *13*, 358−376.

(5) Prasad, A. S. Discovery of human zinc deficiency: 50 years later. *J. Trace Elem. Med. Biol.* **2012**, *26*, 66−69.

(6) Black, R. E.; Allen, L. H.; Bhutta, Z. A.; Caulfield, L. E.; de Onis, M.; Ezzati, M.; Mathers, C.; Rivera, J. Maternal and child undernutrition: global and regional exposures and health consequences. *Lancet* **2008**, *371*, 243−260.

(7) de Romaña, L.; Lönnerdal, B.; Brown, K. H. Absorption of zinc from wheat products fortified with iron and either zinc sulfate or zinc oxide. *Am. J. Clin. Nutr.* **2003**, *78*, 279−283.

(8) Trewin, D. *The Australian Wheat Industry. Statistics*; Yearbook Australia: Canberra, 2006; Vol. *1301.0*.

(9) Shahzad, Z.; Rouached, H.; Rakha, A. Combating Mineral Malnutrition through Iron and Zinc Biofortification of Cereals. *Compr. Rev. Food Sci. Food Saf.* **2014**, *13*, 329−346.

(10) Bouis, H. E.; Hotz, C.; McClafferty, B.; Meenakshi, J. V.; Pfeiffer, W. H. Biofortification: A new tool to reduce micronutrient malnutrition. *Food and Nutrition Bulletin* **2011**, *32* (1), S31−S40.

(11) Brown, K. H.; Hambidge, K. M.; Ranum, P. Zinc fortification of cereal flours: current recommendations and research needs. *Food Nutr Bull.* **2010**, *31*, S62−74.

(12) Jou, M. Y.; Du, X. G.; Hotz, C.; Lonnerdal, B. Biofortification of Rice with Zinc: Assessment of the Relative Bioavailability of Zinc in a Caco-2 Cell Model and Suckling Rat Pups. *J. Agric. Food Chem.* **2012**, *60*, 3650−3657.

(13) Welch, R. M.; House, W. A.; Ortiz-Monasterio, I.; Cheng, Z. Potential for improving bioavailable zinc in wheat grain (Triticum species) through plant breeding. *J. Agric. Food Chem.* **2005**, *53* (6), 2176−2180.

(14) Rosado, J. L.; Hambidge, K. L.; Miller, L. V.; Garcia, O. P.; Westcott, J.; Gonzalez, K.; Conde, J.; Hotz, C.; Pfeiffer, W.; Ortiz-Monasterio, I.; Krebs, N. F. The quantity of zinc absorbed from wheat in adult women is enhanced by biofortification. *J. Nutr.* **2009**, *139*, 1920−1925.

(15) Reed, S.; Qin, X.; Ran-Ressler, R.; Brenna, J. T.; Glahn, R. P.; Tako, E. Dietary Zinc Deficiency Affects Blood Linoleic Acid: Dihomo-gamma-linolenic Acid (LA:DGLA) Ratio; a Sensitive Physiological Marker of Zinc Status in Vivo (Gallus gallus). *Nutrients* **2014**, *6* (3), 1164−1180.

(16) Tako, E.; Bar, H.; Glahn, R. P. The Combined Application of the Caco-2 Cell Bioassay Coupled with In Vivo (Gallus gallus) Feeding Trial Represents an Effective Approach to Predicting Fe Bioavailability in Humans. *Nutrients* **2016**, *8*, 732.

(17) Tako, E. B.; Blair, M. W.; Glahn, R. P. Biofortified red mottled beans (Phaseolus vulgaris L.) in a maize and bean diet provide more bioavailable iron than standard red mottled beans: Studies in poultry (Gallus gallus) and an in vitro digestion/Caco-2 model. *Nutr. J.* **2011**, *10* (113).10.1186/1475-2891-10-113

(18) Bao, Y. M.; Choct, M.; Iji, P. A.; Bruerton, K. Trace mineral interactions in broiler chicken diets. *Br. Poult. Sci.* **2010**, *51*, 109−117.

(19) Cui, H.; Xi, P.; Junliang, D.; Debing, L.; Guang, Y. Pathology of lymphoid organs in chickens fed a diet deficient in zinc. *Avian Pathol.* **2004**, *33*, 519−524.

(20) Wang, X.; Fosmire, G.; Gay, C. V.; Leach, R. M., Jr. Short-term zinc deficiency inhibits chondrocyte proliferation and induces cell apoptosis in the epiphyseal growth plate of young chickens. *J. Nutr.* **2002**, *132*, 665−673.
(21) Knez, M.; Stangoulis, J. C. R.; Zec, M.; Debeljak-Martacic, J.; Pavlovic, Z.; Gurinovic, M.; Glibetic, M. An initial evaluation of newly proposed biomarker of zinc status in humans - linoleic acid: dihomo-γ-linolenic acid (LA:DGLA) ratio. *Clinical Nutrition ESPEN* **2016**, *15*, 85−92.
(22) Muñoz-Huerta, R. F.; Guevara-Gonzalez, R. G.; Contreras-Medina, L. M.; Torres-Pacheco, I.; Prado-Olivarez, J.; Ocampo-Velazquez, R. V. A review of methods for sensing the nitrogen status in plants: advantages, disadvantages and recent advances. *Sensors* **2013**, *13* (8), 10823−10843.
(23) Watson, M. E.; Galliher, T. L. Comparison of Dumas and Kjeldahl methods with automatic analyzers on agricultural samples under routine rapid analysis conditions. *Commun. Soil Sci. Plant Anal.* **2001**, *32*, 2007−2019.
(24) Dionex. *Application Note 65sAnalysis of Inositol Phosphates*; Dionex Corp.: Sunnyvale, CA, 1990.
(25) Lehrfeld, J. High-performance liquid chromatography analysis of phytic acid on a pH-stable, macroporous polymer column. *Cereal Chem.* **1989**, *66*, 510−515.
(26) Lehrfeld, J. HPLC separation and quantification of phytic acid and some inositol phosphates in foods: Problems and solutions. *J. Agric. Food Chem.* **1994**, *42*, 2726−2731.
(27) Subcommittee on Poultry Nutrition; Committee on Animal Nutrition; Board on Agriculture; Nation Research Council. *Nutrient Requirements of Poultry*; National Academies Press: Washington, DC, 1994.
(28) Tako, E.; Glahn, R. P.; Knez, M.; Stangoulis, J. C. The effect of wheat prebiotics on the gut bacterial population and iron status of iron deficient broiler chickens. *Nutr. J.* **2014**, *13*, 58.
(29) Hou, T.; Kolba, N.; Glahn, R.; Tako, E. Intra-Amniotic Administration (*Gallus gallus*) of Cicer arietinum and Lens culinaris Prebiotics Extracts and Duck Egg White Peptides Affects Calcium Status and Intestinal Functionality. *Nutrients* **2017**, *9* (7), 785.
(30) Pacifici, S.; Song, J.; Zhang, C.; Wang, Q.; Glahn, R. P.; Kolba, N.; Tako, E. Intra Amniotic Administration of Raffinose and Stachyose Affects the Intestinal Brush Border Functionality and Alters Gut Microflora Populations. *Nutrients* **2017**, *9* (3), 304.
(31) Mete, A.; Van Zeeland, Y. R.; Vaandrager, A. B.; van Dijk, J. E.; Marx, J. J.; Dorrestein, G. M. Partial purification and characterization of ferritin from the liver and intestinal mucosa of chickens, turtledoves and mynahs. *Avian Pathol.* **2005**, *34*, 430−434.
(32) Passaniti, A.; Roth, T. F. Purification of chicken liver ferritin by two novel methods and structural comparison with horse spleen ferritin. *Biochem. J.* **1989**, *258*, 413−419.
(33) Bouis, H. E.; Welch, R. M. Biofortification—A sustainable agricultural strategy for reducing micronutrient malnutrition in the global south. *Crop Sci.* **2010**, *50*, S-20−S-32.
(34) Davies, N. T.; Olpin, S. E. Studies on the phytate: zinc molar contents in diets as a determinant of Zn availability to young rats. *Br. J. Nutr.* **1979**, *41*, 591−603.
(35) Lonnerdal, B. Dietary factors influencing zinc absorption. *J. Nutr.* **2000**, *130* (5), 1378S−1383S.
(36) Chiplonkar, S.; Khadilkar, A.; Pandit-Agrawal, D.; Kawade, R.; Kadam, N.; Ekbote, V.; Sanwalka, N.; Khadilkar, V. Influence of micronutrient status and socioeconomic gradient on growth indices of 2−18-year-old Indian girls. *J. Pediatr Endocrinol Metab.* **2013**, *26* (9−10), 825−32.
(37) Hess, S. Y.; Peerson, J. M.; King, J. C.; Brown, K. H. Review Use of serum zinc concentration as an indicator of population zinc status. *Food Nutr Bull.* **2007**, *28* (3), S403−29.
(38) Lowe, N. M.; Fekete, K.; Decsi, T. Methods of assessment of zinc status in humans: a systematic review. *Am. J. Clin. Nutr.* **2009**, *89* (9), 2040S−2051S.
(39) King, J. C.; Brown, K. H.; Gibson, R. S.; Krebs, N. F.; Lowe, N. M.; Siekmann, J. H.; Raiten, D. J.; Biomarkers of Nutrition for Development (BOND)—Zinc Review. *J. Nutr.* **2015**.*146*858S10.3945/jn.115.220079
(40) Sandstead, H. H. Review Zinc deficiency. A public health problem? *Am. J. Dis. Child.* **1991**, *145* (8), 853−859.
(41) Ayala, S.; Brenner, R. R. Essential fatty acid status in zinc deficiency. Effect on lipid and fatty acid composition, desaturation activity and structure of microsomal membranes of rat liver and testes. *Acta Physiol Lat Am.* **1983**, *33* (3), 193−204.
(42) Horrobin, D. F.; Cunnane, S. C. Interactions between zinc, essential fatty acids and prostaglandins: relevance to acrodermatitis enteropathica, total parenteral nutrition, the glucagonoma syndrome, diabetes, anorexia nervosa and sickle cell syndrome. *Med. Hypotheses* **1980**, *6*, 277−96.
(43) Dufner-Beattie, J.; Wang, F.; Kuo, Y. M.; Gitschier, J.; Eide, D.; Andrews, G. K. The acrodermatitis enteropathica gene ZIP4 encodes a tissue-specific, zinc-regulated zinc transporter in mice. *J. Biol. Chem.* **2003**, *278* (35), 33474−33481.
(44) Hashimoto, A.; Nakagawa, M.; Tsujimura, N.; Miyazaki, S.; Kizu, K.; Goto, T.; Komatsu, Y.; Matsunaga, A.; Shirakawa, H.; Narita, H.; Kambe, T.; Komai, M. Properties of Zip4 accumulation during zinc deficiency and its usefulness to evaluate zinc status: a study of the effects of zinc deficiency during lactation. *Am. J. Physiol Regul Integr Comp Physiol.* **2016**, *310* (5), R459−68.
(45) Lichten, L. A.; Cousins, R. J. Mammalian Zinc Transporters: Nutritional and Physiologic Regulation. *Annu. Rev. Nutr.* **2009**, *29*, 153−176.
(46) Geiser, J.; Venken, K. J.; De Lisle, R. C.; Andrews, G. K. A mouse model of acrodermatitis enteropathica: loss of intestine zinc transporter ZIP4 (Slc39a4) disrupts the stem cell niche and intestine integrity. *PLoS Genet.* **2012**, *8* (6).e100276610.1371/journal.p-gen.1002766
(47) Iyengar, V.; Pullakhandam, R.; Nair, K. M. Coordinate expression and localization of iron and zinc transporters explain iron-zinc interactions during uptake in Caco-2 cells: implications for iron uptake at the enterocyte. *J. Nutr. Biochem.* **2012**, *23* (9), 1146−1154.
(48) Pfaffl, M. W.; Windisch, W. Influence of zinc deficiency on the mRNA expression of zinc transporters in adult rats. *J. Trace Elem. Med. Biol.* **2003**, *17* (2), 97−106.
(49) Knez, M.; Graham, R. D.; Welch, R. M.; Stangoulis, J. C. R. New Perspectives on the Regulation of Iron Absorption via Cellular Zinc Concentrations in Humans. *Crit. Rev. Food Sci. Nutr.* **2017**, *57* (10), 2128−2143.
(50) Cragg, R. A.; Christie, G. R.; Phillips, S. R.; Russi, R. M.; Kury, S.; Mathers, J. C.; Taylor, P. M.; Ford, D. A novel zinc-regulated human zinc transporter, hZTL1, is localized to the enterocyte apical membrane. *J. Biol. Chem.* **2002**, *277* (25), 22789.
(51) Graham, R. D.; Knez, M.; Welch, R. M. How Much Nutritional Iron Deficiency in Humans Globally Is due to an Underlying Zinc Deficiency? *Adv. Agron.* **2012**, *115*, 1−40.
(52) Yamaji, S.; Tennant, J.; Tandy, S.; Williams, M.; Singh Srai, S. K.; Sharp, P. Zinc regulates the function and expression of the iron transporters DMT1 and IREG1 in human intestinal Caco-2 cells. *FEBS Lett.* **2001**, *507* (2), 137−141.

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