

METABOLISM AND NUTRITION

Effect of Dietary Folic Acid Supplementation on Egg Folate Content and the Performance and Folate Status of Two Strains of Laying Hens

K. Hebert,* J. D. House,*†¹ and W. Guenter*

*Department of Animal Science and †Department of Human Nutritional Sciences,
University of Manitoba, Winnipeg, MB, R3T 2N2, Canada

ABSTRACT Enrichment of eggs with folate is possible when dietary folic acid levels are increased. However, development of optimal strategies for the production of folate-enriched eggs requires knowledge as to differences due to strain of bird and a greater understanding of the factors limiting egg folate deposition. To this end, a study was designed to determine the response of two leghorn strains that differ in production performance. Hyline W36 and W98 hens ($n = 6$ per diet) received a barley-based ration containing 0, 2, 4, 8, 16, 32, 64, or 128 mg/kg of crystalline folic acid for 21 d. Response criteria included production parameters, measures of blood folate status, and egg folate content. Significant ($P < 0.05$) main effects of folate supplementation were observed for egg folate content and plasma folate, which increased, and homo-

cysteine concentrations, which decreased with supplementation; performance, however, was not affected. The Hyline W98 strain had significantly ($P < 0.05$) higher total egg and yolk weights and feed consumption when compared with the W36. Significant ($P < 0.05$) ration \times strain interactions were observed for egg and yolk weight, egg folate content, and plasma homocysteine. The higher egg mass producing strain, Hyline W98, benefited from increased folic acid through a reduction in plasma homocysteine concentrations, suggesting that this strain has a higher requirement for folate than the W36 strain. Overall, egg folate content is maximized when crystalline folic acid is supplemented to the diet at 2 mg/kg or higher. Higher levels of egg folate are not achieved due to the saturation of the precursor pool for egg folate deposition.

(Key words: folic acid, egg, laying hen, homocysteine)

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INTRODUCTION

Folate is a collective term for a group of compounds with a pteroylglutamic acid backbone but differing oxidation states (i.e., folic acid, 5-methyltetrahydrofolate) whose primary function includes one-carbon transfer reactions (Selhub and Rosenberg, 1996). Examples of one-carbon transfer reactions include the remethylation of homocysteine, glycine-serine interconversion, and purine synthesis (Selhub and Rosenberg, 1996). Increased awareness of the importance of this B-vitamin has arisen due to the fact that supplemental folic acid has been shown to reduce a woman's risk for having a baby with a neural tube defect (Czeizel and Dudas, 1992; Scott, 1999). Additionally, folate deficiency results in an increase in plasma homocysteine concentrations, with the latter being linked to an increased risk for cardiovascular disease (Boushey et al., 1995; Refsum et al., 1998), Alzheimer's disease (Morris, 2003), and osteoporosis (McLean et al., 2004). Therefore

considerable attention has been given to the development of strategies to increase human folate intakes, most notably the programs to fortify cereal grain products with folic acid undertaken by many countries, including Canada and the US. Although the program has proven effective in reducing the rates of neural tube defects (Choumenkovitch et al., 2002), complementary strategies are required to help ensure that all segments of the population are consuming adequate folate, especially in light of recent trends toward the reduction of carbohydrate levels in the diet. A folate-enriched egg may serve as such vehicle.

Eggs naturally contain folate at levels approximating 22 μg per large egg (USDA, 2004), or roughly 6% of the adult RDA for folate (Institute of Medicine 1998). We have previously shown that the folate content of eggs can be increased by approximately threefold by increasing the level of crystalline folic acid in the laying hen diet to 4 mg/kg (House et al., 2002). In that study, there was evidence of a biphasic response in egg folate levels, as levels reached plateau values between 4 and 16 mg of folic acid/kg; however, egg folate levels significantly increased above the plateau when crystalline folic acid was supplemented at 32 mg/kg of diet. However, as this was the highest inclusion level tested, further speculation was not possible.

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¹To whom correspondence should be addressed: J_House@umanitoba.ca.

In our previous studies (House et al., 2002), we used a single strain of laying hen, the Hyline W36 (selected for high feed efficiency). In order to progress toward best management practices to optimize egg folate content, the potential for differences due to strain must be evaluated. To this end, a study was designed to determine the optimal dietary folic acid level required for maximal egg folate deposition and to determine the potential for differences in how two different strains respond. The potential for further increases in egg folate content was assessed by including dietary folic acid levels that spanned those previously tested, up to a maximal level of 128 mg/kg. In order to gain knowledge as to the factors limiting egg folate deposition, measures of folate status, including plasma folate and homocysteine concentrations, were also determined.

MATERIALS AND METHODS

General

Single-Comb White Leghorn laying hens² (Manitoba Perfect Pullets), Hyline W98, an early-maturing, large egg mass strain (Hyline, Inc., 2004), and Hyline W36, a traditional strain (efficient egg layer with excellent livability; Hyline, Inc., 2003), were used in this experiment. Hens were kept in confinement housing under semicontrolled environmental conditions and exposed to a 16-h photoperiod. Ninety-six birds were housed individually; the cage dimensions were 25.4 × 40.64 cm, providing 1,032 cm² per bird. Feed and water were available ad libitum. Animal care approval was received from The University of Manitoba's Animal Care Protocol Review Committee, and the birds were managed in accordance with recommendations established by the Canadian Council on Animal Care (1984).

Diets

The basal diet was a barley-based ration (Table 1). The diet was formulated to meet the requirements of laying hens consuming 100 g of feed/d (NRC, 1994). In accordance with industry standards (BASF, 2000), the basal diet included no crystalline folic acid. Analysis of the basal diet by a commercial lab³ indicated a folate level of 1.45 mg/kg, an amount in excess of the NRC (1994) requirement (0.25 mg/kg).

Experimental Approach

For 2 wk prior to the commencement of the study, 256 healthy hens of each strain were monitored for egg production, and the 48 highest-producing hens of each

TABLE 1. Composition of the basal barley-based laying hen ration

Ingredient	%
Barley (11.9% CP)	58.8
Soybean meal (45.0% CP)	12.6
Canola meal (37.6% CP)	10.0
Fish meal (72% CP)	2.0
Vegetable oil	5.6
Limestone	8.8
Biophos (monocalcium phosphate)	0.8
Vitamin premix ¹	1.0
Mineral premix ²	0.5
Calculated nutrient composition	
Crude protein, %	18.0
Metabolizable energy, kcal/kg	2,703
Calcium, %	3.75
Phosphorus (available), %	0.4
Folate, ³ mg/kg	1.45

¹Provided (per kg of diet): vitamin A, 8,225 IU; vitamin B₁₂, 11.2 µg; calcium pantothenate, 4.4 mg; choline chloride, 110; vitamin D₃, 1,000 IU; vitamin E, 5.46 IU; ethoxyquin, 125 mg; dl-methionine, 500 mg; niacin, 7.6 mg; riboflavin, 2.2 mg.

²Provided (per kg of diet): MnO, 165 mg; ZnO, 55 mg; salt (iodized), 4.78 g.

³Measured by Covance Laboratories Inc., Madison, WI (AOAC, 2000).

strain were selected for the experiment. At 22 wks of age (peak production of 92 to 96%), the selected hens were placed individually into battery cages and were randomly assigned to receive 1 of 8 dietary folic acid levels (n = 6 per treatment). The birds were given a 2-wk adaptation period followed by a 7-d collection period in which the barley diets supplemented with 0, 2, 4, 8, 16, 32, 64, or 128 mg of crystalline folic acid/kg⁴ were provided. Feed consumption for each cage unit was determined at the completion of the trial to calculate average daily feed intake and feed efficiency. Egg production was recorded daily for each cage unit and an average egg production rate (hen-day percent) was calculated. Ninety-six eggs (1 egg per replication; 6 replications per treatment per strain) were randomly collected per treatment each day for 3 d (288 eggs total). The eggs were weighed to give an average egg weight for the treatment period and processed for egg folate determination.

At the end of the 3-wk period, a 1-mL blood sample was collected via wing venipuncture from 48 birds (3 replications per strain). Blood samples were collected with a 2-mL sterile syringe containing 50 µL of a porcine heparin saline solution (68.6 USP). The samples were cooled on ice and immediately centrifuged at 12,000 × g for 5 min.⁵ Plasma was retained and stored at -80°C until analysis.

Extraction and Analysis of Egg Yolk Folate Content

All chemicals used in the extraction and analysis of folate were purchased from Sigma Chemical Co. The extraction and analysis of the egg yolk folate content was performed as described previously (House et al., 2002). In brief, eggs were weighed, placed in boiling water for 10 min, cooled, and the yolks separated, weighed and

²Rosenort, Manitoba, Canada.

³Covance Laboratories Inc., Madison, WI.

⁴Sigma Chemical Co., Oakville, Ontario, Canada.

⁵CR 3000, Jouan, Fisher Scientific, Nepean, Ontario, Canada.

TABLE 2. Performance, egg folate content, and indices of folate status in laying hens receiving diets with graded levels of crystalline folic acid: main effect of ration¹

Item	Dietary folic acid level, mg/kg							SEM ²	P-value	
	0	2	4	8	16	32	64			128
Hen-day production, %	92.9	98.0	96.4	96.4	96.4	96.0	95.2	95.6	1.8	0.6866
Feed consumption, g/bird per day	90.7	92.7	93.2	95.3	94.1	94.0	94.2	92.5	1.8	0.7727
Feed efficiency, g of feed/g of egg	1.61	1.67	1.68	1.72	1.63	1.69	1.67	1.63	0.04	0.466
Egg weight, g	56.4	55.4	55.4	55.9	58	55.7	56.2	57.2	0.9	0.4508
Yolk weight, g	13.6	13.2	13.0	14.0	13.7	13.4	14.0	13.6	0.3	0.2350
Egg folate, µg/egg ³	16.7	37.5	38.6	40.6	38.3	44.0	41.2	38.8	3.3	<0.0001
Plasma folate, ng/mL ⁴	15.2 ^d	32.6 ^{cd}	36.9 ^{bc}	38.7 ^{bc}	41.6 ^{abc}	49.1 ^{ab}	48.6 ^{abc}	59.4 ^a	3.6	<0.0001
Plasma homocysteine, µM ³	12.59	10.01	10.80	10.76	9.99	10.27	9.73	8.55	0.75	0.0478

¹Data are presented as least square means.

²SEM = standard error of the mean.

³Means not subjected to multiple comparisons procedure due to significant ration × strain interaction.

⁴Data within a row that do not share letters are significantly different ($P < 0.05$) by Tukey's procedure.

retained for analysis by storing at -80°C . Previous research has documented that >95% of the folate in egg is located in the yolk (Sherwood et al., 1993), and we have confirmed this (data not shown). Egg folate in the form of 5-methyltetrahydrofolate, the sole form of folate in eggs (Seyoum and Selhub, 1998), was extracted into an ascorbate buffer (pH 7.8). The extracts were analyzed for 5-methyltetrahydrofolate via reverse-phase HPLC with fluorescence detection,⁶ using the method of Vahteristo et al. (1997). An external standard curve with purified 5-methyltetrahydrofolate was used to quantify egg folate concentrations. The inter- and intraassay CV for determinations was <2%, and recovery of 5-methyltetrahydrofolate added to egg yolk was 98.9%. The content of egg folate was expressed as micrograms of folic acid per egg.

Analysis of Blood Plasma

Plasma folate concentrations were determined through the use of a competitive binding assay, Quantaphase II B12/folate Radioassay purchased from Bio-Rad⁷ (no. 191-1041), according to the manufacturer's recommended protocol. Plasma homocysteine was determined by reverse-phase HPLC with fluorescence detection, using the method of Araki and Sako (1987), as modified by Gilfix et al. (1997).

Statistical Analyses

An 8×2 (ration × strain as main effects) factorial arrangement of a completely randomized design was used to analyze the data. Performance data collected were subjected to ANOVA using SAS Analyst (SAS Inst., Inc., 1998). Differences between means were determined using Tukey's Honest Significance Difference. Differences with an α level of $P < 0.05$ were considered to be statistically significant. A regression analysis was run on plasma folate and homocysteine concentrations. It was not neces-

sary to log transform the data for plasma folate and homocysteine, since a plot of residuals vs. predicted values did not reveal any heterogeneity in the variability.

RESULTS

Data for the main effects of ration are given in Table 2. Those parameters that exhibited significant ration × strain interactions are given in Table 3. With respect to the main effect of ration, increasing dietary folic acid levels had a significant ($P < 0.05$) impact on egg folate content and plasma homocysteine concentrations, but a significant ration by strain interaction existed for these two variables. Only plasma folate was significantly different due solely to ration (with no strain or ration × strain interaction effects apparent). Plasma folate levels increased ($P < 0.05$) from a low of 15.2 ng/mL for birds consuming the basal diet with no added folic acid, up to a high of 59.4 ng/mL for birds consuming diets with 128 mg/kg of folic acid. At folic acid inclusion levels of 16 mg/kg and above, plasma folate concentrations were not significantly different from each other, providing evidence that plasma folate levels can be saturated.

With respect to the main effect of strain, there were no significant differences between the W36 and the W98 hens for hen-day production, feed efficiency, plasma folate, or plasma homocysteine. Significant ($P < 0.05$) differences were found for feed consumption (W36 = 88.7; W98 = 97.9; SEM = 0.9 g·head⁻¹·d⁻¹), egg weight (W36 = 53.7; W98 = 58.9; SEM = 0.5 g), yolk weight (W36 = 13.3; W98 = 13.8; SEM = 0.2 g), and egg folate content (W36 = 34.1; W98 = 39.8; SEM = 1.6 µg/egg), with the latter 3 variables exhibiting significant ration × strain interactions. The differences in egg folate content reflected the larger egg size produced by the Hyline W98 hens. The Hyline W98 hens ate significantly more feed compared to the Hyline W36 hens (97.9 vs. 88.7 g·head⁻¹·d⁻¹; SEM = 0.92; $P < 0.0001$).

Significant ($P < 0.05$) ration by strain interactions were observed for egg weight, yolk weight, egg folate content, and plasma homocysteine concentration (Table 3). Hyline W98 hens consuming diets containing the highest level of folic acid (128 mg of folic acid/kg of diet) produced

⁶Shimadzu, Mantech, Guelph, Ontario, Canada.

⁷Hercules, CA.

TABLE 3. Performance, egg folate content and indices of folate status in laying hens receiving diets with graded levels of crystalline folic acid: variables with significant ($P < 0.05$) ration \times strain interactions¹

Dietary folic acid level, mg/kg	Egg weight, g		Yolk weight, g		Plasma homocysteine, μ M		Egg folate, μ g/egg	
	W36	W98	W36	W98	W36	W98	W36	W98
0	55.8 ^{abcd}	57.1 ^{abcd}	14.2 ^{ab}	13.1 ^{ab}	10.45 ^{ab}	14.73 ^a	16.6 ^b	16.8 ^b
2	53.2 ^{cd}	57.7 ^{abcd}	13.5 ^{ab}	12.9 ^{ab}	10.69 ^{ab}	9.34 ^b	32.8 ^{ab}	42.2 ^{ab}
4	53.3 ^{cd}	57.4 ^{abcd}	12.8 ^{ab}	13.2 ^{ab}	9.81 ^{ab}	11.78 ^{ab}	48.2 ^a	31.0 ^{ab}
8	52.4 ^d	59.3 ^{abc}	13.2 ^{ab}	14.8 ^a	12.67 ^{ab}	8.82 ^b	31.6 ^{ab}	49.5 ^a
16	54.7 ^{bcd}	61.3 ^{ab}	13.1 ^{ab}	14.3 ^{ab}	11.08 ^{ab}	8.90 ^b	29.5 ^{ab}	47.1 ^a
32	53.2 ^{cd}	58.3 ^{abcd}	13.1 ^{ab}	13.7 ^{ab}	11.57 ^{ab}	8.98 ^b	45.8 ^a	42.2 ^a
64	54.9 ^{bcd}	57.6 ^{abcd}	14.0 ^{ab}	14.0 ^{ab}	11.20 ^{ab}	8.26 ^b	35.8 ^{ab}	46.7 ^a
128	52.2 ^d	62.2 ^a	12.7 ^b	14.6 ^{ab}	8.40 ^b	8.70 ^b	34.5 ^{ab}	42.8 ^a
SEM ²	1.3		0.4		1.06		4.5	
<i>P</i> -value	0.047		0.01		0.015		0.008	

^{a-d}Data within columns and rows comprising a ration \times strain data set for a given variable that do not share letters are significantly different ($P < 0.05$) by Tukey's procedure.

¹Data are presented as least square means.

²SEM = standard error of the mean.

eggs that had significantly higher egg weights than Hyline W36 hens receiving the higher levels of folic acid. This may be due to the fact that egg weights were significantly lower for the W36 hens consuming diets containing 128 vs. 0 mg of folic acid/kg of diet. For yolk weight, significant differences were observed between those eggs produced by Hyline W98 hens consuming 8 mg of folic acid/kg of diet vs. the Hyline W36 hens consuming diets containing 128 mg of folic acid/kg of diet. Increasing dietary folic acid supplementation did not affect plasma homocysteine concentrations for the Hyline W36. However, plasma homocysteine concentrations were significantly reduced for the W98 hens consuming the higher levels of folic acid. The folate content of eggs produced by Hyline W98 hens were more responsive to increasing dietary folic acid supplementation, when compared to the W36 hens, in terms of maximal egg folate content achieved. Finally, while not reaching the significance threshold of $P < 0.05$ established, it is valuable to note that the ration \times strain interaction for egg production approached significance ($P = 0.0625$). The Hyline W98 hens appear to respond to increasing dietary folic acid supplementation by increasing their egg production up to an inclusion level of 64 mg of folic acid/kg, at which point egg production declined.

DISCUSSION

In agreement with our previous study (House et al., 2002) and the work of others (Sherwood et al., 1993), egg folate concentrations responded to increasing levels of dietary folic acid supplementation between 0 and 2 mg of crystalline folic acid per kilogram of diet. Maximal egg folate levels were achieved when dietary folate concentrations exceeded the recommendation of 0.25 mg of folate/kg of diet given by NRC (1994), by a factor of eightfold or higher. In our previous study (House et al., 2002), we observed that egg folate concentrations increased above a plateau value when the level of folic acid in the diet was 32 mg/kg of diet, the highest level tested. Those

results prompted the current study aimed at determining whether a biphasic response existed for egg folate content. However, results from the current work did not find evidence of this biphasic response, as increasing folate levels up to 128 mg of folic acid/kg did not lead to consistent increases in egg folate content over and above those achieved at plateau.

Consideration of the plasma folate data provides a partial explanation of why egg folate levels reached a plateau value. Plasma serves as the precursor pool for egg yolk deposition. Miller and White (1986) have identified the existence of a binding protein-mediated mechanism for the transfer of riboflavin into the egg yolk, but a similar mechanism has yet to be elucidated for folate. Plasma folate concentrations may serve as the limiting factor for egg folate concentrations (Sherwood et al., 1993), as both compartments appear to saturate at dietary folic acid concentrations between 2 and 4 mg/kg of diet. Plasma folate concentrations are higher in birds consuming 128 mg of folic acid/kg diet than those consuming 4 mg/kg; however, this increase is not sufficient to translate into higher egg folate concentrations. Therefore, a greater understanding of the factors regulating the absorption of dietary folic acid and its appearance in the systemic circulation in the form of 5-methyltetrahydrofolate in the laying hen, is warranted.

In studies with rats and in vitro intestinal cell model systems, folic acid has been shown to be absorbed from the gut via a membrane-bound folate transport system (Said, 2004) that accepts both oxidized and reduced forms of the monoglutamated forms of folate. This process has been shown to be saturable in a number of model systems (Said, 2004), and as such represents a potential control point for plasma folate concentrations. However, the 5-methyltetrahydrofolate form is believed to be the primary circulating form of folate in the plasma, and therefore the existence of other control points can not be dismissed. In the enterocyte, folic acid is reduced via a 2-step process involving the enzyme dihydrofolate reductase to yield tetrahydrofolate (Henderson, 1990). Tetrahydrofolate is

then transported to the liver, where it is converted to the 5-methyltetrahydrofolate form. Attachment to binding proteins within hepatocytes and transport via the systemic circulation is the putative mechanism whereby folate is transported to the developing yolk (Henderson, 1990). Therefore multiple control points exist to regulate plasma folate levels, and further studies are required to elucidate the key control point(s), especially in an avian model system.

5-Methyltetrahydrofolate participates in a reaction involving the folate-dependent remethylation of the sulfur AA homocysteine to yield methionine. Increasing the crystalline folic acid level from the 0 mg/kg present in the basal diet resulted in a significant reduction in plasma homocysteine concentrations in the HyLine W98 hens. As homocysteine is a sensitive marker of folate status (Miller et al., 1994; House et al., 2003), this reduction may indicate that the HyLine W98 hens have a higher requirement for folate as compared to the W36 hens and, indeed, a higher requirement than that given by NRC (1994). However, the short term nature of the current studies precludes further insight into folate requirements of these birds. Indeed, when compared with the W36 hens, the W98 hens did produce heavier eggs (approximately 5 g) and consume more feed (approximately 10 g/d), independent of folic acid supplementation. The production of heavier eggs also translated into greater egg folate contents (on a per egg basis). As the HyLine W98 hens have been selected for early maturity, large egg mass and high egg production, perhaps this strain is more sensitive to dietary folic acid supplementation.

Understanding the factors that influence the level of folate in eggs is critical for ensuring the delivery of a product to consumers that has a consistent level of this key nutrient. The purported link between increased folic acid intake, reduced plasma homocysteine concentrations, and reductions in the risk for chronic diseases, such as cardiovascular disease, may increase the demand for products shown to be enriched in this vitamin. We have previously documented that the folate in eggs has a relative bioavailability $\geq 100\%$ when determined using a rat bioassay (House et al., 2003). Furthermore, the form of folate in eggs, 5-methyltetrahydrofolate, is a form that does not mask the symptoms of cobalamin deficiency unlike the crystalline folic acid form that is used to enrich cereal grain products (Institute of Medicine, 1998). These attributes position the egg as a good vehicle for the delivery of folate into the human diet.

In summary, this study supports the potential for fortifying eggs with folic acid by supplementation of crystalline folic acid to barley-based diets at a minimum of 2 mg/kg of diet. The saturation of plasma folate concentrations represents a putative control point for egg folate concentrations. Overall, performance was not affected by folate supplementation. However, a strain of hens selected for large egg mass benefited from increased dietary folic acid through reductions in plasma homocysteine concentrations, with a trend toward increased production. The latter data point to a need to reassess folate requirements

for high-producing strains of birds. Additional research is required to assess production factors, such as level of production, age of flock, and dietary factors that are likely to influence egg folate content in order to determine the optimal strategies for the production of folate-enriched eggs.

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