The Enrichment of Eggs with Folic Acid through Folic Acid Supplementation of the Laying Hen Diet

A Thesis

Presented to

The Faculty of Graduate Studies

of

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by

Katherine Ann Hebert

In partial fulfillment of requirements

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Abstract

Three experiments were designed to assess optimal dietary folic acid level required for maximal egg folate deposition, potential differences due to strain, production factors (level of production, age of flock) and dietary factors that were likely to influence egg folate content.

In Experiment 1, Hyline W98 and W36 laying hens (n=6/ diet) received a barley-based ration, containing 0, 2, 4, 8, 16, 32, 64, or 128 mg/ kg of crystalline folic acid for 21 days to determine the optimal dietary level. There was a significant ($P<0.05$) increase in total egg folate content from 0 to 2 mg folic acid/ kg diet (16.7 to 37.5 μg/egg, respectively), after which egg folate content plateau.

In Experiment 2, Hyline W98 and W36 laying hens (n=156/ trt) received a barley-based ration, containing 0 or 4 mg/ kg of crystalline folic acid for eleven 4-week periods to determine the consistency of folate deposition throughout the production cycle. There was a significant ($P<0.05$) increase in total egg folate content within 28 days, after which it remained constant at 46.9 μg/egg ± 3.90. No strain differences were observed.

In Experiment 3, Bovan White laying hens (n=12/ diet) received a barley-, barley plus β-glucanase-, corn- or wheat-based ration, containing 0 or 4 mg/ kg of crystalline folic acid for 21 days to determine the influence of dietary ingredients on egg folate content. Corn-based diets had significantly ($P<0.05$) higher total egg folate content than wheat-based diets (37.2 vs 28.1 μg/egg), however the barely-based diets were not significantly different from the corn-based diets.

These studies suggest that egg folate content can be increased three-fold when supplementing diets with 4 mg crystalline folic acid/ kg diet.

Acknowledgements

I would like to acknowledge the Manitoba Egg Producers, Canadian Egg Marketing Agency and Clark Hy-Line Inc. for their support, without which this thesis would not have been possible.

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I am grateful to my advisory committee members, Dr. Gary Crow and Dr. Harold Aukema, for ensuring there was an end to this.

A heart felt thank you to 346 Belvidere, office mates and friends who were ready to orchestrate "Humpty Dumpty's Great Fall". I would also like to thank Ricky Araneda, for her invaluable technical support and lessons on life. I am also grateful to all of the technical staff at the Department of Animal Science, Harry Muc, Janice Haines, Karen Carrette, Jerry Levandoski and Hae Suk Yoon.

And finally to my brother and Dad—this chickenologist finally has a real job.

Foreword

This thesis is written in manuscript style. The three manuscripts will be submitted to Poultry Science. Currently Manuscript One has been accepted for publication and is in press. The authors of these manuscripts are K. Hebert, J.D. House and W. Guenter, Department of Animal Science, University of Manitoba, Winnipeg, Canada, R3T 2N2.

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Abbreviations

5-MTHF	5-methyl-tetrahydrofolate
10-formyl-THF	10-formyl-tetrahydrofolate
AH	albumen height
CL	cystathionine-γ-lyase
CBS	cystathionine-β-synthase
CEMA	Canadian Egg Marketing Agency
CG	chorionic gonadotrophin
CV	coefficient of variance
CVD	cardiovascular disease
DNA	deoxyribonucleic acid
DTT	dithiothreitol
DV	daily value
EDTA	ethylenediaminetetra-acetic acid
EP	hen-day production
EW	egg weight
FC	feed consumption
FE	feed efficiency
folyl conjugase	exocarboxypeptidase folyl γ-glutamyl hydrolase
FSH and LH	pituitary gonadotropins: follicle stimulating and luteinizing hormone
HCY	homocysteine
HPLC	high pressure liquid chromatography

HU	haugh unit
MTHFR	methylene-THF-reductase
NRC	Nutrient Research Council
NSP	non-starch polysaccharides
NTD	neural tube defects
PABA	para aminobenzioc acid
RCP	riboflavin carrier protein
RDA	recommended dietary allowance
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SBDF	7-flurobenzo-2-oxa-1,3-dizole-4-sulfonic acid
SEM	standard error of the mean
SHMT	serine hydroxymethyltransferase
TCEP	tris (2-carboxyethyl)-phosphine hydrochloride
THF	tetrahydrofolic acid
US	United States

1.0 Introduction

For centuries the egg was known as a perfectly packaged, portion controlled, highly nutritious food providing the highest quality protein, and source of all necessary vitamins except vitamin C, and minerals. Differentiated diets for laying hens can be utilized to enhance the levels of certain beneficial compounds like vitamins, such as folic acid.

Due to the recent awareness of the need for increased consumption of folate by humans, it is necessary to consider the incorporation of natural sources of this vitamin in their diets. It is recommended that women of childbearing age consume 400 μg of folic acid per day to reduce the occurrence of neural tube defects, such as spina bifida and anencephaly (Czeizel and Dudas, 1992). Folate is also a cofactor in the remethylation of homocysteine to form methionine (House et al., 1999). A folate deficiency results in an increase in the serum level of homocysteine, a situation now recognized to increase the risk for cardiovascular disease (Boushey et al., 1995). Therefore it is important that humans have adequate intake of folic acid.

Foods naturally contain marginal levels of folic acid. A large egg contains approximately 24 μg of folate (USDA, 2005). By increasing the folate content of eggs, the egg could be marketed as an excellent dietary source of folate. This would provide the public with the option of including eggs as part of a nutritional breakfast to meet the necessary dietary requirements of folic acid instead of consuming crystalline formulated vitamin pills.

Because of changing dietary habits, annual total egg consumption in Canada has dropped from 23 dozen per person in 1960 to 14.3 dozen in 1995 (Agriculture and

Agri-Food Canada, 2005). However, in the past few years, egg consumption has increased and in 2003, it reached 15.6 dozens per person, a 1.3% increase over 2002 (CEMA, 2005). The fortification of eggs with folate could appeal to consumers, as their demand for "natural" products with health-promoting properties increases. This has the potential to increase demand for eggs and improve the market potential for this valuable farm commodity.

Supplementing the diets of laying hens with 4 mg crystalline folic acid/ kg diet will result in an increase in the folate deposited in eggs by approximately 3-fold (House et al., 2002). This study suggested there was a bi-phasic response in egg folate level. Between 4 and 16 mg folic acid/ kg, egg folate levels plateau; at 32 mg folic acid/ kg there was a significant increase in egg folate level. This was the highest inclusion level tested and further speculation was not possible. House et al., (2002) also used a single strain of laying hen, the Hyline W36 (selected for high feed efficiency) and a barley-based ration in this study.

The broad objective of this thesis is to determine the factors that influence the production of folate-enriched eggs. Three trials were designed to assess optimal dietary folic acid level required for maximal egg folate deposition, potential differences due to strain, measures of folate status in hens (including plasma folate and homocysteine concentrations), production factors, such as level of production, age of flock and dietary factors that were likely to influence egg folate content. Further details of specific objectives are presented in each manuscript that constitute this thesis.

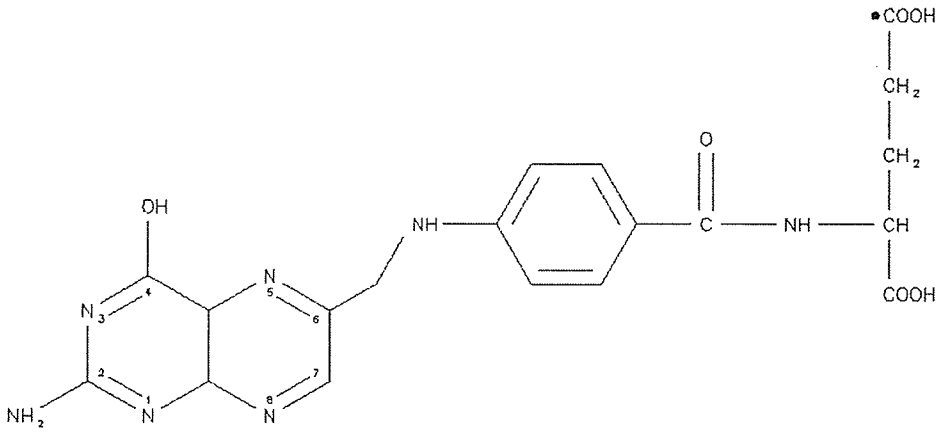
2.0 Literature Review

2.1 Folic Acid

2.1.1 Chemical Structure and Forms

Pteroylglutamic or pteroylmonoglutamic acid, commonly known as folic acid is a water soluble B-vitamin, involved in single-carbon transfer reactions and it exists in many chemical forms (Sauberlich, 1987). Folate and folacin are terms commonly used to describe compounds with similar chemical structure and nutritional properties as folic acid. The structure of folic acid is shown in **Figure 1**. There are three distinctive subunits of folic acid: (1) pteridine linked to (2) para aminobenzoic acid (PABA) forming pteroic acid, and is joined to (3) glutamic acid (Wagner, 1984). When one or more glutamic acid residues bond together, pteroylpolyglutamates are formed (Rodriguez, 1978).

Figure 1. Structure of folic acid.



Pteroylpolyglutamates are the form of folate commonly found in food and human tissue. 5-methyl-tetrahydrofolate (5-MTHF) and 10-formyl-tetrahydrofolate (10-formly-THF) are most predominant in food (Groff and Gropper, 2000) and 5-MTHF in plasma (Sauberlich, 1987). Due to the stability of the oxidized state of folic

acid, this form is commonly used in pharmaceutical supplements and food fortification (Scott, 1999). In order for the oxidized form of folic acid to be metabolically active, it must be reduced by metabolic systems in the gut and other tissues. The forms found in foods and the human body are reduced forms (Rodriguez, 1987). For example, 5,6-dihydrofolic acid is in the partially reduced form and 5,6,7,8-tetrahydrofolic acid is in the reduced form.

2.1.2 Sources of Folate

Folate is found in a variety of foods (**Table 1**, USDA, 2005). Liver, mushrooms and green leafy vegetables such as asparagus, brussels sprouts and spinach provide an excellent source of folate (Perloff and Butrum, 1977). Animal feed sources of folic acid are primarily derived from oilseed meals (soybean meal) and animal by-products (Perloff and Butrum, 1977). The majority of food folates occur as reduced polyglutamyl derivatives of tetrahydrofolic acid (THF) (Herbert, 1999). Very little monoglutamate (free folate) is found in foods or feedstuffs (Herbert, 1999). 5-MTHF and 10-formyl-THF are the predominant forms found in food (Herbert, 1999). Substantial losses in folic acid content can occur as a result of leaching and chemical degradation during cooking (Bailey, 1992).

2.1.3 Absorption and Transport

Since the majority of food folates occur as reduced polyglutamates, they must be cleaved to the mono- or diglutamate forms for absorption (Herbert, 1999). Exocarboxypeptidase folyl γ-glutamyl hydrolase (folyl conjugase) is responsible for this reaction (Sauberlich, 1987). Conjugase activity is concentrated in the mucosa of

Table 1. Food sources of folate.

Food	Folate (µg per usual serving)	Folate (µg/100 g)
Liver, beef, cooked	221	260
Cold cereals (Kellogg's Rice Krispies)	256	776
Navy beans, cooked	255	140
Asparagus	89	148
Spinach, raw	58	193
Broccoli, raw	20	65
Avocados, California	55	63
Brussel Sprouts, cooked	94	60
Orange Juice	45	18
Artichokes	61	51
Corn, canned	103	49
Oranges	39	30
Eggs	24	48
Cauliflower	57	57
Green peas, canned	75	44
Beets, cooked	40	80
Winter squash, cooked	41	20
Peanut butter	12	75
Grapefruit	25	10
Mangos	29	14
Blackberries	36	25
Green beans	41	33
Tomato juice	49	20
2% fat milk	12	5
Rice, white, long-grain, cooked	153	97
Bananas	24	20
Strawberries	4	22
Yogurt, whole milk	16	7
Sweet potatoes, baked in skin	9	6

Adapted from USDA, 2005

the proximal small intestine, both intracellularly and in association with the brush border (McDowell, 2000). Folate absorption is impaired by the loss of conjugase activity by a nutritional zinc deficiency (Bender, 1992) or naturally occurring conjugase inhibitors, such as orange juice and bananas (Bhandari and Gregory, 1990).

Monoglutamate derivatives of folic acid are taken up at the cellular level, via active transport, diffusion and folate-binding proteins (Herbert, 1999). Polyglutamates cannot cross biological membranes (Herbert, 1999). Glucose stimulates a sodium coupled, carrier mediated process, which actively transports folic acid across the jejunum, at a maximum pH of 6.0 (Groff and Gropper, 2000). Unlike active transport, diffusion is not a saturable process and absorption is linearly related to lumenal folate concentrations (Russell et al., 1979). Folate-binding proteins have been identified in plasma, milk and several other tissues (erythrocytes, leukocytes, intestinal mucosa, kidney, liver, placenta) (Henderson, 1990).

2.1.4 Metabolism

Folate metabolism can be broken down into three aspects: (1) pterin ring reduction, (2) polyglutamyl side chain reactions and (3) acquisition of single-carbon units. The pterin ring of folic acid and dihydrofolic acid must be fully reduced to THF, in order to accept single-carbon units. This is accomplished by 7,8-dihydrofolate reductase primarily in the liver and kidney. THF, reduced form, is the predominant form of folate in portal plasma (Wagner, 1984). Dietary folic acid reduced by dihydrofolate reductase, is absorbed and transported to the liver as THF (Bailey and Gregory III, 1999). THF is converted primarily to 5-MTHF and then transported to peripheral tissues (Burns and Jackson., 1982a). 5-MTHF can be

formed by a variety of pathways. THF receives a CHO group via 10-formyl-THF synthetase, resulting in 10-formyl-THF. 10-formyl-THF then receives a methyl group via methenyl-THF cyclohydrolase to form 5,10-methenyl-THF. 5,10-methylene-THF is produced from 5,10-methenyl-THF via methylene-THF dehydrogenase. 5,10-methylene-THF is reduced by methylene-THF reductase to form 5-MTHF. THF can also be regenerated by removing a methyl group from 5-MTHF via methionine synthase. Thymidylate synthase removes a CH_2 group from 5,10-methylene-THF (this becomes the thymidine methyl group) and THF is regenerated (Bailey and Gregory III, 1999). Serine hydroxymethyltransferase also transfers a one-carbon unit from serine to THF to form 5,10-methylene-THF and glycine (Bailey and Gregory III, 1999). A portion of the 5,10-methylene-THF produced undergoes enzymatic reduction to 5-MTHF by methylene-THF reductase (Bailey and Gregory III, 1999).

Folyl polyglutamate synthetase links glutamyl residues of monoglutamyl transport forms of folic acid together, forming metabolically active polyglutamyl forms that cannot cross cell membranes (Anderson et al., 1992). Single carbon units substitute at the N-5 and/or N-10 positions of the pterine ring, result in many metabolically active derivatives of folate, such as 5-M THF, 5-formyl-THF, 10-formyl-THF, 5,10-methylene-THF (Herbert, 1999). Serine hydroxymethyltransferase cleaves serine, resulting in the main source of single-carbon fragments needed to form these folate derivatives (Bailey and Gregory III, 1999). Each folate dervivative is a donor of its single-carbon unit in metabolism.

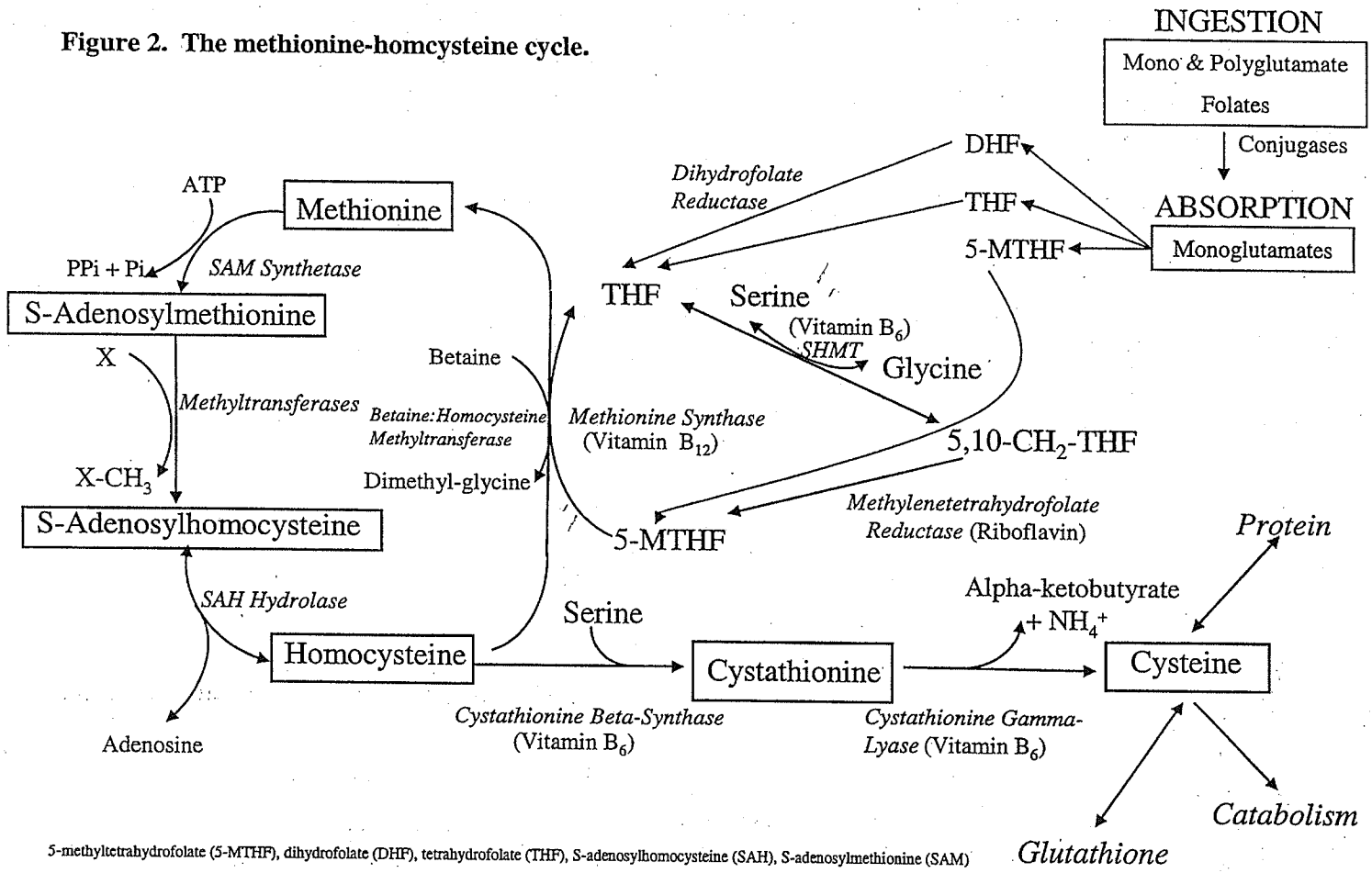
2.1.5 Functions

Folic acid is a vital co-factor for enzymes involved in one-carbon metabolism of methyl and methylene groups (Rodriguez, 1978). Only the fully reduced tetrahydro forms of folates accept or donate single-carbon units (Wagner, 1995). Folate distribution and metabolism occurs between the cytosol and the mitochondria of cells (Wagner, 1995 and Girgis et al., 1997). These one carbon units are usually produced during amino acid metabolism and are used in metabolic interconversions of amino acids and in the biosynthesis of purine (adenine and guanine) and pyrimidine (thymine) components of nucleic acids, and thus necessary for DNA replication and cell division (Steinberg, 1984). THF regeneration is required for the formation of 5,10-methylene-THF and 10-formyl-THF which are used directly in purine and pyrimidine synthesis via methionine synthetase (Bailey and Gregory, III, 1999). The amino acid methionine is essential in the synthesis of proteins and polyamines. Methionine is also the precursor to the universal methyl donor, S-adenosylmethionine (SAM), involved in numerous enzymatic reactions crucial for metabolism (Selhub, 1999). Folate is also a cofactor for methionine synthetase, an enzyme that recycles homocysteine into methionine (**Figure 2**).

2.1.6 Folate and the Methionine-Homocysteine Cycle

In order to understand how folate could negatively influence plasma homocysteine concentrations, one must first be familiar with the entire methionine-homocysteine metabolic cycle and its dependence on B vitamins (**Figure 2**). The methionine-homocysteine metabolic cycle is composed of three basic pathways: transmethylation,

Figure 2. The methionine-homcysteine cycle.



5-methyltetrahydrofolate (5-MTHF), dihydrofolate (DHF), tetrahydrofolate (THF), S-adenosylhomocysteine (SAH), S-adenosylmethionine (SAM)

remethylation and transsulfuration (Stipanuk, 1999). The transmethylation pathway involves the conversion of methionine, through two intermediates, SAM and S-adenosylhomocysteine (SAH), to homocysteine. Homocysteine will either follow the remethylation pathways for methionine regeneration or the transulfuration pathway for the synthesis of cysteine a non-essential amino acid. Approximately 50% of the homocysteine enters the transulfuration pathway (Mayer et al., 1996). Many factors can influence the direction of the homocysteine flow, requiring the body to maintain methionine and homocysteine homeostasis. For example, if methionine intake increases, more homocysteine will be directed towards cysteine synthesis and less towards methionine regeneration, thereby avoiding excessive methionine (Stipanuk, 1999). To illustrate this concept, one study compared methionine intakes of two groups and showed that individuals with adequate methionine intake exhibited a 36 per cent homocysteine remethylation rate as compared to a 67 per cent remethylation rate for those consuming a sulfur amino acid-free diet (Storch et al., 1990). The "methionine sparing" effect of cysteine acts contrary to the effects of high methionine intake, directing more homocysteine towards methionine regeneration while avoiding excessive cysteine synthesis (Stipanuk, 1999). Hence regardless of the factors that influence the movement of homocysteine, the flow will be in the direction that maintains amino acid levels at homeostasis.

As shown in **Figure 2**, folate, vitamin B_6 (pyridoxine), B_{12} (cyanocobalamin) and B_2 (riboflavin) play intimate roles in methionine-homocysteine metabolism. Folate, in the form 5-MTHF, is required as the primary methyl donor to homocysteine

along the remethylation pathway. The loss of a methyl group for methionine synthesis converts 5-MTHF to the non-methylated THF storage form that can be reconfigured to methylene-THF and eventually lead to the resynthesis of 5-MTHF.

Vitamin B_6, B_{12} and B_2 act as enzyme cofactors in the folate-recycling pathway, ensuring the complete functionality of the remethylation process. Vitamin B_6 is a cofactor for serine hydroxymethyltransferase (SHMT), vitamin B_{12} acts with methionine synthase and vitamin B_2 functions as a coenzyme to methylene-THF-reductase (MTHFR), all required for 5-MTHF and methionine regeneration (D'Angelo and Selhub 1997). Vitamin B_6 is also required in the transsulfuration pathway as a required cofactor for cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CL), two enzymes essential for the synthesis of cysteine (Nygård et al., 1999). Whether needed as a methyl donor or as a coenzyme, folate, vitamin B_6, B_{12} and B_2 are crucial to the full operation of the methionine-homocysteine cycle.

2.1.7 Hyperhomocysteinemia

According to a recent literature review, up to two-thirds of reported cases of hyperhomocysteinemia are due to B-vitamin deficiencies (Taylor et al., 2000). Numerous studies have demonstrated that deficiencies of folate, B_6, B_{12} and B_2 either alone or combined, will lead to hyperhomocysteinemia and an increased risk of cardiovascular disease (CVD) (Robinson et al., 1998, Lobo et al., 1999, Minet et al., 2000).

Of the four B-vitamins, folate has been considered the most important in terms of its ability to control hyperhomocysteinemia (Verhoef et al., 1996, McKay et al., 2000). As mentioned previously, 5-MTHF is the primary methylating agent

converting homocysteine to methionine via the remethylation pathway (**Figure 2**). Since a limited amount of folate can be stored in the body, a constant dietary source is essential to maintain the remethylation process. A folate deficiency limits methionine regeneration, inhibiting the movement of homocysteine through this pathway. The remethylation inhibition and the inability of the transsulfuration pathway to compensate for the reduced remethylation activity results in an elevated level of homocysteine (Nygård et al., 1999).

Nygård et al., (1999) reported that plasma total HCY levels are a strong predictor of mortality in patients. There was a strong graded dose-response relationship between the total HCY level and overall mortality. As patients aged their total HCY level also increased. With each additional 20 years of age, total HCY level increased by 1.3 μmol per litre (Nygård et al., 1997).

Reviews of the literature by Selhub et al., (1993), Kang et al., (1987), Refsum et al., (1998), Nygård et al., (1999) and Taylor et al., (2000) have reported elevations in total blood HCY concentrations in patients with low circulating folate. In addition, a strong, inverse relationship between folate deficiency and the increased risk of heart disease (via elevated HCY) has been shown. Morrison et al., (1996) indicated that individuals with less than 3.0 nmoles of folate per litre of serum were 1.69 times more likely to develop CVD than individuals with folate levels greater than 13.6 nmole/ L (acceptable serum folate $\geq$13.5 nmoles/ L, folate deficiency $\leq$6.7 nmoles/ L) (Combs,1998). However, Lewis et al., (1992) argued that a considerable proportion of the adult American population had elevated plasma HCY levels due to inadequate plasma folate levels and the lower acceptable plasma folate level should

be about 15 nmol/ L. Rimm et al. (1998) supported these findings, showing that women with the lowest folate intake had the greatest risk of myocardial infarctions and mortality and that each 100 μg per day increase in folate decreased the risk of heart disease by 5.8 per cent. These studies are but two of many that support the relationship between folate deficiency and hyperhomocysteinemia in the onset of CVD.

In general, the use of B-vitamins to treat hyperhomocysteinemia has been shown successful and most studies report an inverse relationship between B-vitamins supplementation and circulating HCY concentrations (McKay et al., 2000). According to numerous researchers, folate is regarded as the B-vitamin primarily responsible for the observed HCY concentration-lowering effect (Ubbink et al., 1994, McKay et al., 2000). Bellamy et al., (1999) reported that subjects given folate supplements had reduced HCY levels, from 14.9 μM to 8.7μM, and significantly improved brachial artery flow-mediated vasodilation. HCY has been implicated in preventing normal arterial dilation by inhibiting the action of nitric oxide while folate supplementation is shown to maintain endothelial function and reduces the risk of heart disease (Bellamy et al., 1999). While reports that 0.5 to 5.0 mg of daily folate intake will consistently lower plasma HCY levels by 25 per cent, doses as low as 100 to 400 μg/day are almost as effective and fall within a range easily achievable through nutritional intervention such as food fortification (McDowell and Lang, 2000). In fact, it has been proposed that food fortification would reduce the annual mortality from coronary heart disease in the United States (US) by 50,000 individuals (Swain et al., 1997).

2.1.8 Neural Tube Defects (NTD)

The most common neural tube defects are spina bifida (an incomplete closure of the spinal cord and spinal column), anencephaly (severe underdevelopment of the brain), and encephalocely (when brain tissue protrudes out of the skin from an abnormal opening in the skull). All of these defects occur during the first 28 days of pregnancy, usually before a woman is aware she is pregnant.

Repeated studies have shown that women consuming 400 μg of folic acid daily prior to conception and during early pregnancy reduce the risk of occurrent NTDs up to 60 per cent (Werler et al., 1993 and Czeizel et al., 1992) and the recurrent risk of having a child with NTD by 72 per cent (MRC, 1981). Since only 50 per cent of all pregnancies are planned, this is why it is very important for all women of child bearing age to consume folic acid, not just the ones planning to become pregnant.

It has been documented that a subgroup of women with pregnancies afflicted with NTD had higher total HCY concentrations than the healthy control women (Steegers-Theunissen et al., 1994). The same researchers suggest that total plasma HCY concentration can be used as a marker for folate deficiency or folate metabolism defect and therefore a risk factor for giving birth to a child with NTD. In a study performed by Scott et al., (1994), when subjects went from high-normal folate status to low-normal folate status there was a 10-fold increase in NTD risk.

In 1992, the Food and Drug Administration recommended fortifying the nation's food supply with folic acid, a strategy that has worked well in the past with other beneficial substances including iodine (in salt), vitamin D (in milk) and thiamine (in flour and bread). Folic acid began being added to breads and other grain

products of the US and Canada in January of 1998 at a rate of 140 μg/ 100g. Research has proven that the occurrence of NTD can be reduced with folic acid supplementation (Brouwer et al., 1999). It is recommended that women of child bearing age consume 400 μg folic acid/ day (CFDP, 1992). The mandatory folic acid fortification has reduced the number of cases of spina bifida and anencephaly annually by an estimated 19 per cent (Honein et al, 2001).

The importance of folic acid in the human diet cannot be underestimated. The development of other strategies to increase human folate intake are required to help ensure that all segments of the population are consuming adequate folate, especially in light of recent trends towards the reduction of carbohydrate levels in the diet. The fortification of eggs with folic acid may elevate this potential problem. As side from the production of a folate-enriched egg, folate nutrition is equally important for poultry.

2.2 Folate Nutrition

2.2.1 Folic Acid Deficiency and Toxicity in Poultry

A folic acid deficiency in poultry is characterized by macrocytic (megaloblastic) anemia and leukopenia (Hogan et al., 1940). The anemia has been characterized by a decrease in haemoglobin concentrations and number of erythrocytes, leucocytes and thrombocytes, together with increases in erythrocyte size and proportion of heterophils relative to lymphocytes (Maxwell et al., 1988). Cell growth, tissue regeneration and tissues with a rapid turnover (epithelial lining, gastrointestinal tract, epidermis and bone marrow) are principally affected.

Consequences of folic acid deficiency are poor feathering (Maxwell et al., 1988; Luckey et al., 1946), slow growth (Maxwell et al., 1988; Jukes et al., 1947; Luckey et al., 1946), an anemic appearance (Siddons, 1978), perosis (Daniel et al., 1946) and cervical paralysis (necks become stiff and extended) with turkey poults (Jukes et al., 1947; Miller et al., 1967). Siddons (1978) described chickens with waxy white combs and pale mouth mucous membranes as anemia develops. The oestrogen-induced growth of the oviduct in mature fowl is also impaired with folic acid deficiencies (Burns and Jackson, 1982b). This is due to the role of folic acid in purine, nucleic acid and protein synthesis. Also when birds are subject to a disease challenge such as reovirus, birds consuming diets low in folic acid had higher lesion scores (Cook et al., 1984). A deficiency in folic acid also increases erythrocyte phosphoribosylpyrophosphate concentrations in chicks (Rennie et al., 1993). Maxwell et al., (1988) described marginal deficiencies when no physical symptoms are observed, such as macrocytic anemia, abnormal nuclear bodies in erythrocytes, and numerous mitoses and hypersegmented granulocytes. These are changes consistent with folate deficiencies in man and other mammals (Herbert, 1999).

A deficiency in folic acid can impair egg production and results in decreased hatchability (Leeson et al., 1979), associated with increased embryonic mortality (Sunde et al., 1950b), usually during the last few days of incubation. Sunde et al., (1950b) noted these embryos illustrated cases of syndactyly, deformed upper mandibles, parrot beaks and a bending of the tibiotarsus.

Poultry are very tolerant to extremely high concentrations of folic acid. At 5,000 times the normal intake of folic acid, toxicity resulting in renal hypertrophy has been described (Leeson et al., 2001).

2.2.2 Folic Acid Requirements of Laying Hens

There is limited information available on the folic acid requirements of laying hens. A large proportion of studies examining nutrient requirements were conducted prior to 1950. Since that time there have been dramatic changes in dietary ingredients, dietary processing, bird type, management and incubation techniques in the poultry industry. Although there is intestinal microbial synthesis of folic acid, this is inadequate to meet the needs of the bird. This is due to their short gastro-intestinal tract and the majority of synthesis taking place in the later portion of the tract, decreasing the potential for absorption (Leeson et al., 2001). **Table 2** summarizes the folic acid requirements of turkey breeders, turkeys, starting and market broilers and layers based on available research from 1946 onward. The National Research Council (1994) reports 0.25 mg/kg of diet for laying hens and 0.55 mg/kg of diet for broilers. In a survey conducted of 53 commercial layer barns in the United States (BASF, 1993), found that producers supplement their hens with folic acid from 0- 0.59 mg/kg, the average being 0.25 mg/kg. The requirement decreases with age due to reduced growth rate and reduced deoxyribonucleic acid synthesis (Naber et al., 1957; Balek and Morse, 1976).

The reported requirements for broilers vary greatly (Maxwell et al., 1988; Wong, 1977), ranging from less than 0.27 mg/kg to more than 2 mg/kg. This wide variation can be attributed to great difficulties in assaying the folate vitamers and to

Table 2. Documentation of the folic acid requirements of poultry.

Folic Acid (mg/kg)	Age (days)	Response Criteria	Breed	Reference
Turkey Breeders				
0.42	180	poult performance, concentration of pteroylglutamic acid in egg & blood	Bronze	Schweigert et al., 1948
0.7	32-48	poult yield	Bronze females	Kratzer et al., 1956
0.35	32-48	egg production, egg fertility, feed conversion	Large White females	Miller & Balloun, 1967
1.23	32-48	poult yield, survival	Large White females	Miller & Balloun, 1967
1.0				NRC, 1994
Turkeys				
0.8	0-6	growth, anemia prevention	Bronze	Jukes et al., 1947
2.0	0-3	growth, cervical paralysis	Jersey Buff	Russell et al., 1947
0.8				Scott et al., 1948
5.51		egg & poult weight	Large White females	Robel, 1993
0.7				NRC, 1994
Starting & Growing Market Broilers				
≤ 0.5	1-28	growth		Saxena et al., 1954
≤ 0.3	1-21 & 1-28	growth, perosis	Rhode Island Red x White Plymouth Rock	Young et al., 1955
0.88-1.43	1-35	growth	New Hampshire	March & Biley, 1956
0.12-0.27	0-24	growth, normal hematocrit (casein-gelatin diet)		Wong et al., 1977
0.34-0.49	0-24	growth, normal hematocrit (0.7% glycine, casein diet)		Wong et al., 1977
1.69	0-24	growth, normal hematocrit (low glycine, casein diet)		Wong et al., 1977
1.2-1.7	0-21	normal erthrocyte PRPP concentration		Rennie et al., 1993
1.3	0-21	growth, feed efficiency (low choline diet)	Arbor Acre x Arbor Acre	Ryu et al., 1995
1.2	0-21	growth, feed efficiency (diet with 1,300 mg/kg choline)	Arbor Acre x Arbor Acre	Ryu et al., 1995
1.45	0-21	growth, feed efficiency	Arbor Acre x Arbor Acre	Ryu et al., 1995
1.7-2.0	0-42	growth, feed conversion (mash diet)	Cobb	Whitehead et al., 1997; Whitehead et al., 1995
2.5-3.0	0-42	growth, feed conversion (pelleted feed)	Cobb	Whitehead et al., 1997; Whitehead et al., 1995

Folic Acid (mg/kg)	Age (days)	Response Criteria	Breed	Reference
Starting & Growing Market Broilers				
0.75	20	growth, feed efficiency, normal hematocrit		Oloyo et al., 1999
0.55	0-42			NRC, 1994
0.5	42-56			NRC, 1994
Leghorn Type Chickens in Egg Production				
0.25	1-28	growth, feathering, normal hematocrit & hemoglobin	White Leghorn cockerals	Luckey et al., 1946
0.12		egg production		Taylor et al., 1947
0.12-0.42	180	egg production	White Leghorn	Schweigert et al., 1948
0.25		egg production	White Leghorn	Cravens & Halpin, 1949
0.5		hatchability	White Leghorn	Cravens & Halpin, 1949
0.2	N/A	hatchability	White Leghorn	Couch & German, 1950
$\leq$ 0.25	180	maintain body weight, egg production	White Leghorn	Sunde et al., 1950a,b
0.5	180	hatchability	White Leghorn	Sunde et al., 1950a,b
1.0	180	chick livability	White Leghorn	Sunde et al., 1950a,b
0.8	0-35	growth, feed efficiency	White Leghorn	Schweigert et al., 1947; March & Biely, 1955
0.3	0-28	growth, feed efficiency	White Leghorn	Young et al., 1955
0.46-0.96	0-28	growth, feed efficiency (choline deficiency)	Broiler strain	Young et al., 1955
0.33-1.45	0-35	growth, feed efficiency (depending on protein level)	New Hampshire	March & Biely, 1956
0.3	0-18	growth, feed efficiency (18% protein)	Broiler strain	Creek & Vasaitis, 1963
0.45	0-18	growth, feed efficiency (25-27% protein)	Broiler strain	Creek & Vasaitis, 1963
1.5	0-18	growth, feed efficiency (37.5% protein)	Broiler strain	Creek & Vasaitis, 1963
0.25			Commercial Layers	BASF, 1993
0.55	0-42		Leghorn type chickens	NRC, 1994
0.25	42 +		Leghorn type chickens	NRC, 1994

folate interaction with other nutrients (choline, zinc, cyanocobalamin) and other dietary components (protein).

Young et al. (1955) reported that a choline deficiency increased the folic acid requirement of chicks from 0.46 mg/kg to 0.96 mg/kg. This is supported by Pesti et al., (1991) and Ryu et al., (1995) and contrasts the findings of Whitehead et al., (1997;1995). A deficiency in cyanocobalamin also increases the requirement for folic acid (McDowell, 2000), since cyanocobalamin is required for the regeneration of THF from 5-MTHF.

A relationship between folic acid requirement and dietary protein level and source has also been reported. As the protein level of the diet increases so does the folic acid requirement (March and Biely, 1956). Creek and Vasaitis (1957) confirmed that the folic acid requirement had to be increased from 0.3 mg/kg at 18% protein to 0.45 mg/kg at 25 to 27% protein and 1.5 mg/kg at 37.5% protein due to accentuated formation of uric acid with excessive dietary protein. Estimates of folic acid requirements are also dependent on protein source. Wong et al., (1977) reported that if isolated soybean protein was the main protein source, the requirement was less than 0.44 mg/kg. The requirement was between 0.34 and 0.49 mg/kg, with casein supplemented with 7 g glycine/kg but, the requirement was less than 0.27 mg/kg when casein-gelatin was the protein source. Whitehead et al., (1997;1995) observed that in broilers up to 3 weeks of age body weight and feed efficiency conversion were improved by supplementing the diets with folic acid (0.75, 1.5 and 2.0 mg folic acid/kg), optimal results were obtained at 1.5 mg/kg. Wheat contains about 0.2 mg

available folate/kg and corn 0.5 mg/kg. Whitehead et al., (1997;1995) recommended that the folic acid requirement of young broilers is between 1.7 to 2.0 mg/kg.

Robel (1993) studied the effect of folic acid supplementation on the hatchability of fertile turkey eggs and poult weight. The National Research Council's (1994) recommendation for turkey breeders is 1.0 mg folic acid/ kg of diet. However, rapid embryonic development may require higher supplementation, due to the role of folic acid in cellular development. Hatchability was not increased but egg and poult weights were significantly increased in eggs containing 6 to 7 μg folic acid/g of dried egg, from hens consuming 5.51 mg folic acid/ kg of diet. The folic acid requirement for hatchability is greater than that for egg production (Sunde et al., 1950a,b).

Feed additives also influence the folic acid requirements of poultry. It has been shown that sulfa drugs (Luckey et al., 1946; Daniel et al., 1946) and mycotoxins increase this requirement. Luckey et al., (1946) found when adding 25 mg of synthetic folic acid/ kg to a folic acid deficient diet prevented the reduced growth, poor feathering, low hemoglobin and hematocrit values which are normally associated with this diet. However when 1% succinylsulfathiazole was included in the basal diet, the chicks receiving 25 mg/kg folic acid supplementation did not give a complete response and still displayed signs of deficiency. Luckey et al., (1946) reported that folic acid was three times as effective without the addition of sulfa drugs.

The addition of antibiotics to the diet increases vitamin synthesis by microbes, reduces bacterial competition with the host for these nutrients by other microbes and improved absorption of these nutrients (Teeri et al., 1958; Waibel et al., 1952). Teeri

et al., (1958) reported that the addition of oxyteracycline at 10 g/ton to the diet of laying hens significantly ($P<0.05$) increased the concentration of folic acid in egg yolk. There were no adverse effects on egg production, egg weight, hen weight or hatchability. A 26.7% increase in folic acid deposition in egg yolk was reported by Waibel et al., (1952) when supplementing diets with 200 mg/ kg penicillin, for a 30-47 day period. At 83-174 days after penicillin feeding began a 12.6% increase was reported.

Since folic acid is essential for growth in birds, the outlined research above can easily be applied to laying hens, suggesting the NRC requirements are inadequate.

2.3 Folic Acid Absorption and Distribution

2.3.1 Folic Acid Absorption

The absorption of folic acid by laying hens is consistent with the process described in Section 2.1.3. The first two important events in folate absorption are (1) the hydrolysis of polyglutamates to monoglutamic forms of folate by folate hydrolase (Herbert, 1999) and the absorption of monoglutamic folate from the gut (Anderson, et al., 1992; Henderson, 1990), since polyglutamates cannot cross biological membranes.

The monoglutamic folic acid in the intestinal cells is reduced to dihydrofolate and THF via dihydrofolate reductase (Henderson, 1990). THF is then transported form the intestinal cells via a membrance-bound transport system to the liver (Henderson, 1990). In the liver THF is converted to 5-MTHF, attached to folate-binding proteins and then released into plasma (Henderson, 1990).

2.3.2 Distribution of Folates in Laying Hens

The livers of laying hens have a higher concentration of folates in comparison to other tissues (Sherwood et al., 1993). We believe that the folate is then transported via folate-binding transport proteins from the plasma to the egg.

Folate deposits in eggs are i) bound to the yolk fraction (Sherwood et al., 1993), ii) present as 5-MTHF (Seyoum and Selhub, 1998; Tolomelli, 1981), and iii) present as the monoglutamate (Seyoum and Selhub, 1998; Tolomelli, 1981). The folate concentration in yolk is much greater than that of albumen. Sherwood et al., (1993) determined by radioactivity that ~1% of folate was found in the albumen. Therefore, egg yolk folate concentrations may be regulated by intestinal folate uptake.

2.3.3 Deposition of Folate in Eggs

For several decades the developing egg has intrigued biologists. The biosynthetic processes fundamental to the maturation of the egg can be divided into two categories: (1) autosynthetic –those occurring within the maturing oocyte itself and (2) heterosynthetic –those that are initiated outside the oocyte. The liver, the oviduct and the follicle are the three main sites of product synthesis for the maturing oocyte. Pituitary gonadotropins (FSH and LH) activate the follicle cells, which are responsible for the formation of egg proteins and oestrogen. Oestrogen regulates the activity of the liver and oviduct. The liver is the major source of yolk proteins, carrier proteins for cholesterol and other very low density lipoproteins and water soluble vitamins, such as folate and riboflavin. Ovalbumin, conalbumin, lysozyme and

ovomucois, glycoproteins of the egg white, are provided by the oviduct (McKnight, 1978).

Distinct carrier proteins are responsible for the deposition of water-soluble vitamins, such as biotin, riboflavin and thiamine (White et al., 1986, Muniyappa & Adiga, 1979). It is believed that micronutrient transport proteins like vitellogenin (an energy-rich phosphate storage, calcium-transport protein) are also under the influence of oestrogens. These carrier proteins have a greater affinity for their respective vitamin than the coenzyme forms.

Adiga and Murty (1983) proposed that the production of the carrier protein in the maternal liver is mediated by hormonal stimulus transmitted by the feto-placental unit, either directly and/or through chorionic gonadotrophin (CG). CG is responsible for the stimulation of the ovary to produce steroids, which then stimulates the liver. The carrier protein with tightly bound vitamins are secreted from the liver into the blood, and permeate intact through the placental barrier.

Dietary folate is metabolized in the liver cells to 5-MTHF and then released into plasma (Henderson, 1990). There is little information on the metabolism and deposition of folate in chickens. Even though flavins and folates are functionally distinct, they do have structural resemblance in the pterin ring system. Thus it is conceivable that they would have similar carrier systems. Riboflavin-binding proteins have been isolated from hen plasma, egg yolk and egg white (Miller and White, III, 1986). Riboflavin-binding proteins are synthesized in the liver and secreted into the bloodstream (White and Merrill, 1988). The vitamin-protein complex is then deposited in the yolk (White and Merrill, 1988). We can speculate

that similarly folate is transported via folate-binding transport proteins from the plasma to the egg.

2.4 Development of the Folate Enriched Egg

Due to the recent awareness of the need for increased consumption of folate, a water soluble vitamin, by humans, it is necessary to consider the incorporation of natural sources in their diets. It is recommended that women of childbearing years consume 400 μg of folic acid per day to reduce the occurrence of neural tube defects, such as spina bifida and anencephaly (Czeizel and Dudas, 1992). Folate is also a cofactor in the remethylation of HCY to form methionine (House et al., 1999). A folate deficiency results in an increase in the serum level of HCY and in humans is a predictor for an increase in the occurrence of cardiovascular disease (Boushey et al., 1995). Therefore it is important that humans have adequate intake of folic acid.

Foods naturally contain marginal levels of folic acid. It is found mostly in leafy green vegetables like kale and spinach, orange juice, and enriched grains. However, a large egg contains approximately 24 μg of folate (USDA, 2005). By increasing the folate content of eggs, the egg could be marketed as an excellent dietary source of folate. This would provide the public with the option of including eggs as part of a nutritional breakfast to meet the necessary dietary requirements of folic acid instead of consuming crystalline formulated vitamin pills.

It has been previously shown that supplementing the diets of laying hens with crystalline folic acid will result in an increase in the folate deposited in the eggs (House et al., 2002; Sherwood et al., 1993). Sherwood et al., (1993) reported that supplementing commercial laying hen ration above 1.5 mg folic acid/ kg resulted in a

saturation of yolk folate content. House et al., (2002) also observed the most sensitive response of egg folate content to be between 0 and 2 mg folic acid/ kg diet, after which egg folate levels appeared to reach a plateau. At an inclusion level of 32 mg folic acid/ kg, House et al., (2002) reported a further significant increase in egg folate content, suggesting a biphasic response.

Based on limited research on the production of folate-enriched eggs, the overall objective of this thesis was to determine the factors that influence the production of folate-enriched eggs.

Chapter 3

MANUSCRIPT 1

Effect of Dietary Folate on Laying Hen Performance Traits, Folate and Homocysteine Concentration of Blood Plasma and Egg Folate Content.

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3.1 Abstract

A study was designed to determine the optimal dietary folic acid level for maximal egg folate deposition, and the potential for differences in egg folate deposition due to strain of birds. Hyline W36 and W98 hens (n=6/ diet) received a barley-based ration, containing 0, 2, 4, 8, 16, 32, 64 or 128 mg/kg of crystalline folic acid for 21 days. Response criteria included production parameters, measures of blood folate status and egg folate content. There was no significant difference ($P<0.05$) in performance parameters due to folate supplementation. Egg folate content significantly ($P<0.0001$) increased from 16.7 μg/egg with an unsupplemented diet to 37.5 μg/egg when supplemented with 2 mg folic acid/ kg of diet. Hyline W98 had significantly ($P<0.05$) higher total egg folate content, feed consumption, egg weight, yolk weight and lower percent yolk of egg weight than the W36. There was a significant ($P<0.05$) increase in plasma folate concentrations and a significant ($P<0.05$) decrease in plasma homocysteine (HCY) concentrations, a marker of folate status, with supplementation. A significant ($P<0.05$) ration x strain interaction was evident for HCY, as Hyline W98 hens exhibited a decrease in HCY plasma concentration from 0 to 2 mg folic acid/ kg diet. This strain appears to be more responsive to folate supplementation by decreasing their plasma HCY levels.

Funding: Manitoba Egg Producers, CEMA, Clark Hy-line Inc.

Keywords: Folate, Egg, Homocysteine

3.2 Introduction

The fortification of eggs with nutrients that appeal to consumers, as their demand for "natural" products with health-promoting properties increases, will result in an increased demand for eggs and an improvement in the market potential for this valuable farm commodity. Differentiated diets for laying hens can be utilized to enhance the levels of certain beneficial compounds like omega-3 fatty acids (Farrell, 1998) and vitamins (Naber, 1993).

Pteroylglutamic or pteroylmonoglutamic acid, commonly known as folic acid is a water soluble B-vitamin, involved in single-carbon transfer reactions and exists in many chemical forms (Sauberlich et al., 1987). Folate and folacin are terms commonly used to describe compounds with similar chemical structure and nutritional properties as folic acid. Folate is also a cofactor for methionine synthetase, an enzyme that recycles homocysteine into methionine (House et al., 1999).

Due to the recent awareness of the need for increased consumption of folate by humans, it is necessary to consider the incorporation of natural sources in their diets. It is recommended that women of childbearing years consume 400 μg of folic acid per day to reduce the occurrence of neural tube defects, such as spina bifida and anencephaly (Czeizel and Dudas, 1992). A folate deficiency results in an increase in the serum level of homocysteine and an increase in the occurrence of cardiovascular disease (Boushey et al., 1995). Therefore it is important that humans have adequate intake of folic acid. Folic acid began being added to breads and other grain products

of the US and Canada in January of 1998 at a rate of 140 μg/ 100g, based on the recommendation of the Food and Drug Administration.

Foods naturally contain marginal levels of folic acid. A large egg contains approximately 24 μg of folate (USDA, 2005). By increasing the folate content of eggs, the egg could be marketed as an excellent dietary source of folate. This would provide the public with the option of including eggs as part of a nutritious breakfast to meet the necessary dietary requirements of folic acid instead of consuming crystalline formulated vitamin pills.

Previous research illustrated the potential for egg folate fortification (Sherwood et al., 1993; House et al., 2002). However, these studies were conducted with a maximum supplementation level of 32 mg crystalline folic acid/ kg diet and demonstrated the potential to increase the folic acid content of eggs two- to four- fold (House et al., 2002).

In the egg industry today producers have the opportunity to choose from a number of different strains. Strains vary not only in appearance but in production capabilities and in response to different management practices. Previous researchers have used Hyline W36 and Dekalb XL in egg folate fortification studies. It is important to compare two strains under identical management practices to highlight any differences due to strain.

Based on the results of published research, a study was designed to determine the optimal dietary folic acid level required for maximal egg folate deposition, and the potential for strain differences that may exist. This was achieved by saturating the digestive tract with dietary folate levels three times that of previous studies reported

in the literature. Plasma folate and plasma homocysteine concentrations will be examined with respect to level of folate supplementation and the relationship between plasma and egg folate levels will be determined.

3.3 Hypotheses and Objectives

3.3.1 Hypotheses

Alternative Hypothesis: Egg folate level will be affected by laying hen strain and dietary folate treatment.

Null Hypothesis: Egg folate level will not be affected by laying hen strain or dietary folate treatment.

3.3.2 Objectives

The purpose of this research was to determine the optimal production conditions for the production of folate-fortification of eggs. This will be accomplished through the following objectives:

i) To determine the optimal level of dietary folate supplementation for maximal egg folate deposition.

ii) To determine whether strain differences exist for maximal egg folate deposition.

iii) To determine the homocysteine and folate status of plasma in laying hens fed graded levels of folate.

iv) To determine the relationship between plasma and egg folate levels as a function of uptake from the hen's intestine.

3.4 Materials and Methods

3.4.1 General

Single-Comb White Leghorn laying hens (Manitoba Perfect Pullets, Rosenort, MB, Canada), Hyline W98, an early maturing, large egg mass strain, and Hyline W36, a traditional strain (efficient egg layer with excellent livability), were used in this experiment. Hens were kept in confinement housing under semi-controlled environmental conditions and exposed to 16-hr photoperiod. Ninety-six birds were housed individually; the cage dimensions were 25.4 cm by 40.64 cm, providing 1,032 cm^2 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).

3.4.2 Diet and Experimental Protocol

The basal diet was a barley-based ration (**Table 3**). The diet was formulated to meet the requirements of laying hens consuming 100g of feed /d (NRC, 1994). In accordance with industry standards (BASF, 2000), the basal diet included no crystalline folic acid.

For two weeks prior to the commencement of the study, 256 healthy hens of the Hyline W36 and W98 strains each received a standard barley-based ration with no additional folic acid supplementation. These hens were monitored for egg production, and the 48 highest producing hens of each strain were selected for the experiment. At 22 weeks of age (peak production 92-96%), the selected hens were

Table 3. Composition of basal barley-based laying hen ration.

Ingredient	%
Barley (11.9% CP)	58.80
Soybean Meal (45.0% CP)	12.60
Canola Meal (37.58% (CP)	10.00
Fish Meal (72% CP)	2.00
Vegetable Oil	5.57
Limestone	8.73
Biophos (monocalcium phosphate)	0.80
Vitamin premix[1]	1.00
Mineral premix[2]	0.50
Calculated nutrient composition	
Crude protein	18.0
Metabolizable energy, kcal/kg	2703
Calcium	3.75
Phosphorus (available)	0.40
Folate[3] (mg/kg)	1.45

[1]Provided (per kg of diet): vitamin A, 8225 IU; vitamin D3, 1000 IU; vitamin E, 5.46 IU; vitamin B12, 11.2 μg; calcium panthothenate, 4.4 mg; choline chloride, 100 mg; ethoxyquin, 125 mg; dl-methionine, 500 mg; niacin, 7.6 mg; riboflavin, 2.2 mg.

[2]Provided (per kg of diet): Mn, 52 mg; Zn, 60 mg; salt (iodized), 4.78 g.

[3]Measured by Covance Laboratories Inc., Madison, WI via Official Methods of Analysis of AOAC International (2000) 17th ed., AOAC International, Gaithersburg, MD, USA, Official Methods 960.46, 992.05.

placed individually into battery cages and were randomly assigned to receive one of eight dietary folic acid levels (n=6 per treatment). The birds were given a two-week adaptation period followed by a seven day collection period in which the barley diets supplemented with 0, 2, 4, 8, 16, 32, 64, or 128 mg crystalline folic acid / kg (Sigma Chemical Co., Oakville, ON, Canada) were provided. Feed consumption (FC) for each cage unit was determined at the completion of the trial to calculate average daily feed intake and feed efficiency (FE). Egg production (EP) was recorded daily for each cage unit and an average egg production rate (hen-day percent) was calculated. Ninety-six eggs (1 egg/ replication; 6 replications per treatment per strain) were randomly collected per treatment per day for three days (288 eggs total). The eggs were weighed to give an average egg weight for the treatment period and processed for egg folate determination.

To determine plasma folate and homocysteine levels, at the end of the production period, a 1 ml blood sample was collected via wing venipuncture from forty-eight birds (6 replicates from each treatment; 3 per strain). Blood samples were collected with a 2 ml sterile syringe containing 50 µl of a porcine heparin saline solution (68.6 USP) to prevent clotting. The samples were cooled on ice and immediately centrifuged at 12,000 x g for 5 minutes (Jouan, CR 3000). Plasma was then pipetted into microcentrifuge tubes and stored at –80°C until analysis.

3.4.3 Extraction of Egg Yolk Folate

All chemicals used in the extraction and analysis of folate were purchased from Sigma Chemical Co. (Oakville, ON, Canada). It has been previously shown that folate deposits in eggs are i) bound to the yolk fraction (Sherwood et al., 1993), ii)

present as 5-MTHF (Seyoum and Selhub, 1998), and iii) present as the monoglutamate (Seyoum and Selhub, 1998). As a result of these findings, supported by House et al., (2002), further preparation was not required to deconjugate glutamate residues or to quantify other forms of folate.

On sample days, the eggs were collected before 10:00 am and labelled with cage number and ration. The eggs were then weighed and placed in a stock pot. Cold water was added to cover the eggs with one inch of water. The pot was covered and burner set to high. Once the water began to boil, the burner temperature was reduced by half to ensure a gentle boil for 10 minutes. The water was then drained and the eggs cooled rapidly in ice water for 5 minutes. The yolks were then separated from the white, individually bagged and stored at –80°C until analyzed.

The egg yolk folate extraction procedure is described by House et al., 2002. Approximately 0.5 g of egg yolk was weighed into glass tubes with lids. Ten millilitres of ascorbate buffer (pH 7.8) (see appendix for preparation) was added to each tube. The tubes were topped up with nitrogen gas, caps secured, vortexed and placed in a boiling water bath for 60 minutes. The tubes were then centrifuged at 4,000 x g for 30 minutes (Jouan CR3000). The supernatant from each tube was removed and placed in a corresponding 25 ml volumetric flask. Another 10 ml of ascorbate buffer was added to each tube. The tubes were vortexed and centrifuged as before. The supernatants were then pooled, and brought to a final volume of 25 ml with ascorbate buffer. The extracts were then mixed thoroughly. A sample from each flask was placed into a microcentrifuge tube and stored at -20°C until analyzed.

3.4.4 Analysis of Egg Yolk Folate Content

As previously described by Vahteristo et al., (1997), the concentration of 5-MTHF in the egg yolk extracts was determined by reverse-phase high pressure liquid chromatography (HPLC) with fluorescence detection (Shimadzu, Mantech, Guelph, ON, Canada). An external standard curve with purified 5-MTHF was used to quantify egg folate concentrations. The inter- and intra-assay coefficient of variance (CV) for determinations was <2%, and recovery of 5-MTHF added to egg yolk was 98.9%. The content of egg folate was expressed as micrograms of folic acid per egg.

3.4.5 Analysis of Blood Plasma

The analysis of blood plasma was conducted to determine the relationship between plasma and egg folate levels. Plasma folate was determined by using a competitive binding assay, Quantaphase II B12/Folate Radioassay purchased from Bio-Rad (Hercules, CA; #191-1041). All reagents, standards and samples were allowed to equilibrate to room temperature prior to beginning the assay. The plasma samples were diluted with Red Cell Folate Reagent Diluent (Bio-Rad, Hercules, CA; #191-1060) to ensure the results would fall in the centre of the standard curve. The reaction tubes (12 x 75 mm) were appropriately labelled and 200 µl of each standard, control or plasma sample were added to the corresponding tubes. To all tubes 1 ml of working tracer (dithiothreitol (DTT) dissolved in deionized water) was added and vortexed. Total count tubes were prepared by adding 1 ml of working tracer (^{125}I-folate) to the appropriate reaction tube. The test tube rack was covered with aluminum foil and placed in a boiling water bath at 100°C for 20 minutes. The boiling inactivates endogenous proteins and the reduced folate and its analogs are

stabilized by DTT. To return the reaction tubes to room temperature the test tube rack was then placed in a cold water bath for 10 minutes. To the blank tubes 100 µl of blank reagent was added. To all the tubes except the blank tubes and total count tubes 100 µl of microbead reagent (immobilized folate binding protein) was added. All tubes were then vortexed and incubated at room temperature for 1 hour. During incubation, based on their concentrations the endogenous and labelled vitamers compete for the limited number of binding sites. The tubes were then centrifuged (Jouan, CR 3000) for 10 minutes at 1500 x g and immediately decanted to discard the supernatant. The labelled and unlabelled vitamins bound to the immobilized binding proteins are concentrated at the bottom of the reaction tube in the form of a pellet and the unbound vitamins in the supernatant discarded. After decanting, the tubes were immediately returned to their upright position. Finally, the radioactivity associated with the pellet was counted for 1 minute using a gamma counter (LKB Wallax, 1282 Compugamma). The folate concentrations contained in the samples were determined by using a standard curve that was prepared with the assay and mathematically fit to the curve.

Plasma homocysteine was determined by HPLC as described by Shoveller et al., (2004). A 0.1 mM D,L-homocysteine standard was prepared by dissolving 27.04 mg D,L-homocysteine in 100 ml of 0.1 M potassium borate buffer (see appendix for preparation). The 0.1 mM D,L-homocysteine solution was used as 100% concentration, from this a standard curve of 0, 25, 50, 75 and 100% was prepared, using 0.1 M potassium borate buffer as the diluent for a final volume of 1 ml. Into microcentrifuge tubes 150 µl of each standard in duplicate and sample was pipetted.

To all microcentrifuge tubes 20 µl of tris (2-carboxyethyl)-phosphine hydrochloride (TCEP) solution (see appendix for preparation) was added, and the sample vortexed (IEC, Micromax). The microcentrifuge tubes were then incubated at room temperature for 30 minutes. Following incubation 125 µl of 0.6 M perchloric acid (see appendix for preparation) was added to each microcentrifuge tube to cause protein denaturation and the tubes were then centrifuged at 10,000 rpm for 10 minutes. Following centrifuging 100 µl of each standard and sample were pipetted into new microcentrifuge tubes. To all microcentrifuge tubes 200 µl of 2 M potassium borate buffer (pH 10.5) (see appendix for preparation) and 100 µl of 7-flurobenzo-2-oxa-1,3-dizole-4-sulfonic acid (SBDF) (see appendix for preparation) was added and the tubes were vortexed. The microcentrifuge tubes were then placed in a 60°C heating block (Fisher Isotemp 145D, #11-715-145D, Ottawa, ON, Canada) for 1 hour, and subsequently cooled at 4°C for 20 minutes. Finally, approximately 200 µl of the standard/ sample was transferred to HPLC vials for analysis. An isocratic elution system was used, consisting of 0.1 M sodium acetate (see appendix for preparation), pH 5.5, with 2% methanol. The flow rate was 1.0 ml/min and the plasma homocysteine concentrations were determined by fluorescence detection (Shimadzu, Mantech, Guelph, ON) with an excitation of 385 nm and emission of 515 nm with reference to an external standard curve.

The collected production data was then used to calculate hen-day production (EP), FC, FE, percent yolk of egg weight and folate transfer efficiency, based on the following equations.

3.4.6 Calculations

Hen-Days =

Cumulative Number of days each hen was present during the period.

Hen-Day Production (%) =

$$\frac{\text{(Total Number of Eggs Laid in the Period)}}{\text{(Number of Hen-Days)}} \times 100$$

Feed Consumption (g/hen/d) =

$$\frac{\text{Total Amount of Feed Fed (g) in the Period} - \text{Feed Weigh Back (g)) at the End of the Period}}{\text{(Number of Hen-Days)}} / \text{\# Days in the Period}$$

Feed Efficiency (g feed/ g egg) =

$$\frac{\text{(Feed Consumption (g/hen/d))}}{\text{(Average Egg Weight (g) for the Period X Rate of Production (\%))}}$$

Percent Yolk of Egg Weight =

$$\frac{\text{(Average Yolk Weight (g))}}{\text{(Average Egg Weight (g))}} \times 100$$

Folate Transfer Efficiency =

$$\frac{\text{(Egg Folate Content (µg/egg)}}{\text{(Egg Weight (g)) X (Feed Efficiency) X (Folic Acid Supplementation}} \times 100$$

(g feed/ g egg)) Level) (µg/100g))

3.4.7 Statistical Analysis

An 8 x 2 (ration x strain as main effects) factorial arrangement of a completely randomized design was used to analyze the data. Performance data collected were subjected to analysis of variance using SAS Analyst (SAS Institute Inc., 1998). Differences between means were determined using Tukey's Honestly 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 necessary to log transform the data for plasma folate and homocysteine, since a plot of residuals versus predicted values did not reveal any heterogeneity in the variability.

3.5 Results

The main effects of ration and strain and their interactions for different performance traits are shown in **Table 4**. Significant ($P<0.05$) ration differences existed for egg folate content, plasma HCY concentration and plasma folate concentration. Significant ($P<0.05$) strain differences were found for FC, egg weight, yolk weight, percent yolk of egg weight, and egg folate content. Significant ($P<0.05$) ration by strain interactions were observed for egg weight, yolk weight, egg folate content, and plasma HCY concentration. A possible ration by strain interaction ($P=0.0625$) for EP was also apparent.

The performance data and egg folate content of laying hens receiving diets with graded level of crystalline folic acid are shown in **Table 5**. The Hyline W98 hens had significantly ($P<0.0001$) higher FC than the Hyline W36 hens, 97.9 vs. 88.7 g/h/d. Similarly, Hyline W98 hens had significantly ($P<0.05$) larger egg and yolk weights than the Hyline W36 hens (58.9 vs. 53.7 g and 13.8 vs. 13.3 g, respectively). Hyline W98 hens also had a significantly (12%; $P<0.0001$) higher egg folate content than the Hyline W36 hens, 39.8 vs. 34.1 μg/egg (2.9 μg vs. 2.6 μg/ g yolk). Although not significant the mean hen-day production and feed efficiency for the Hyline W98 and W36 were 96.5, 95.2 ± 0.89 and 1.66, 1.67 ± 0.019 respectively.

Egg folate content and indices of folate status in laying hens receiving diets with graded levels of crystalline folic acid are shown in **Table 6**. Egg folate content significantly ($P<0.0001$) increased from 16.7 μg/egg with an unsupplemented diet to 37.5 μg/egg when supplemented with 2 mg folic acid/ kg of diet. There was no

Table 4. P-values of main effects and interactions of ration and strain on different performance traits.

Parameters	Ration	Strain	Str*Rat
Hen-day Production (%)	0.6866	0.3081	0.0625
Feed Consumption (g/h/d)	0.7727	<0.0001	0.7825
Feed Efficiency (g feed/g egg)	0.4660	0.5869	0.1628
Egg Weight (g)	0.4508	<0.0001	0.0468
Yolk Weight (g)	0.2350	0.0280	0.0102
% Yolk of Egg Weight	0.0804	<0.0001	0.3515
Egg Folate Content (μg/egg)	<0.0001	0.0240	0.0154
Plasma HCY (μM)	0.0048	0.1434	0.0077
Plasma Folate Concentration (ng/mL)	<0.0001	0.7299	0.4962

Table 5. Performance summary of Hyline W98 and W36 laying hens receiving diets with graded levels of crystalline folic acid- Main effects of strain[1].

	Strain			
Parameters	W36	W98	SEM	p-value
Feed Consumption (g/h/d)	88.7	97.9	0.92	<0.0001
Egg Weight (g)	53.7	58.9	0.46	<0.0001
Yolk Weight (g)	13.3	13.8	0.15	0.0280
% Yolk of Egg Weight	24.80	23.50	0.21	<0.0001
Egg Folate Content (µg/egg)	34.1	39.8	1.62	0.0240

[1]Data are presented as least square means

Table 6. Egg folate content and indices of folate status in laying hens receiving diets with graded levels of crystalline folic acid- Main effects of ration[1].

	Parameters		
Dietary Folic acid Supplementation (mg/kg)	Egg Folate Content (μg/egg)	Plasma HCY (μM)	Plasma Folate Concentration (ng/mL)
0	16.7^{b}	12.59^{a}	15.23^{d}
2	37.5^{a}	10.01^{a,b}	32.59^{c,d}
4	38.6^{a}	10.80^{a,b}	36.85^{b,c}
8	40.6^{a}	10.756^{a,b}	38.67^{b,c}
16	38.3^{a}	9.99^{a,b}	41.63^{a,b,c}
32	44.0^{a}	10.27^{a,b}	49.11^{a,b}
64	41.2^{a}	9.73^{a,b}	48.60^{a,b,c}
128	38.8^{a}	8.55^{b}	59.40^{a}
SEM	3.25	0.750	3.580
p-value	<0.0001	0.0478	<0.0001

Means within a column with different letter superscript are significantly different (P<0.05)

[1]Data are presented as least square means

further significant increase in egg folic acid content up to an inclusion level of 128 mg/ kg of diet **(Table 6)**.

A significant ($P<0.05$) decrease in plasma HCY concentration was observed between the control diet and supplementation of 128 mg folic acid/ kg diet. Plasma HCY levels appear to stabilize at a folic acid supplementation level of 2 mg/ kg of diet. The HCY levels of the birds fed 2 to 64 mg folic acid/ kg diet were not significantly different from either the 0 and 128 mg folic acid/ kg diet. Plasma HCY concentrations were unaffected by increasing dietary folic acid supplementation from 0 to 64 mg/ kg. However, at dietary supplementation of 128 mg/ kg there was a significant ($P<0.05$) decrease in plasma HCY concentration in comparison to hens fed the unsupplemented diet. Plasma folate concentration increased with increasing dietary folic acid supplementation. However, plasma folic acid concentration does not increase at the rate of dietary folic acid supplementation **(Table 6)**. There was an inverse relationship between plasma folate concentration and plasma HCY concentration.

Ration by strain interaction effects on the performance of hens are shown in **Table 7**. There appears to be a trend in hen-day production (EP) ($P=0.0625$). The Hyline W98 appear to respond to increasing dietary folic acid supplementation by increasing their EP up to an inclusion level of 64 mg folic acid/ kg at which EP declines. This demonstrates the value of providing the Hyline W98 with additional folic acid to increase EP up at an inclusion level of 32 mg/ kg. Ration by strain interactions were significant for egg weight, yolk weight, egg folate content and plasma HCY concentration.

Table 7. Performance summary and egg folate content of Hyline W98 and W36 laying hens receiving diets with graded level of crystalline folic acid- Variable with significant (P<0.05) ration*strain interactions[1].

	Hen-Day Production (%)		Egg Weight (g)		Yolk Weight (g)		Egg Folate Content (μg/egg)	
	Strain		Strain		Strain		Strain	
Dietary Folic acid Supplementation (mg/kg)	W36	W98	W36	W98	W36	W98	W36	W98
0	99.21	98.51	$55.8^{a,b,c,d}$	$57.1^{a,b,c,d}$	$14.2^{a,b}$	$13.1^{a,b}$	16.6^{b}	16.8^{b}
2	97.62	98.41	$53.2^{b,d}$	$57.7^{a,b,c,d}$	$13.5^{a,b}$	$12.9^{a,b}$	$32.8^{a,b}$	$42.2^{a,b}$
4	94.44	98.41	$53.3^{b,d}$	$57.4^{a,b,c,d}$	$12.8^{a,b}$	$13.2^{a,b}$	48.2^{a}	$31.0^{a,b}$
8	96.83	96.03	52.4^{d}	$59.3^{a,b,c}$	$13.2^{a,b}$	14.8^{a}	$31.6^{a,b}$	49.5^{a}
16	95.24	97.62	$54.7^{b,c,d}$	$61.3^{a,c}$	$13.1^{a,b}$	$14.3^{a,b}$	$29.5^{a,b}$	47.1^{a}
32	96.03	96.03	$53.2^{b,d}$	$58.3^{a,b,c,d}$	$13.1^{a,b}$	$13.7^{a,b}$	45.8^{a}	42.2^{a}
64	96.06	94.44	$54.9^{b,c,d}$	$57.6^{a,b,c,d}$	$14.0^{a,b}$	$14.0^{a,b}$	$35.8^{a,b}$	46.7^{a}
128	96.83	94.44	52.2^{d}	62.2^{a}	12.7^{b}	$14.6^{a,b}$	$34.5^{a,b}$	42.8^{a}
SEM	2.51		1.31		0.44		4.58	
p-value	0.0625		0.0468		0.0102		0.0154	

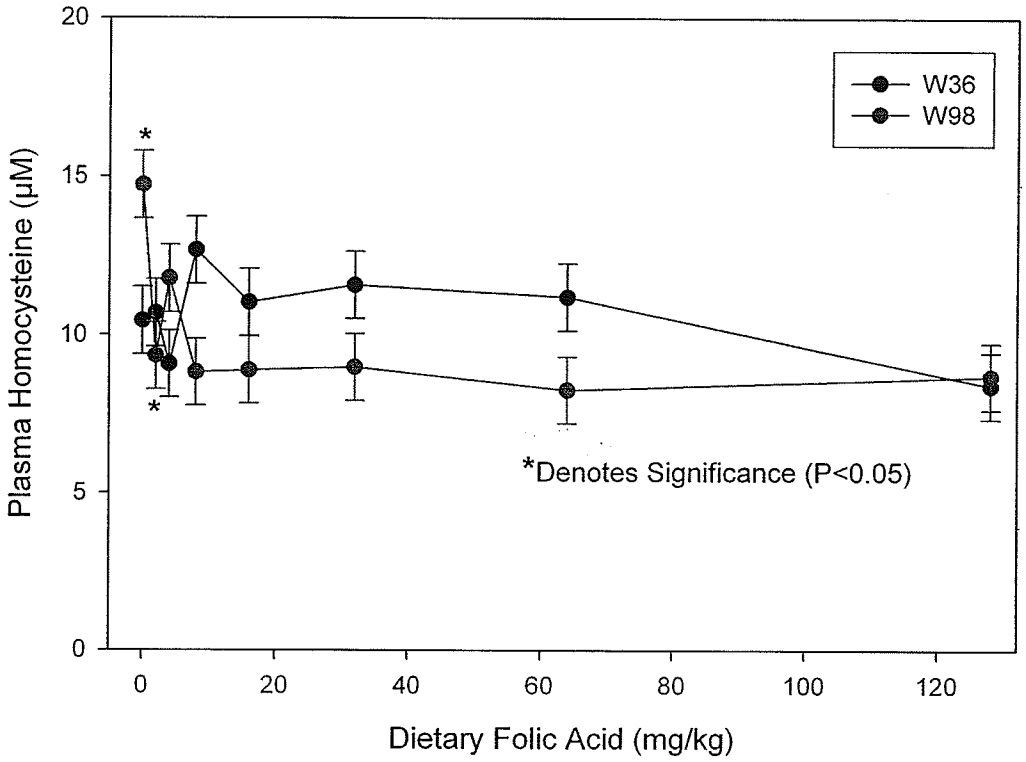
Means within each parameter with a different letter superscript are significantly different (P<0.05)

[1]Data are presented as least square means

Hyline W98 had significantly ($P<0.05$) higher egg weights when consuming 8, 16 and 128 mg folic acid/ kg diet than Hyline W36. Overall, increasing folic acid supplementation from 0 to 128 mg/ kg diet resulted in a decrease in egg and yolk weights for the Hyline W36 and an apparent increase in egg and yolk weights for the Hyline W98 hens.

Increasing dietary folic acid supplementation did not affect plasma HCY concentrations for the Hyline W36 **(Figure 3)**. However, increasing dietary folic acid supplementation to 2 mg/ kg, significantly decreased plasma HCY concentration of the Hyline W98 from 14.7 μM to 9.3 μM. The Hyline W98 hens were also more responsive to increasing dietary folic acid supplementation by increasing their egg folate content to a maximum at 8 mg/ kg diet, while the Hyline W36 egg folate content was unaffected with increasing dietary supplementation above 4 mg/ kg.

Figure 3. The effect of dietary folic acid on plasma homocysteine concentrations of Hyline W98 and W36 laying hens.



Data reported as LS Means +/- Standard Error
Strain*Ration Interaction P<0.0077
All means are composed of at least 3 replicates

3.6 Discussion

3.6.1 Effect of Dietary Folate on the Folate Content of Eggs

This study supports the work of House et al., (2002) and Sherwood et al., (1993), that egg folate concentrations are sensitive to dietary folate levels. In this study the folate content of eggs was increased 2-fold by supplementing crystalline folic acid to cereal-based laying hen rations. There was a significant increase in total egg folate content from 0 to 2 mg folic acid / kg diet, at which egg folate levels plateau (**Table 6**). This is supported by Sherwood et al. (1993), who supplemented commercial cereal based laying hen rations between 0 and 7 mg folic acid/ kg diet. These authors suggested that above 1.5 mg folic acid/ kg diet resulted in a saturation of yolk folate content (Sherwood et al., 1993). House et al., (2002) also observed the most sensitive response in egg folate content between 0 and 2 mg folic acid/ kg, after which egg folate levels appeared to reach a plateau. At an inclusion level of 32 mg folic acid/ kg, House et al., (2002) reported a further significant increase in egg folate content, suggesting a biphasic response. However, in our study increasing dietary supplementation up to 128 mg folic acid/ kg of diet failed to define such a response.

The Hyline W98 hens exhibited an overall higher total egg folate content compared to the W36 hens. This can be attributed to the fact that the W98 produce larger eggs than the W36, and therefore would produce eggs with a higher total egg folate content. However, when we compare the Hyline W98 and W36 egg folate content on a μg/ g yolk basis, there is no significant difference between strains (2.9 vs. 2.6 μg/ g yolk, respectively).

3.6.2 Deposition of Folate in Eggs

Dietary folate is absorbed from the gut via a membrane-bound folate transport system (Said et al., 2000), metabolized in the liver cells to 5-MTHF and then released into plasma (Henderson, 1990). There is little information on the metabolism and deposition of folate in chickens. Even though flavins and folates are functionally distinct, they do have structural resemblance in the pterin ring system. Thus it is conceivable that they would have similar carrier systems. Riboflavin-binding proteins have been isolated from hen plasma, egg yolk and egg white (Miller and White, III, 1986). Riboflavin-binding proteins are synthesized in the liver and secreted into the bloodstream (White and Merrill, 1988). The vitamin-protein complex is then deposited in the yolk (White and Merrill, 1988). We can speculate that folate is then transported via folate-binding transport proteins from the plasma to the egg.

With increasing dietary folic acid supplementation egg yolk folate content and plasma folate concentrations saturate. The system that transfers folate from plasma across the oocyte vitelline membrane to the yolk does not appear to be contributing to this saturation. Systems that supply and remove plasma folates may be limiting in further increases in egg folate content, such as the metabolic conversion of monoglutamic folate to reduced methyl folate (primarily 5-MTHF) within intestinal cells prior to the release into the portal circulation and deposited into the egg. Sherwood et al., (1993) speculated that the folate egg yolk saturation was not due to limits on the system that transfers folate from plasma across the oocyte vitelline membrane to the yolk because plasma folate concentrations are proportional to egg

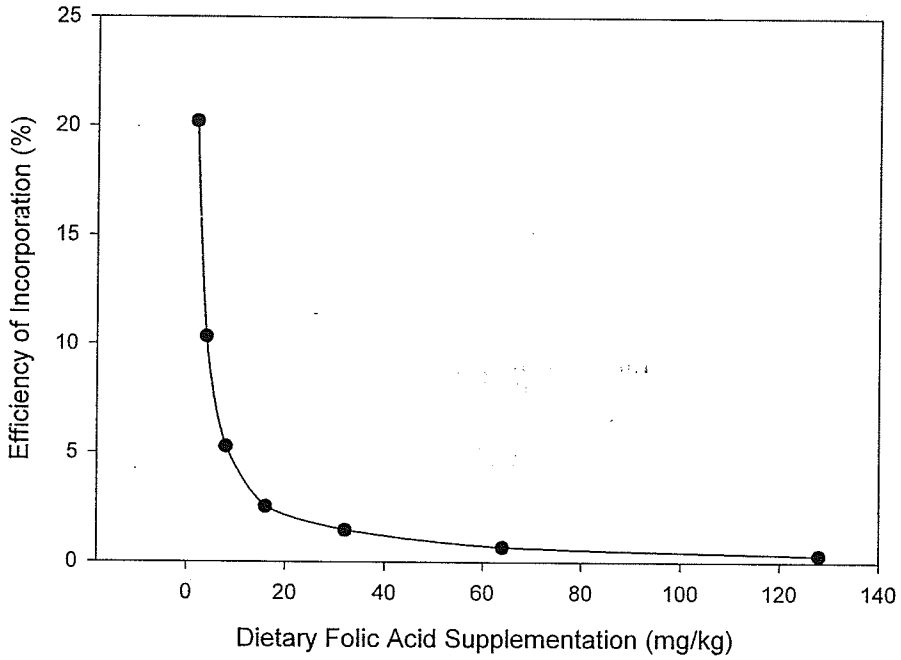
folate concentrations. Perhaps there is a maximal amount of folate the plasma can sustain or there are limitations on the supply of plasma folates.

Figure 4 illustrates the efficiency of folic acid incorporation from the diet into the egg. Efficiency of incorportation decreases dramatically with increasing dietary supplementation.

When hens were fed four times the dietary requirement for riboflavin, Naber and Squires (1993) reported no further increase in the riboflavin content of eggs. Riboflavin carrier protein (RCP) was first isolated form egg yolk in 1970 by Clagett. A particular strain of chickens were found to lack this protein (a disease called avian riboflavinuria), these eggs failed to hatch (Clagett, 1970). However, growth rate, onset of sexual maturity and egg production of the parental birds were unaffected, implying that the physiological function of RCP is exclusively to deposit riboflavin in the maturing egg. Injecting riboflavin into the eggs saved the affected progeny (Clagett, 1970). White et al., (1986) suggested that the limiting factor in riboflavin transfer to the egg involves riboflavin binding proteins in the blood and some unknown membrane receptor. White et al., (1992) studied hens that were unable to produce riboflavin binding protein. These hens exhibited normal riboflavin metabolism other than the fact they were unable to deposit riboflavin in their eggs.

The absence of a dramatic rise in plasma folate concentration in our study implies that folate transportation from the gut is mediated by a membrane-bound folate transport system (Anderson, et al., 1992; Henderson, 1990) rather than a plasma folate-binding transport protein. This is confirmed by Said et al., (2000), who

Figure 4. The efficiency of dietary folic acid incorporation into eggs of laying hens.



described a carrier-mediated folate uptake across the brush-border membrane derived from rat jejunum. This process was shown to be saturable. The folate deficient rats uptake of folic acid by jejunal brush-border membrane vesicles plateau was higher than the pair-fed control rats (Said et al., 2000). This could be possibly due to the increased availability of monoglutamate folate in the folate deficient rats due to increased folate hydrolase activity. Therefore, egg yolk folate concentrations may be regulated by intestinal folate uptake.

The monoglutamic folic acid in the intestinal cells is reduced to dihydrofolate and THF via dihydrofolate reductase (Henderson, 1990). THF is then transported form the intestinal cells via a membrance-bound transport system to the liver (Henderson, 1990). In the liver THF is converted to 5-MTHF, attached to folate-binding proteins and then released into plasma (Henderson, 1990). We believe that the folate is then transported via folate-binding transport proteins from the plasma to the egg. There may be a limit to the number of folate-binding proteins available to deposit folic acid in the yolk, resulting in the saturation of both plasma folate and egg folate concentrations.

Burns and Jackson (1982a) reported a reduction in the activity of dihydrofolate reductase in the folate deficient chick. Thus impaired folate status would reduce the amount of THF available for transport from the intestinal cells to the liver, ultimately impairing the production of 5-MTHF. Burns and Jackson (1982a) also reported that a folate deficiency in chickens resulted in an increased activity of serine hydroxymethyltransferase, 10-formyl-THF synthetase and 5,10-

methylene-THF dehydrogenase. Increased activity of these enzymes result in an increased formation of 5,10-methylene-THF and 10-formyl-THF, which are used directly in purine and pyrimidine synthesis via methionine synthetase (Bailey and Gregory, III, 1999). In plasma, 5-MTHF is the predominant form (Sauberlich et al., 1987), which cannot be directly utilized for purine biosynthesis. Since chickens synthesize uric acid (a purine compound) for nitrogen detoxification and elimination, during a folate deficiency the increased enzyme activity may allow a more rapid interconversion of any available folate and reduce the effects of folate depletion. This could help explain the plateau of available plasma 5-MTHF, if the hens are under the stress of folate deficiency. However, it is unlikely that the hens in our study are folate deficient since the basal diet supplied folic acid five times the NRC (1994) requirement for laying hens.

3.6.3 Effect of Dietary Folate on the Folate Content of Blood Plasma

Plasma folate concentrations became saturated with increasing dietary folate supplementation (**Table 6**). Plasma folate levels appear to saturate at 16 mg folic acid/ kg diet. This contradicts the finding by Sherwood et al., (1993), who reported that blood folate concentrations became saturated when hens were supplemented between 0 and 7 mg folic acid/ kg diet. The values that Sherwood et al., (1993) reported for plasma folate concentrations were higher than those in our study (at supplementation of 2 folic acid/ kg; 36 vs. 32 ng folate/ mL plasma, at supplementation of 4 mg folic acid/ kg; 47 vs. 37 ng folate/ mL plasma). This suggests there was significantly more available dietary folate in the research by Sherwood et al., (1993) compared to our study, and therefore the plateau of plasma

folate was realized sooner. This could be attributed to the difference in the base diets, Sherwood et al., (1993) fed a corn and soybean meal-based diet, whereas in our study a barley-based diet was fed. The viscosity attributed with barley-based diets (Brufau et al., 1994; Iji, 1999) may negatively impact the amount of available folate for uptake by intestinal cells.

Numerous studies have outlined the impact of cereal non-starch polysaccharides (NSP), associated with the endosperm cell wall of grain, on intestinal development and function (Iji, 1999; Brufau et al., 1994). β-glucans are the primary NSP in barley (Rotter et al., 1990; White et al., 1983). NSP cannot be hydrolysed by the enzymes that are produced by the bird. NSP have an antinutritional effect, preventing the access of endogenous enzymes to the nutrients contained within the grain. The uptake of folate from the gut may further be impaired due to the increased viscosity of barley-based rations (Brufau et al., 1994; Iji, 1999). The high viscosity of the digesta reduces the passage time and impairs diffusion of digestive enzymes to the substrates and the mixing of gut content (Antoniou et al., 1981; Antoniou and Marquardt, 1982). Thus viscosity interferes with the digestion and absorption of nutrients in the ration. The use of barley in laying hen diets has been shown to have a negative effect on productivity due to gut viscosity (Iji, 1999). Gut viscosity could impair the uptake of folate, thus decreasing potential plasma folate concentrations and egg folate content. Further studies are required to examine the effect of different dietary ingredients and enzyme supplementation on gut folate uptake.

3.6.4 Effect of Dietary Folate on the Homocysteine Content of Blood Plasma

5-MTHF is the primary methylating agent converting HCY to methionine via the remethylation pathway. Since there is limited storage of folate in the body, a constant dietary source is essential to maintain the remethylation process. A folate deficiency limits methionine regeneration, inhibiting the movement of HCY through this pathway. Remethylation inhibition and the inability of the transsulfuration pathway to compensate for the reduced remethylation activity results in an elevated level of HCY (Nygård et al., 1997).

Plasma HCY concentrations tended to decrease with increasing dietary folate supplementation. A significant ($P<0.05$) decrease in HCY concentration was only observed between the control diet and supplementation of 128 mg folic acid/ kg diet, which coincided with the highest plasma folate concentration. House et al., (2003) described a significant increase in plasma HCY in rats consuming a folate-devoid diet for five weeks, at which time there was no further increase in plasma HCY. McKay et al., (2000) also described an inverse relationship between B-vitamin supplementation and circulating HCY concentrations in human subjects. Other researchers (Selhub et al., 1993; Kang et al., 1987; Refsum et al., 1998; Nygård et al., 1997 and Taylor et al., 2000) have reported elevations in total blood HCY concentrations in human patients with low circulating folate concentration.

A significant ($P<0.05$) ration x strain interaction was evident for HCY, as Hyline W98 hens exhibited a decrease in HCY plasma levels from 0 to 2 mg folic acid/ kg diet (**Figure 3**). Hyline W98 may have a higher folic acid requirement, since HCY was decreased by increased supplementation. Hyline W98 hens are a heavier, early

maturing strain, that produce larger eggs in comparison to the Hyline W36 hens. Hyline W98 hens tend to have a slightly reduced livability to 80 weeks compared to the Hyline W36 (93 vs. 96%) (Hyline Inc., 2003 and 2004). In the short term, production was not affected by increased folate supplementation; however long term, a folate deficiency may impact deoxyribonucleic acid (DNA) synthesis and reduce responsiveness to an immune challenge (Steinberg, 1984).

Nygård et al., (1999) reported that plasma total HCY levels are a strong predictor of mortality in human subjects. There was a strong graded dose-response relationship between the total HCY level and overall mortality. As patients aged their total HCY level also increased (Nygård et al., 1999). A similar trend may be observed in laying hens.

Since the current study was short term, it is necessary to consider the effects of folic acid supplementation during the entire production cycle, as the hens age and rate of production changes. Hyline W36 hens do not appear to respond to increased folic acid supplementation, since no significant difference in plasma HCY levels was observed.

3.6.5 Effect of Dietary Folate on Egg Production and Egg Size

In the short term, increased folate supplementation did not significantly affect egg production, FC, FE, egg weight, yolk weight or percent yolk of egg weight. The reported folic acid requirement of laying hens vary greatly, ranging from less than 0.27 mg/ kg to more than 2.0 mg/ kg (Maxwell et al., 1988; Wong et al., 1977). The current NRC (1994) folic acid requirement for laying hens is 0.25 mg/ kg, based on measures of productivity and egg quality. The analyzed level of folic acid in the

unsupplemented diet (1.45 mg/ kg) is well in excess of the NRC requirement, thus we did not expect to see a positive response on production due to increased folate supplementation.

Sherwood et al., (1993) reported that hens consuming a folate deficient diet laid eggs that were 7% smaller than the controls. This supports our study verifying that our hens were receiving adequate folic acid. However, our data suggest that Hyline W98 hens have a greater requirement for folate to maximize egg production (**Table 7**). Contrary to our data, House et al., (2002) reported FC significantly increased at a supplementation level of 32 mg folic acid/ kg and may be attributed to the short collection period (5 day) of that trial.

In summary, this study supports the potential for fortifying eggs with folic acid by supplementation of crystalline folic acid to barley-based diets at levels in excess of 2 mg/ kg diet. Overall, performance was not affected by folate supplementation. The Hyline W98 compared to the Hyline W36 have higher FC, egg and yolk weights and lower percent yolk of egg weight. As expected plasma folate concentrations increased and HCY concentrations decreased with supplementation. The Hyline W98 benefit from increased folate supplementation by decreasing their plasma HCY content, suggesting the folic acid requirement of the Hyline W98 is higher than the Hyline W36. 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.

Chapter 4

MANUSCRIPT 2

Effect of Dietary Folate Supplementation on the Performance and Efficiency of Egg Folate Deposition Throughout the Production Cycle of Hyline W98 and W36 Laying Hens.

To be Submitted to

Poultry Science for Publication

4.1 Abstract

With previous research to document that supplementing diets with 4 mg crystalline folic acid /kg increased egg folate content three-fold that of a regular egg, a study was designed to determine the age and rate of production effects of laying hens on egg folate level, and the potential for differences due to strain of birds. Hyline W36 and W98 hens (n=156/ treatment) received a barley-based ration, containing 0 or 4 mg/kg of crystalline folic acid for eleven 4-week periods. Response criteria included production parameters, egg quality measurements and measures of egg folate content. There was no significant difference ($P<0.05$) in performance parameters or egg quality measurements due to folate supplementation. However, the hens consuming folate enriched diets had significantly ($P<0.05$) improved livability. Hyline W98 had significantly ($P<0.05$) higher feed consumption (FC), egg weight, yolk weight, body weight and lower percent yolk of egg weight than the W36 strain. Throughout the production cycle hen-day production (EP) and feed efficiency (FE) increased to peak and then decreased. FC, egg weight, yolk weight and percent yolk of egg weight significantly ($P<0.05$) increased, and specific gravity significantly ($P<0.05$) decreased throughout the production cycle. Egg folate content significantly ($P<0.0001$) increased from period 1 to 2. A significant ($P<0.05$) ration x strain interaction was evident for EP and FC, as Hyline W98 hens exhibited higher EP and FC than W36 with supplementation level of 4 mg/kg. A significant ($P<0.05$) strain x period interaction was evident for EP, FC, FE, egg weight, yolk weight and egg folate content. Hyline W98 had a significant ($P<0.05$) increase in egg folate content in period 8. A significant ($P<0.05$) ration x period interaction was evident for FC, FE

egg folate content and total uncollectable eggs. Overall, performance was not affected by long-term folate supplementation. This study supports the potential for consistently fortifying eggs with folic acid throughout the production cycle of laying hens.

Funding: Manitoba Egg Producers, CEMA, Clark Hy-line Inc.

Keywords: Folate, Egg, Production

4.2 Introduction

Most vitamin enriched eggs are enriched with vitamin E, due to its antioxidant property for protecting polyunsaturated fats, such as omega-3s, although it is possible to enrich eggs with other vitamins. Naber (1993) reviewed the potential efficiency of vitamin transfer from feed to eggs. When hens were supplemented at one or two times the National Research Council (1994) requirement, the vitamin transfer efficiencies ranged from 5 to 80%. The vitamins were grouped into four categories based on transfer efficiencies; low (vitamin K_1, thiamine, folacin), medium (vitamin D_3 and vitamin E), high (riboflavin, pantothenic acid, biotin, vitamin B_{12}) and very high (vitamin A).

Based on the findings in Manuscript One and by House et al., (2002), supplementing laying hen diets with 4 mg crystalline folic acid /kg can increase egg folate up to three-fold that of a regular egg. This level of supplementation exceeds the National Research Council (1994) requirement of 0.25 mg folic acid / kg feed for laying hens by eight fold.

Hens, like other animals, experience changes due to aging. Liljedahl et al., (1984) observed a decline in egg quality and most performance traits of layers, with the exception of egg weight which increased with age. Naber and Squires (1993) noted a decrease in riboflavin and vitamin A deposited in the eggs of laying hens over 42 weeks of production. Robel (1983) noted a decrease in the efficiency of pyridoxine, vitamin B_{12}, calcium and folic acid transfer to turkey eggs over a 70 week trial. In order to produce a consistent folate enriched egg, production and age effects must be evaluated.

There may also be other benefits of prolonged folic acid supplementation for the hens. There has been a great deal of research into the understanding of vitamin nutrition, homocysteine and vascular disease in humans. Vitamin intervention with folic acid will lower plasma homocysteine (McKay et al., 2000), thus lowering the incidence of stroke, coronary heart disease and peripheral vascular disease (Bellamy et al., 1999). Swain et al., (1997) proposed that folate fortification of food would reduce the annual US mortality from coronary heart disease by 50,000 individuals.

With previous short term research to document the potential for fortifying eggs with folate, a study was designed to determine differences due to strain of birds, effect of age and rate of production on egg folate content and other benefits of supplemented folic acid.

4.3 Hypotheses and Objectives

4.3.1 Hypotheses

Alternative Hypothesis:

i) Egg folate level will be affected by laying hen age, rate of production, and laying hen strain.

ii) Egg folate level will affect egg quality.

Null Hypothesis:

i) Egg folate level will not be affected by laying hen age, rate of production and laying hen strain.

ii) Egg folate level will not affect egg quality.

4.3.2 Objectives

The purpose of this research is the study the elements of a production cycle of hens producing a folate-fortified egg. This will be accomplished through the following objectives:

i) To determine the production performance of Hyline W98 and W36 hens fed two different levels of folic acid.

ii) To determine the age and rate of production effects of laying hens on egg folate content and the potential for differences due to strain of bird.

iii) To determine the relationship between egg folate level and egg quality.

4.4 Materials and Methods

4.4.1 General

Single-Comb White Leghorn hens (Manitoba Perfect Pullets, MB), Hyline W98, an early maturing, large egg mass strain, and Hyline W36, a traditional strain (efficient egg layer with excellent livability), were used throughout this experiment. All hens were kept in confinement housing under semi-controlled environmental conditions and exposed to 16-hr photoperiod. Six hundred and twenty-four birds were housed in groups of three; the cage dimensions were 40.64 cm x 40.64 cm, providing 413 cm^2 (64 in^2) per bird. Feed and water were available *ad libitum* for all hens. 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).

4.4.2 Diet and Experimental Protocol

The basal diet was a barley-based ration (please refer to Section 3.4.2). The diet was formulated to meet the requirements of laying hens consuming 100g of feed /d (NRC, 1994). In accordance with industry standards (BASF, 2000), the basal diet included no crystalline folic acid.

In order to study the elements of a production cycle of hens producing a folate fortified egg, 312 hens from each strain (n=156 per treatment) were housed three per cage and four cages per replicate (12 birds/ replication; 13 replications per strain). Each replicate was randomly assigned to receive one of two dietary folic acid levels: the basal diet (no added folic acid / kg) or the basal diet plus 4 mg crystalline folic acid / kg (Sigma Chemical Co., Oakville, ON, Canada). House et al., 2002 reported

this level of folic acid to yield 90% maximal egg folate concentrations in a short term study. The treatments were introduced 14 days before the egg collection period began. The experiment was subdivided into eleven 4-week periods.

Information on body weight, FC, EP, egg weight, and egg quality (external and internal) were recorded. Individual body weight of all hens was taken at the initiation and termination of the experiment to determine the average body weight gain. Mortality was also recorded daily as it occurred. Residual feed was weighed back at the end of each period to determine FC for each replicate and average daily feed intake and FE was then calculated. EP was recorded daily for each replicate and an average egg production rate (hen-day percent) was calculated for each production period.

Fifty-two eggs (1 egg/ replication; 13 replications per treatment per strain) were randomly collected per treatment per day during three consecutive days during the third week of each period. The eggs were weighed to give an average egg weight for the period and processed for egg folate determination. Due to mechanical difficulties with the HPLC, egg folate data for periods 10 and 11 were not determined.

Measurements of egg quality, including egg weight, yolk weight, Albumen Height (AH), and specific gravities were determined at mid production and three months later. All eggs for three consecutive days from each replicate were weighed and specific gravity was determined (Holder and Bradford, 1979). From the eggs collected for specific gravity determination one hundred and four eggs (2 eggs/

replication; 13 replications total) were randomly selected for egg weight, yolk weight, AH and Haugh Unit (HU) measurement.

4.4.3 Extraction of Egg Yolk Folate

Please refer to Section 3.4.3.

4.4.4 Analysis of Egg Yolk Folate Content

Please refer to Section 3.4.4.

4.4.5 Measurements of Egg Quality

Specific gravity was determined by the saline flotation method as described by Holder and Bradford (1979). A series of five solutions were prepared, with saline concentrations ranging from 1.070 to 1.085 in increments of 0.005. The density of each solution was confirmed with a hydrometer prior to use and specific gravity was determined sequentially placing eggs in solutions of increasing salinity. Both eggs and solutions were at 15°C. Eggs that did not float in the 1.085 specific gravity solutions were assigned a specific gravity of 1.090.

AH was measured in millimetres using an electronic albumen height gauge (Queensboro Instruments, Ottawa, ON, Canada) in the middle of the outer, thick layer of albumen equidistant from the outer edge of the albumen and the yolk. Any eggs whose vitalline membranes ruptured after the egg was opened were deleted from samples. HU score (AH corrected for egg weight (EW)) was then calculated by the formula $HU = \log (AH + 7.57 - 1.7\ EW^{0.37})$.

4.4.6 Calculations

Please refer to Section 3.4.6.

Cumulative Mortality (% of total hens housed) =

$$\frac{\text{(Number of Hens Dead Since Beginning of the Trial)}}{\text{(Number of Hens Housed at the Beginning of the Trial)}} \times 100$$

Total Uncollectable Eggs (% of total eggs laid) =

$$\frac{\text{(Total Number of Uncollectable Eggs for the Period)}}{\text{(Total Number of Egg Laid in the Period)}} \times 100$$

4.4.7 Statistical Analysis

A 2 x 2 (ration x strain as main effects) factorial arrangement with split-plot in time was used to analyze the data for the eleven experimental periods of the production trial. Performance data collected were subjected to analysis of variance using SAS Analyst (SAS Institute Inc., 1998). Differences between means were determined using Tukey's Honestly Significance Difference. Differences with an α level of $P<0.05$ were considered to be statistically significant. A regression analysis was run on the data for mortality and percent total uncollectable eggs. It was necessary to arc sin transform the data for mortality and percent total uncollectable eggs, since a plot of residuals versus predicted values revealed heterogeneity in the variability. To verify the significance of the arc sin transformation of mortality, a Chi Square Test was performed.

4.5 Results

The main effects of ration, strain and period and their interactions for different performance traits are shown in **Table 8**. Significant ($P<0.05$) ration differences existed for egg folate content, cumulative mortality, and total uncollectible eggs. However, significant ($P<0.05$) strain differences are shown for FC, egg weight, yolk weight, percent yolk of egg weight and body weight. Significant ($P<0.05$) period differences were found for all measured parameters. Ration by strain interactions ($P<0.05$) were observed for EP and FC, whereas period by strain interactions ($P<0.05$) were found in EP, FC, FE, egg weight, yolk weight, egg folate content, total uncollectable eggs and HU. Significant ($P<0.05$) ration by period interactions were detected for FC, FE, egg folate level, and total uncollectable eggs. There was also a significant ($P<0.05$) strain by ration by period interaction for total egg folate content.

The performance data of laying hens are shown **Table 9**. Compared to the Hyline W36 strain, the Hyline W98 had significantly ($P<0.0001$) higher FC (101.6 vs. 95.3 g/h/d), significantly ($P<0.0001$) larger egg and yolk weights (63.5 vs. 58.9 g and 15.1 vs. 14.8 g, respectively), significantly ($P<0.05$) larger body weight (1.71 vs. 1.53 kg) and total uncollectable eggs (0.24 vs. 0.09%), and significantly ($P<0.05$) lower percent yolk of egg weight (23.69 vs. 25.07%).

The performance data of hens consuming folate enriched diets irrespective of strain is shown in **Table 10.** Egg folate content significantly ($P<0.0001$) increased from 17.4 μg/egg with an unsupplemented diet to 45.0 μg/egg when supplemented with 4 mg folic acid/ kg diet. A significant ($P<0.05$) lower cumulative mortality was

Table 8. P-values of main effects and interactions of ration, strain and period on different performance traits.

Parameters	Ration	Strain	Period	Str*Rat	Str*Per	Rat*Per	Str*Rat*Per
Hen-day Production (%)	0.2079	0.2515	<0.0001	0.0085	<0.0001	0.1413	0.4917
Feed Consumption (g/h/d)	0.2682	<0.0001	<0.0001	0.0306	<0.0001	<0.0001	0.1219
Feed Efficiency (g feed/g egg	0.8874	0.0969	<0.0001	0.3339	<0.0001	<0.0001	0.1318
Egg Weight (g)	0.6942	<0.0001	<0.0001	0.5506	0.0006	0.5102	0.4462
Yolk Weight (g)	0.9382	0.0007	<0.0001	0.8880	0.0036	0.6493	0.8563
% Yolk of Egg Weight	0.6866	<0.0001	<0.0001	0.6001	0.3526	0.8799	0.5390
Egg Folate Content (µg/egg)	<0.0001	0.5338	<0.0001	0.0824	<0.0001	<0.0001	0.0025
Body Weight (kg)	0.5903	<0.0001	<0.0001	0.1705	0.1214	0.3195	0.8459
Cummulative Mortality (% of total hen housed)	0.0013	0.4019	<0.0001	0.2761	0.9423	0.9937	0.9493
Total Uncollectable Eggs[1] (% of total eggs laid)	<0.0001	<0.0001	<0.0001	0.5115	0.0066	0.0372	0.2014
Specific Gravity (>1.085)[2]	0.1615	0.5178	<0.0001	0.8734	0.2289	0.9161	0.1743
Haugh Units[2]	0.0790	0.3544	0.0142	0.3238	0.0206	0.3455	0.8795

[1]Includes cracked and shell-less eggs

[2]Compares egg quality measurements at 6 months and 9 months of production

Table 9. Performance summary of Hyline W98 and W36 laying hens- Main effects of strain[1].

	Strain			
Parameters	W36	W98	SEM	p-value
Feed Consumption (g/h/d)	95.3	101.6	0.18	<0.0001
Egg Weight (g)	58.9	63.5	0.16	<0.0001
Yolk Weight (g)	14.8	15.1	0.05	0.0007
% Yolk of Egg Weight	25.07	23.69	0.07	<0.0001
Initial Body Weight (kg)	1.43	1.59	0.01	<0.0001
Final Body Weight (kg)	1.63	1.82	0.01	<0.0001
Total Uncollectable Eggs[2] (% of total eggs laid)	0.09	0.24	0.002	<0.0001

[1]Data are presented as least square means
[2]Includes cracked and shell-less eggs

Table 10. Egg folate content and cumulative mortality of laying hens receiving diets with or without crystalline folic acid supplementation- Main effects of ration[1].

	Parameters	
Dietary Folic Acid Supplementation (mg/kg)	Egg Folate Content (µg/egg)	Cummulative Mortality (% of total hen housed)
0	17.4	2.56
4	45.0	2.24
SEM	0.71	0.01
p-value	<0.0001	0.0013

[1]Data are presented as least square means

observed between the control diet and the diet supplemented with 4 mg folic acid/ kg diet (2.56 vs. 2.24 %). However, the significance of this finding was not supported with the Chi Square Test due to the very small SEM.

The performance data of hens throughout the production cycle is shown in **Table 11**. Throughout the production cycle EP increased to peak then decreased. A significant ($P<0.0001$) increase in EP was observed between periods one and two. Egg production persisted over 91% for periods two, three and four, decreasing in period 5 to 89% and gradually over time declining to 73% in period eleven.

There was a significant ($P<0.0001$) effect of FC throughout the production cycle. FC increased significantly from period one (84.8 g/h/d) progressing to period three (100.6 g/h/d). There was a significant decrease (95.3 g/h/d) in FC in period five, followed by an increase in period 6 (100.6 g/h/d), after which FC remained constant until period ten, when there was a significant increase (107.0 g/h/d), followed by a significant decrease (101.1 g/h/d) in period eleven.

There was also a significant ($P<0.0001$) period effect for FE throughout the production cycle. FE significantly decreased in period two, improved in period three and continued improving in periods five, six, and ten.

A significant ($P<0.0001$) increase in egg weight throughout the production cycle was observed. Egg weight rapidly increased from periods one to two to three (50.1, 54.7, and 59.4 g respectively). There was a further increase in egg weight in period eight (64.8 g) and ten (66.8 g). Yolk weight changes ($P<0.0001$) basically

Table 11. Performance summary and egg quality measurements of laying hens throughout the production cycle- Main effects of period[1].

	Parameters										
Period	Hen-day Production (%)	Feed Consumption (g/h/d)	Feed Efficiency (g feed/ g egg)	Egg Weight (g)	Yolk Weight (g)	% Yolk of Egg Weight	Egg Folate Content (μg/egg)	Cummulative Mortality (% of total hen housed)	Total Uncollectable Eggs[2] (% of total eggs laid)	Specific Gravity (>1.085)[3]	Haugh Units[3]
1	67.12^{g}	84.8^{g}	1.70^{b}	50.1^{h}	10.5^{h}	20.87^{f}	22.9^{d}	0^{c}	0.41^{a}		
2	$91.44^{a,b}$	$96.9^{e,f}$	1.77^{a}	54.7^{g}	12.3^{g}	22.50^{e}	$30.4^{b,c}$	0.16^{c}	$0.19^{a,b,c}$		
3	92.96^{a}	$100.6^{b,c}$	1.70^{b}	59.4^{f}	14.0^{f}	23.60^{d}	$29.9^{b,c,d}$	0.32^{c}	$0.28^{a,b}$		
4	92.18^{a}	$99.3^{b,c,d}$	$1.64^{b,c}$	$60.6^{e,f}$	14.9^{e}	$24.65^{b,c}$	$31.2^{b,c}$	0.32^{c}	$0.06^{c,d}$		
5	$89.19^{b,c}$	95.3^{f}	$1.56^{e,f}$	$61.1^{e,f}$	14.8^{e}	$24.20^{c,d}$	29.3^{c}	0.32^{c}	$0.12^{b,c,d}$	1.082^{a}	85.04^{a}
6	$86.56^{c,d}$	$100.6^{b,c}$	$1.63^{c,d}$	$61.8^{d,e}$	15.6^{d}	$25.22^{a,b}$	$31.6^{b,c}$	0.32^{c}	$0.12^{b,d}$		
7	$84.51^{d,e}$	$98.9^{c,d,e}$	$1.58^{d,e}$	$62.9^{c,d}$	$16.1^{c,d}$	25.57^{a}	$30^{b,c,d}$	$0.48^{b,c}$	0.02^{d}		
8	81.01^{f}	$98.4^{d,e}$	$1.52^{e,f,g}$	64.8^{b}	$16.4^{b,c}$	$25.32^{a,b}$	39.2^{a}	$0.96^{a,b,c}$	0.06^{d}	1.078^{b}	83.68^{b}
9	$82.25^{e,f}$	$100.1^{b,c,d}$	$1.56^{e,f}$	$64.2^{b,c}$	16.2^{c}	$25.28^{a,b}$	$36.3^{a,b}$	$1.44^{a,b,c}$	$0.13^{b,c,d}$		
10	$81.85^{e,f}$	107.0^{a}	1.50^{g}	66.8^{a}	$16.9^{a,b}$	$25.40^{a,b}$	NA	$2.08^{a,b}$	$0.33^{a,b}$		
11	79.23^{f}	101.1^{b}	$1.52^{f,g}$	66.7^{a}	17.1^{a}	25.61^{a}	NA	2.40^{a}	$0.33^{a,b}$		
SEM	0.6	0.43	0.01	0.38	0.12	0.16	1.51	0.01	0.005	0.01	0.01
p-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0142

[1]Data are presented as lest square means

[2]Includes cracked and shell-less eggs

[3]Period compares egg quality measurements at 6 months and 9 months of production

NA= data not available

Means within a column with different letter superscript are significantly different (P<.05)

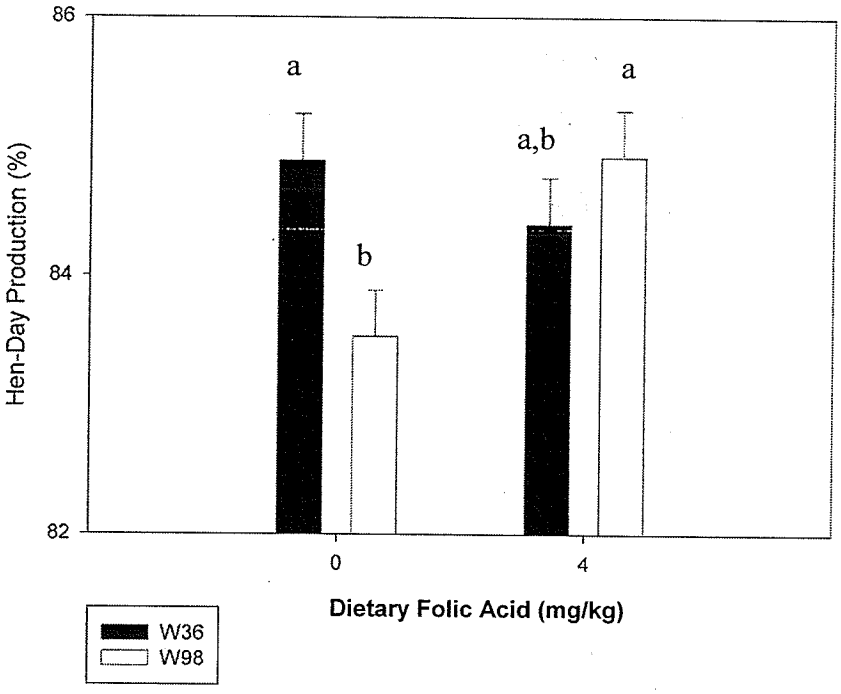
followed the same pattern as egg weight increasing from 10.5 g to a maximum of 17.1 g in period 11. The percent yolk of egg weight significantly ($P<0.0001$) increased each period one to six (20.87, 22.50, 23.60, 24.50, and 25.22 %; respectively). As expected egg and yolk weight increase with maturity of the hen. Throughout the production cycle, the percent of yolk of egg weight increases to a maximum of ~25% at which it plateaus.

Total egg folate content significantly increased in period two, from 22.9 µg/egg to 30.4 µg/egg, and again in period eight, to 39.2 µg/egg. Cumulative mortality (15 birds) was very low over the first seven periods (0.48%) and increased in period eleven to 2.40%.

Egg quality was significantly affected throughout the production cycle. Total uncollectable eggs was significantly ($P<0.0001$) higher at the beginning (periods one, two and three) and end (periods ten and eleven) of the production cycle than the middle. As hens age specific gravities and HU score of their eggs decrease. Specific gravity significantly ($P<0.0001$) decreased from 1.082 in the middle (period six) of the production cycle to 1.078 at the end (period nine) of the production cycle. A similar trend was observed for HU (85.04 vs. 83.68).

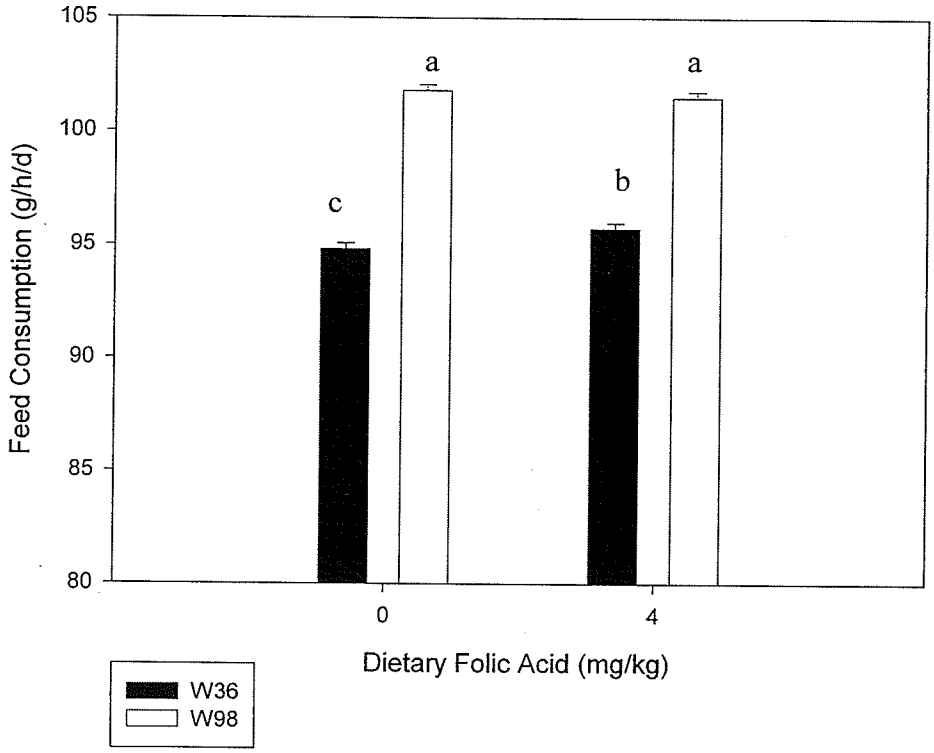
Ration by strain interactions were significant ($P<0.05$) for EP (**Figure 5)** and FC (**Figure 6**). Hyline W36 had significantly higher egg production than the Hyline W98 without folic acid supplementation (84.89 vs. 83.53 %). However, when supplemented with 4 mg folic acid/ kg diet, egg production for the Hyline W98 and W36 were not significantly different. Increasing dietary folic acid supplementation

Figure 5. Significant strain*ration interaction for hen-day production of Hyline W98 and W36 laying hens.



Data reported as LS Means +/- Standard Error
Treatment Effect P<.0001
a,b means not followed by the same superscript are significantly different by P<0.05
All means are composed of 13 replicates

Figure 6. Significant strain* ration interaction for feed consumption of Hyline W98 and W36 laying hens.



Data reported as LS Means +/- Standard Error
Treatment Effect P<0.0085
a,b means not followed by the same superscript are significantly different by P<0.05
All means are composed of 13 replicates

did not affect egg production for the Hyline W36 hens, contrary to the W98 hens (83.53 vs. 84.93%).

Hyline W36 had significantly lower FC than the Hyline W98 without folic acid supplementation (94.8 vs. 101.8 g/h/d). Increasing dietary folic acid supplementation did not significantly affect FC for the Hyline W98 hens, however, the Hyline W36 significantly increased consumption from 94.8 to 95.7 g/h/d. When supplemented with dietary folic acid at 4 mg/ kg, the Hyline W98 still had significantly higher FC than the Hyline W36 (101.5 vs. 95.7 g/h/d).

The performance summary and egg folate content of hens with significant strain by period interaction is shown in **Table 12**. In period one the Hyline W98 hens had significantly higher EP than the Hyline W36, 75.01 vs. 59.24 %. However, the Hyline W36 peak more rapidly and achieved a higher peak than the Hyline W98 hens. Both strains persisted well throughout the production cycle.

The Hyline W98 also had significantly ($P<0.0001$) higher FC throughout the entire production cycle compared to the Hyline W36. For both strains FC increases with hen maturity. In period ten, there was a significant increase in FC for both strains. The Hyline W36 had significantly ($P<0.0001$) better FE than the Hyline W98 in period one, 1.61 vs. 1.78 g feed/ g egg, however the Hyline W98 had significantly ($P<0.0001$) better FE than the Hyline W36 in period four, 1.59 vs. 1.70 g feed/ g egg). Generally, FE increases with the maturity of the hen, since the hen is eating only for maintenance and production and not growth.

Table 12. Performance summary and egg folate content of Hyline W98 and W36 laying hens throughout the production cycle- Variable with significant (P<0.05) strain*period interactions[1]

	Hen-day Production (%)		Feed Consumption (g/h/d)		Feed Efficiency (g feed/g egg)		Egg Weight (g)	
	Strain		Strain		Strain		Strain	
Period	W36	W98	W36	W98	W36	W98	W36	W98
1	59.24^{l}	75.01^{k}	76.6^{k}	$93.1^{i,j}$	$1.61^{d,e,f,g,h}$	$1.78^{a,b}$	47.7^{l}	52.4^{k}
2	$94.10^{a,b}$	$89.79^{b,c,d}$	92.8^{j}	$101.1^{c,d,e,f}$	$1.75^{a,b,c}$	1.80^{a}	53.1^{k}	56.4^{j}
3	95.00^{a}	$90.92^{a,b,c}$	$97.2^{g,h}$	$104.0^{b,c}$	$1.73^{a,b,c}$	$1.66^{c,d,e}$	56.1^{j}	$62.7^{e,f,g}$
4	$93.45^{a,b}$	$90.92^{a,b,c}$	$96.3^{g,h,i.}$	$102.2^{b,c,d,e}$	$1.70^{b,c,d}$	$1.59^{e,f,g,h,i.}$	$56.9^{i,j}$	$64.3^{c,d,e}$
5	$91.07^{a,b,c}$	$87.32^{c,d,e}$	$93.3^{i,,j}$	$97.3^{g,h}$	$1.59^{e,f,g,h,i,j}$	$1.54^{g,h,i,j,k}$	$58.8^{h,i,,j}$	$63.3^{e,f}$
6	$87.47^{c,d,e}$	$85.65^{d,e,f,g}$	$98.2^{f,g,h}$	$103.1^{b,c,d}$	$1.64^{d,e,f}$	$1.62^{d,e,f,g}$	$60.0^{g,h}$	$63.7^{d,e,f}$
7	$85.15^{e,f,g,h}$	$83.87^{f,g,h,i.}$	$97.0^{g,h}$	$100.8^{d,e,f}$	$1.59^{e,f,g,h,i,,j}$	$1.57^{f,g,h,i,j}$	$61.3^{f,g,h}$	$64.5^{c,d,e}$
8	$81.20^{g,h,i,j}$	$80.81^{h,i,j}$	$95.3^{h,i,,j}$	$101.5^{c,d,e,f}$	$1.52^{i,,j,k}$	$1.52^{h,i,j,k}$	$62.9^{e,f}$	$66.7^{a,c}$
9	$82.28^{g,h,i,j}$	$82.22^{g,h,i,j}$	$97.0^{g,h}$	$103.2^{b,c,d}$	$1.57^{f,g,h,i,j}$	$1.56^{f,g,h,i,j,k}$	$62.1^{e,f,g}$	$66.3^{b,c,d}$
10	$82.39^{f,g,h,i,j}$	$81.31^{g,h,i,j}$	105.1^{b}	108.9^{a}	$1.53^{h,i,,j,k}$	1.47^{k}	$64.3^{c,d,e}$	69.3^{a}
11	$79.74^{i,j}$	$78.72^{j,k}$	$99.4^{e,f,g}$	$102.8^{b,c,d}$	$1.54^{g,h,i,j,k}$	$1.50^{j,k}$	$64.8^{c,d,e}$	$68.7^{a,b}$
SEM	0.85		0.61		0.02		0.53	
p-value	<0.0001		<0.0001		<0.0001		0.0006	

	Yolk Weight (g)		Egg Folate Content (μg/egg)		Total Uncollectable Eggs[2] (% of total eggs laid)		Haugh Units	
	Strain		Strain		Strain		Strain	
Period	W36	W98	W36	W98	W36	W98	W36	W98
1	10.2^{k}	10.8^{k}	20.3^{e}	$25.4^{d,e}$	$0.31^{a,b,c}$	$0.49^{a,b}$		
2	12.2^{j}	12.4^{j}	$31.1^{a,b,c,d,e}$	$29.6^{b,c,d,e}$	$0.13^{c,d,e}$	$0.25^{a,b,c,d}$		
3	$13.6^{i,}$	$14.5^{h,i.}$	$34.1^{a,b,c,d}$	$25.7^{c,d,e}$	$0.18^{a,b,c,d,e}$	$0.39^{a,b,c}$		
4	$14.4^{h,i.}$	$15.4^{e,f,g}$	$27.0^{c,d,e}$	$35.5^{a,b,c,d}$	0.04^{e}	$0.09^{c,d,e}$		
5	$14.6^{g,h}$	$14.9^{f,g,h}$	$29.5^{b,c,d,e}$	$29.1^{b,c,d,e}$	$0.08^{c,d,e}$	$0.17^{a,b,c,d,e}$	85.434^{a}	$84.655^{a,b}$
6	$15.5^{d,e,f}$	$15.6^{e,f}$	$31.5^{a,b,c,d}$	$31.6^{a,b,c,d}$	$0.08^{c,d,e}$	$0.16^{c,d,e}$		
7	$16.1^{b,c,d,e}$	$16.0^{c,d,e}$	$34.0^{a,b,c,d}$	$26.0^{c,d,e}$	0.00^{e}	$0.04^{d,e}$		
8	$16.5^{a,b,c,d}$	$16.2^{a,b,c,d,e}$	$36.7^{a,b,c}$	41.7^{a}	0.00^{e}	$0.11^{c,d,e}$	82.777^{b}	$84.576^{a,b}$
9	$16.2^{a,b,c,d,e}$	$16.2^{b,c,d,e}$	$33.7^{a,b,c,d}$	$39.0^{a,b}$	$0.14^{b,c,d,e}$	$0.11^{c,d,e}$		
10	$16.9^{a,b,c}$	$17.0^{a,b}$	NA	NA	$0.09^{c,d,e}$	$.057^{a}$		
11	$17.0^{a,b}$	17.1^{a}	NA	NA	$0.12^{b,c}$	0.54^{a}		
SEM	0.18		2.02		0.01		0.01	
p-value	0.0036		0.0007		0.0066		0.0206	

[1]Data is presented as least square means [2]Includes cracked and shell-less eggs NA= data not available
Means within each parameter with a different letter superscript are significantly different (P<.05)

The Hyline W98 had significantly ($P<0.05$) higher egg weight throughout the entire production cycle compared to the Hyline W36. However, yolk weight except for period four ($P<0.05$) was similar for both strains. In general, egg and yolk weight increased with the aging of the hen.

The total egg folate content of the Hyline W36 remains constant throughout their production cycle. However, there is a significant ($P<0.05$) increase in egg folate content for the Hyline W98 in period eight, increasing from 26.0 to 41.7 μg/egg. There is no logical reason for the increase for egg folate content in period eight.

Both strains appear to have an increased percentage of total uncollectable eggs at the beginning and end of their production cycle. The Hyline W98 had a significant ($P<0.05$) increase in total uncollectable eggs from period nine to ten, 0.11 to 0.57%.

There was no significant difference in egg quality, as measured by HU, between the Hyline W36 and W98 at mid and late production, although, the Hyline W36 had a significant ($P<0.05$) decrease in HU measurement from mid to late production, 85.4 to 82.8.

The performance summary of hens with significant ration by period interaction is shown in **Table 13**. There was a significantly ($P<0.0001$) lower FC when hens were supplemented with 4 mg folic acid/ kg diet in period one, 88.6 vs. 81.0 g/h/d. Throughout the remainder of the production cycle there was no significant difference in FC between the two diets. Noteworthy is the significant decrease in FC for both rations in period five, and a significant increase in FC in period ten. FE was significantly ($P<0.0001$) increased when hens were supplemented with 4 mg folic acid/ kg diet only in period one, (1.63 vs. 1.77 g feed/ g egg).

Table 13. Performance summary and egg folate content of laying hens throughout the production cycle- Variable with significant (P<0.05) ration*period interactions[1]

	Feed Consumption (g/h/d)		Feed Efficiency (g feed/g egg)		Egg Folate Content (μg/egg)		Total Uncollectable Eggs[2] (% of total eggs laid)	
	Dietary Folic Acid Supplementation (mg/kg)		Dietary Folic Acid Supplementation (mg/kg)		Dietary Folic Acid Supplementation (mg/kg)		Dietary Folic Acid Supplementation (mg/kg)	
Period	0	4	0	4	0	4	0	4
1	88.6^{g}	81.0^{h}	1.77^{a}	$1.63^{b,c,d}$	15.9^{e}	29.8^{c}	$0.35^{a,b,c}$	0.47^{a}
2	$95.5^{e,f}$	$98.4^{b,c,d,e}$	1.77^{a}	1.78^{a}	15.8^{e}	$44.9^{a,b}$	$0.21^{a,b,c,d,e}$	$0.17^{a,b,c,d,e}$
3	$100.4^{b,c}$	$100.8^{b,c}$	$1.70^{a,b}$	$1.70^{a,b}$	13.7^{e}	$46.2^{a,b}$	$0.24^{a,b,c,d,e}$	$0.33^{a,b,c,d}$
4	$98.6^{b,c,d,e}$	$99.9^{b,c,d}$	$1.62^{c,d}$	$1.66^{b,c}$	$18.0^{d,e}$	$44.5^{a,b}$	$0.03^{d,e}$	$0.10^{c,d,e}$
5	94.9^{f}	$95.7^{e,f}$	$1.56^{d,e,f,g}$	$1.57^{d,e,f,g}$	$16.6^{d,e}$	42.0^{b}	$0.13^{a,b,c,d,e}$	$0.11^{c,d,e}$
6	$100.7^{b,c}$	$100.6^{b,c}$	$1.64^{b,c,d}$	$1.62^{b,c,d,e,f}$	$18.7^{d,e}$	$44.4^{a,b}$	$0.05^{c,d,e}$	$0.18^{a,b,c,d,e}$
7	$98.3^{c,d,e}$	$99.6^{b,c,d}$	$1.55^{d,e,f,g}$	$1.60^{c,d,e,f}$	11.8^{e}	$48.2^{a,b}$	$0.03^{d,e}$	0.01^{e}
8	$97.0^{d,e,f}$	$99.7^{b,c,d}$	1.51^{g}	$1.53^{e,f,g}$	$26.8^{c,d}$	$51.6^{a,b}$	$0.03^{d,e}$	$0.08^{c,d,e}$
9	$99.0^{b,c,d}$	$101.1^{b,c}$	$1.55^{d,e,f,g}$	$1.57^{d,e,f,g}$	$19.2^{c,d,e}$	53.4^{a}	$0.11^{b,c,d,e}$	$0.14^{b,c,d,e}$
10	107.7^{a}	106.2^{a}	1.51^{g}	1.49^{g}	NA	NA	$0.37^{a,b,c}$	$0.29^{a,b,c,d,e}$
11	$100.6^{b,c}$	101.5^{b}	1.50^{g}	$1.53^{f,g}$	NA	NA	$0.43^{a,b}$	$0.22^{a,b,c,d,e}$
SEM	0.61		0.02		2.24		0.01	
p-value	<0.0001		<0.0001		<0.0001		0.0372	

[1]Data is presented as least square means [2]Includes cracked and shell-less eggs NA= data not available

Means within each parameter with a different letter superscript are significantly different (P<0.05)

Although there were no more significant treatment differences over time, efficiencies generally improved over time.

Hens consuming a diet supplemented with 4 mg folic acid/ kg had significantly ($P<0.0001$) higher total egg folate content throughout the entire production cycle (periods one through eleven) than hens on the control diet. When consuming an unsupplemented diet, total egg folate content remained constant until period eight, when it increased significantly from 11.8 to 26.8 μg/egg. When consuming a diet supplemented with 4 mg folic acid/ kg diet, total egg folate content significantly increased from period one to two, 29.8 to 44.9 μg/egg, after which no further increase was realized. The total uncollectible egg data was very variable over time but not affected by dietary folate levels.

The egg folate data of hens with significant strain by ration by period interaction is shown in **Table 14**. The Hyline W98 had a significant increase in egg folate content from period seven to eight (12.1 vs. 31.9 μg/egg) without folic acid supplementation. The Hyline W36 had a significant increase in egg folate content when supplemented with 4 mg folic acid/ kg diet from period one to two (25.4 vs. 47.4 μg/egg) and a significant decrease in total egg folate content from periods three to four (54.0 vs. 37.2 μg/egg), where as the Hyline W98 were unresponsive to dietary changes.

Table 14. Egg folate content (µg/egg) of Hyline W98 and W36 laying hens throughout the production cycle- Variable with significant (P<0.05) strain*ration*period interactions[1]

Dietary Folic Acid Supplementation (mg/kg)	0			4	
	Strain			Strain	
Period	W36	W98		W36	W98
1	$15.3^{i,j}$	$16.6^{i,j}$		$25.4^{f,g,h,i,,j}$	$34.2^{d,e,f,g,h}$
2	$14.9^{i,j}$	$16.6^{i,j}$		$47.4^{a,b,c,d,e}$	$42.5^{a,b,c,d,e}$
3	$14.3^{i,j}$	13.0^{j}		$54.0^{a,b}$	$38.3^{b,c,d,e,f,g,h}$
4	$16.7^{i,j}$	$19.2^{h,i,j}$		$37.2^{c,d,e,f,g,h}$	$51.8^{a,b,c}$
5	$17.9^{i,j}$	$15.4^{i,j}$		$41.2^{a,b,c,d,e,f}$	$42.9^{a,b,c,d,e}$
6	$15.9^{i,j}$	$21.6^{g,h,i,j}$		$47.2^{a,b,c,d,e}$	$41.7^{a,b,c,d,e}$
7	11.4^{j}	12.1^{j}		$56.5^{a,b}$	$40.0^{a,b,c,d,e,f,g}$
8	$21.6^{g,h,i,j}$	$31.9^{e,f,g,h,i.}$		$51.8^{a,b,c}$	$51.5^{a,b,c}$
9	$17.7^{i,j}$	$20.8^{g,h,i,j}$		$49.6^{a,b,c,d}$	57.2^{a}
SEM	2.99				
p-value	0.0025				

(P<0.05)

4.6 Discussion

4.6.1 Effect of Dietary Folate and Hen Age on the Folate Content of Eggs

The production trial supports the work reported in Manuscript One, House et al., (2002) and Sherwood et al., (1993), that egg folate concentrations are sensitive to dietary folate levels. In this study the folate content of eggs was increased 2.5-fold by supplementing crystalline folic acid to cereal-based laying hen rations. The three way interaction between strain, ration and period may be due to biological factors such as differences in the genetic make-up of the hens or sampling error.

As the hens supplemented with folic acid matured, egg folate content increased with yolk size. The hen appears to reach maximum egg folate deposition early in production (after 28 days of feeding) and it remains basically consistent throughout the lay period (46.9 ± 3.9 μg/egg). Producers can be confident in supplying a consistent product with little variability throughout the productioncycle. Similarly, Squires and Naber (1993) reported that upon riboflavin supplementation, two to three times the NRC (1994) requirement, differences in egg yolk riboflavin content was apparent after one week on the experimental diets and riboflavin yolk content remained consistent for the remainder of the experiment (24 weeks).

Without dietary folic acid supplementation (analyzed level 1.45 μg/kg), total egg folate content remains constant and significantly lower than the eggs from folate supplemented diets, until period eight, at which there is a significant increase. When hens are supplemented with 4 mg folic diet/ kg diet, folic acid supplementation significantly increases in period two and remains unchanged throughout the rest of the production cycle. This is contrary to hens supplemented with riboflavin for 42

weeks, egg riboflavin concentrations decreased with age of the hens (Naber and Squires, 1993). Similarly, folic acid content of turkey eggs have also been shown to decrease over time (23 week trial) (Robel, 1983). Perhaps if we had the egg folate data from the last two periods, we would also see a decrease in total egg folate content. Older birds may have a decreased ability to absorb vitamins or may have a lower efficiency of folate transfer to the ovaries and magnum via folate-binding proteins. Riboflavin studies have shown that riboflavin transfer across membranes (White et al., 1986) or the hormonal induction of liver riboflavin-binding protein synthesis decreases with age. Further research is required to determine if egg folate content is influenced by similar age effects.

4.6.2 Effect of Dietary Folate on the Livability of Hens

Folic acid supplementation at 4 mg/ kg diet may improve the overall livability of laying hens irrespective of strain. However, due to the very low overall mortality during this trial (2.40%; 8 vs. 7 hens for the control vs. the supplemented diet), it is difficult to be confident in this finding. In a commercial operation mortality of 4-5% is common throughout the production cycle (Hy-Line Inc., 2004; 2003). To verify this significance a Chi Square Test was conducted which determined that in fact there was no significant difference in mortality between diets. Since the unsupplemented diet contained 5.8-times the folic acid than the requirement stated in the NRC (1994), it was expected to see a benefit to the hen beyond the requirement for production and egg fortification.

The data reported in Manuscript One revealed that plasma homocysteine concentrations tended to decrease with increasing dietary folate supplementation.

The Hyline W98 also exhibited a decrease in HCY plasma levels when supplemented with 2 mg folic acid/ kg diet. From the combination of these two findings it is speculated that a reduction in hen plasma HCY due to folate supplementation could result in improved hen livability. In the short term, production may not be affected by increased folate supplementation; however long term, a folate deficiency may impact DNA synthesis and reduce responsiveness to an immune challenge (Steinberg, 1984).

Nygård et al., (1999) reported that plasma total HCY levels are a strong predictor of mortality in human subjects. There was a strong graded dose-response relationship between the total HCY level and overall mortality. As patients aged their total HCY level also increased (Nygård et al., 1999; Selhub et al., 1993). As the hens age, their plasma HCY levels may also increase, resulting in the need for additional folate supplementation later in the production cycle. Further studies are required to test this hypothesis, document the cause of death and monitor hen plasma HCY levels throughout the production cycle.

4.6.3 Effect of Dietary Folate and Hen Age on Egg Quality

Specific gravity and HU, as measures of egg quality were not significantly affected by dietary folic acid. In an experiment in which folic acid and vitamin B_{12} was removed from the diet of hens in late production, there were no adverse effects on performance, except that egg weight was significantly reduced and, because of this, egg shell quality (more specifically specific gravity) was significantly increased (Keshavarz, 2003).

Egg weight increases with the age of hens (Ledur et al., 2002; Fletcher et al., 1983), and the proportion of yolk is less in small eggs than in larger ones. Egg size increases proportionally with yolk size, and the solids content of eggs from older hens, which lay larger eggs, may be significantly different than those from younger birds (Rossi and Pompei, 1995). In an experiment with different strains, Rose et al., (1966) found significant differences in percentage of solids. Differences in percentage of solids were attributed to the differences in egg weight and genetic differences.

The percentage of total uncollectable eggs appears to be U-shaped, highest at the beginning and the end of the production cycle. This could be attributed to the increased incidence of double yolkers at the beginning of the production cycle and the large egg size at the end of the production cycle. As a result egg quality decreased with age of the birds, Ledur et al., (2002) also noted similar findings. Reduced egg shell quality associated with aging is not due to a reduced ability of the hen to absorb or mobilize calcium for shell formation (Roland et al., 1975; Keshavarz and Nakajima, 1993). As the hens age, absolute daily retention of calcium (Keshavarz and Nakajima, 1993) and shell weight remain constant (Roland et al., 1975). However, increased egg size results in reduced shell quality because the hen is distributing a constant amount of calcium in the shell over a larger surface area. Specific gravity describes shell strength and relates to the egg's resistance to breakage (Grunder et al., 1989). Since specific gravity decreased in latter production of the hens, this could attribute to the increased number of total uncollectible eggs, and increased number of cracks, at the end of the production cycle. The height of the

albumen (HU) decreases with the age of the bird, even though the egg weight and total amount of albumen increased (Hill and Hall, 1980; Liljedahl et al., 1984; Silversides, 2001).

4.6.4 Effect of Dietary Folate on Strain Performance

There are apparent genetic differences in strains of hens as a result of intense genetic selection programs for egg size and production. The Hyline W98 appear to respond to and benefit from additional folic acid supplementation by increasing ($P<0.05$) EP from 83.5 to 84.9%. A similar trend was noted in Manuscript One. Genetic improvements in this strain of hen may allow these hens to be more sensitive and responsive to dietary changes. Strains selected for large egg mass may have a higher folic acid requirement than previously believed.

Throughout the production cycle the Hyline W36 also responded to increased folic acid supplementation by significantly increasing their FC from 94.8 to 95.7 g/h/d. In a short term trial with Hyline W36, House et al., (2002) reported FC significantly increased at a supplementation level of 32 mg folic acid/ kg. Since folic acid is tasteless and odourless (Leeson and Summers, 2000) and hens have rudimentary taste buds (Mason and Clark, 2000) it is not believed that taste would influence FC. The folic acid supplementation may be an appetite stimulant, although further research is necessary to confirm this.

The strain differences described in this trial are consistent with how the Hyline W98 are marketed to producers. The Hyline W98 is a larger, heavier hen that produces larger eggs. As a result this hen also has a higher yolk weight and a smaller percent yolk of egg weight. The Hyline W98 therefore has a higher percentage of

total uncollectable eggs compared to Hyline W36, possibly due to the larger egg size early on (Hy-line, 2004) and later in production compromises egg shell quality and results in more cracks.

The fluctuations in FC may be attributed to weather changes throughout the production cycle. Leeson and Summers (2000) described the feed intake of layers at 18°C and 30°C. FC significantly decreased with increasing temperature. The hot weather experienced in the period four (July and August) of this trial likely decreased FC and the cold weather (January and February) during periods ten and eleven increased FC. FE is expected to improve throughout the production cycle, as the hens mature they are no longer eating for growth but for maintenance.

In summary, this study supports the potential for consistently fortifying eggs with folic acid by supplementing barley-based rations with crystalline folic acid throughout the production cycle of laying hens. Further experimentation is required to determine whether there is a decrease in folate deposition after nine periods of the production cycle. Overall, performance was not affected by folate supplementation. The potential to improve the livability of hens supplemented with additional folic acid needs to be explored further. Additional research is required to assess dietary factors that are likely to influence egg folate content.

Chapter 5

MANUSCRIPT 3

Effect of Wheat-, Barley- and Corn-Based Diets on Total Egg Folate Content.

To be Submitted to

Poultry Science for Publication

5.1 Abstract

A study was designed to determine the effect of dietary cereal type on egg folate content. White Bovan hens (n=12/ diet) received a barley-, barley plus β-glucanase-, corn- or wheat-based ration, containing 0, or 4 mg/kg of crystalline folic acid for 21 days. Response criteria included production parameters and yolk colour. There was no significant difference ($P<0.05$) in performance parameters due to folate supplementation. Egg folate content significantly ($P<0.0001$) increased 2.9-fold; from 16.7 μg/egg with an unsupplemented diet to 48.6 μg/egg when supplemented with 4 mg folic acid/ kg of diet. There was a significant ($P<0.0001$) difference in yolk colour, with corn rations producing the darkest yolks and wheat the palest. The type of cereal in a diet significantly ($P<0.05$) influenced total egg folate content. Corn-based diets had significantly higher total egg folate content than wheat-based diets (37.2 vs 28.1 μg/egg); whereas, the barley-based diet resulted in an intermediate egg folate level (30.4 μg/egg). However, β-glucanase supplementation of a barley-based diet did not significantly affect total egg folate content (34.8μg/egg vs. 30.4μg/egg).

Funding: Manitoba Egg Producers, CEMA

Keywords: Folate, Egg, Cereal

5.2 Introduction

It has been previously shown that the folate content of eggs can be increased by approximately 3-fold by increasing the level of crystalline folic acid in the laying hen diet to 4 mg/kg (Manuscript One, Two and House et al., 2002). In these studies, diets were barley-based. To develop best management practices for optimizing egg folate content the use of different dietary ingredients also must be evaluated.

Rye, oats and barley are not extensively used in poultry diets. This is due to the presence of antinutritive factors such as β-glucan in barley and oats (Hesselman and Aman 1986; Campbell et al., 1986) and pentosan in rye (Fengler and Marquardt, 1988). These soluble non-starch polysaccharides (NSP) increase the viscosity of the digesta in the gut, preventing the access of endogenous enzymes to the nutrients contained within the grain. The high viscosity of the digesta reduces the passage time and impairs diffusion of digestive enzymes to the substrates and the mixing of gut content (Antoniou et al., 1981; Antoniou and Marquardt, 1982). Thus viscosity interferes with the digestion and absorption of nutrients in the ration.

When feeding barley, wheat and oats to poultry it is common to supplement diets with enzymes (Bedford and Schulze, 1998). These supplemented feed enzymes target the NSP in feedstuffs. Feed enzyme supplementation has been shown to improve the NSP availability, reduce the negative impact of these indigestible residues, increase the amount of nutrients available for absorption from wheat and barley diets and improve bird performance (Annison and Choct, 1991; Sebastian et al., 1998).

Based on the impact dietary ingredients could have on folate availability, a study was designed to determine the influence of dietary ingredients and enzyme supplementation of a barley-based diet on total egg folate deposition. Corn-, barley-, barley plus β-glucanase- and wheat-based diets were evaluated.

5.3 Hypotheses and Objectives

5.3.1 Hypotheses

Alternative Hypothesis: Egg folate level will be affected by cereal and enzyme supplementation.

Null Hypothesis: Egg folate level will not be affected by cereal and enzyme supplementation.

5.3.2 Objectives

The purpose of this research was to determine the affect of basal dietary ingredient on the folate-fortification of eggs. This will be accomplished through the following objectives:

i) To determine the influence of dietary ingredients on deposition of egg folate.

ii) To determine the influence of enzyme supplementation of a barley-based diet on egg folate deposition.

5.4 Materials and Methods

5.4.1 General

Single-Comb Bovan White laying hens (Keystone Hatchery, Niverville, MB, Canada) were used in this experiment. Hens were kept in confinement housing under semi-controlled environmental conditions and exposed to 16-hr photoperiod. Ninety-six birds were housed individually; the cage dimensions were 25.4 cm by 40.64 cm, providing 1,032 cm^2 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).

5.4.2 Diets and Experimental Protocol

The basal diet was a barley-based ration (**Table 15**). The diets were formulated to meet the requirements of laying hens consuming 100g of feed / d (NRC, 1994). In accordance with industry standards (BASF, 2000), the basal diet included no crystalline folic acid.

For two weeks prior to the commencement of the study, 256 healthy Bovan White hens received a standard barley-based ration with no additional folic acid supplementation. These hens were monitored for EP, and the 96 highest producing hens were selected for the experiment. At 22 weeks of age (peak production 92-96%), the selected hens were placed individually into battery cages and were randomly assigned to receive one of eight diets; corn, wheat, barley, barley plus enzyme each supplemented with 0 or 4 mg crystalline folic acid/ kg (Sigma Chemical Co., Oakville, ON, Canada) (n=12 per treatment). The enzyme cocktail (Canadian

Table 15. Composition of the experimental laying hen rations.

Ingredient	%		
Base Ingredient	Barley[4]	Wheat	Corn
Wheat (13.8% CP)	0.00	**67.16**	0.00
Corn (8.1% CP)	0.00	0.00	**57.27**
Barley (11.9% CP)	**57.57**	0.00	0.00
Soybean Meal (45.0% CP)	13.50	8.00	18.50
Canola Meal (37.58% (CP)	10.00	10.00	10.00
Fish Meal (72% CP)	2.00	2.00	2.00
Vegetable Oil	5.75	1.52	0.91
Limestone	8.75	8.83	8.75
Biophos (monocalcium phosphate)	0.88	0.95	1.06
Vitamin premix[1]	1.00	1.00	1.00
Mineral premix[2]	0.50	0.50	0.50
Calculated nutrient composition			
Crude protein	18.0	17.9	18.0
Metabolizable energy, kcal/kg	2703	2702	2702
Calcium	3.75	3.75	3.75
Phosphorus (available)	0.40	0.40	0.40
Folate[3] (mg/kg)	1.45	1.45	1.65

[1]Provided (per kg of diet): vitamin A, 8225 IU; vitamin D3, 1000 IU; vitamin E, 5.46 IU; vitamin B_{12}, 11.2 µg; calcium panthothenate, 4.4 mg; choline chloride, 100 mg; ethoxyquin, 125 mg; dl-methionine, 500 mg; niacin, 7.6 mg; riboflavin, 2.2 mg.

[2]Provided (per kg of diet): Mn, 52 mg; Zn, 60 mg; salt (iodized), 4.78 g.

[3]Measured by Covance Laboratories Inc., Madison, WI via Official Methods of Analysis of AOAC International (2000) 17th ed., AOAC International, Gaithersburg, MD, USA, Official Methods 960.46, 992.05.

[4]The enzyme premix was supplemented at a level of 0.2% of the diet. Enzyme blend per kg of diet: glucanase, 700 IU; xylanase, 400 IU; cellulase 10 IU.

Bio-Systems Inc, Calgary, AB, Canada) consisted of β-glucanase, xylanase and cellulase as the main enzymes, and with a broad spectrum side activities. The enzyme premix was supplemented at a level of 0.2% of the diet yielding 700 IU of β-glucanase, 400IU of xylanase and 10 IU of cellulase per kg of complete feed . The birds were given a two-week adaptation period followed by a seven day collection period. FC for each cage unit was determined at the completion of the trial (7 days) to calculate average daily feed intake and FE. 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/ replication; 12 replications per treatment) were randomly collected per treatment per day for three days (288 eggs total). The eggs were weighed to give an average egg weight for the treatment period and processed for egg folate determination. Ninety-six eggs (1 egg/ replication; 12 replications per treatment) were randomly collected per treatment at the end of the trial to determine yolk colour measured by a Roche Colour Fan. The colour fan was opened, the leaves arranged in the correct sequence and the yolk passed along the scale until the most suitable sheet was found. As recommended by Vuilleumier (1968) the fan was viewed from above and held in such a manner that the surface did not appear glossy.

5.4.3 Extraction of Egg Yolk Folate

Please refer to Section 3.4.3.

5.4.5 Analysis of Egg Yolk Folate Content

Please refer to Section 3.4.5.

5.4.6 Calculations

The collected production data was then used to calculate hen-day production (EP), FC, FE, and percent yolk of egg weight, based on the equations detailed in Section 3.4.6.

5.4.7 Statistical Analysis

A 4 x 2 (cereal x folate as main effects) factorial arrangement of a completely randomized design was used to analyze the data. Performance data collected were subjected to analysis of variance using SAS Analyst (SAS Institute Inc., 1998). Differences between means were determined using Tukey's Honestly Significance Difference. Differences with an α level of $P<0.05$ were considered to be statistically significant.

5.5 Results

The main effects of cereal and folate supplementation for different performance traits are shown in **Table 16**. There was no significant difference ($P<0.05$) in performance parameters due to folate supplementation or dietary ingredient. Egg folate content significantly ($P<0.001$) increased 2.9-fold from 16.7 μg/egg with an unsupplemented diet to 48.6 μg/egg when supplemented with 4 mg folic acid/ kg of diet (**Table 17**). There was a significant ($P<0.0001$) difference in yolk colour between the different cereal based diets (**Table 18**). The corn based diets produced yolks that were significantly darker than the barely based diets, which were significantly darker than the wheat based diets. The respective yolks scored 7, 4, and 2 on the Roche Colour Fan. The type of cereal in a diet significantly ($P<0.05$) influenced total egg folate content (**Table 19**). Corn based diets had significantly higher total egg folate content than wheat based diets (37.2 vs 28.1 μg/egg) and the barley-based diet resulted in intermediate (30.4 μg/egg) levels of egg folate. β-glucanase supplementation of a barley-based diet did not significantly affect total egg folate content (34.8 μg/egg vs. 30.4 μg/egg).

Table 16. P-values of main effects (wheat, corn, barley, barley plus β-glucanase) and interactions of cereal and folate on different performance traits of Bovan White laying hens.

Parameters	Cereal	Folate	Cer*Fol
Hen-day Production (%)	0.1208	0.2313	0.3925
Feed Consumption (g/h/d)	0.8232	0.5784	0.5527
Feed Efficiency (g feed/g egg)	0.3617	0.5901	0.5192
Egg Weight (g)	0.5239	0.1544	0.3362
Yolk Weight (g)	0.5182	0.4393	0.2199
Haugh Units	0.3850	0.5920	0.4194
Yolk Colour (Roche Score)	<0.0001	0.6479	0.5849
Egg Folate Content (µg/egg)	0.0104	<0.0001	0.6230

Table 17. Egg folate content (µg/egg) of Bovan White laying hens receiving diets with or without crystalline folic acid supplementation- Main effects of ration[1]

Dietary Folic Acid Supplementation Inclusion (mg/kg)	Egg Folate Content (µg/egg)
0	16.7
4	48.6
SEM	1.49
p-value	<0.0001

[1]Data presented as least square means

Table 18. Yolk colour score of Bovan White laying hens receiving diets with or without crystalline folic acid supplementation- Main effects of cereal[1].

Base Ingredient	Roche Color Fan Score
Corn	7^{a}
Barley	4^{b}
Barley + β-glucanase	4^{b}
Wheat	2^{c}
SEM	0.14
p-value	<0.0001

Means with a different letter superscript are significantly different ($P<0.05$)

[1]Data is presented as least square means

Table 19. Egg folate content (µg/egg) of Bovan White laying hens receiving diets with or without crystalline folic acid supplementation- Main effects of cereal[1].

Base Ingredient	Egg Folate Content (µg/egg)
Corn	37.2[a]
Barley	30.4[a,b]
Barley + β-glucanase	34.8[a,b]
Wheat	28.1[b]
SEM	0.20
p-value	0.01

Means with a different letter superscript are significantly different ($P<0.05$)

[1].Data is presented as least square means

5.6 Discussion

5.6.1 Effect of Dietary Folate on the Folate Content of Eggs

This study supports the work of House et al., (2002), Sherwood et al., (1993), Manuscript One and Two, that egg folate concentrations are sensitive to dietary folate levels. In this study the folate content of eggs was increased 2.9-fold by supplementing 4 mg crystalline folic acid per diet irrespective of the cereal base (**Table 17**). Based on our earlier research of Manuscript One, and that of Sherwood et al., (1993) and House et al., (2002), the egg folate content saturates at less than 4 mg/ kg diet.

5.6.2 Effect of Dietary Ingredients on the Folate Content of Eggs

The type of cereal in a diet significantly ($P<0.05$) influenced total egg folate content **(Table 19)**. The corn-based diet had significantly higher total egg folate content than the wheat-based diet. Although the corn-based diet contained a higher endogenous folate content than the barley- and wheat-based diets, we do not believe that this would impact the total egg folate content since the availability of endogenous sources of folate is questionable. Numerous studies have outlined the impact of cereal non-starch polysaccharides (NSP), associated with the endosperm cell wall of grain, on intestinal development and function (Iji, 1999; Brufau et al., 1994). β-glucans are the primary NSP in barley (Rotter et al., 1990; White et al., 1983) and arabinoxylan (pentosan) is the primary NSP in wheat (Andrewartha et al., 1979). NSPs cannot be hydrolysed by the enzymes that are naturally produced by the bird. NSPs have an antinutritional effect, preventing the access of endogenous enzymes to the nutrients contained within the grain. The uptake of folate from the gut appears to

be impaired due to the increased viscosity of barley-based rations (Brufau et al., 1994; Iji, 1999) and wheat-based diets (Andrewartha et al., 1979). High viscosity of the digesta reduces the passage time and impairs diffusion of digestive enzymes to the substrates and the mixing of gut content (Antoniou et al., 1981; Antoniou and Marquardt, 1982). Thus viscosity interferes with the digestion and absorption of nutrients in the ration. Gut viscosity may therefore impair the uptake of folic acid by the enterocyte's membrane-bound transport system. Folic acid is then reduced to THF via dihydrolfolate reductase within the enterocycte (Henderson, 1990). THF is then transported to the liver. Within the liver, THF is converted to 5-MTHF and attached to folate binding proteins and secreted into the bloodstream (Henderson, 1990).

Plasma serves as the folate pool for egg yolk deposition. As hypothesized in Manuscript One and by Sherwood et al., (1993) plasma folate concentrations may limit egg folate concentrations. Compared to the results of laying hen plasma folate concentrations in Manuscript One, Sherwood et al., (1993) reported a plateau of laying hen plasma folate content at a lower supplementation level. This could be attributed to the difference in the base diets, Sherwood et al., (1993) fed a corn and soybean meal-based diet, whereas in our study a barley-based diet was fed. It is hypothesized that the viscosity attributed with barley-based diets (Brufau et al., 1994; Iji, 1999) may have negatively impacted the amount of available folate for uptake by intestinal cells. This study verifies that the total egg folate content of a corn-based diet is higher than a wheat-based diet. Therefore, gut viscosity may impair folic acid

uptake and thus decrease potential plasma folate concentrations and egg folate content.

There was not a significant ($P<0.05$) increase in total egg folate content when the barley-based ration was supplemented with β-glucanase. Enzymes, such as β-glucanase, have been developed to reduce the negative effects of NSP and improve the feeding value of cereal-based diets. It is believed that the enzyme breaks β-glucans into smaller polymers (Annison and Choct, 1991), and thus, alters the ability of these polysaccharides to form highly viscous solutions that inhibit nutrient diffusion and transport. The application of β-glucanase to barley-based diets has improved bird performance and increased nutrient digestibility in numerous studies (Petterson et al., 1990; Friesen et al., 1992; Marquardt et al., 1994). The lack of response to enzyme supplementation may be attributed to the short period of feeding, generally feed additive trials are conducted for three months to allow the adaptation of gut microbes.

5.6.3 Effect of Dietary Ingredients on the Laying Hen Performance

In this study, performance parameters were not significantly affected by dietary cereal base or enzyme supplementation (**Table 16**). Coon et al., (1988) also reported no significant difference in EP and egg weights from hens consuming corn and barley based rations. These authors also reported increased FC and decreased FE of layers fed diets containing barely compared with that of birds fed the corn diet, which is supported by the work of Lillie and Denton (1968) and Campbell and Guenter (1985). The use of barley in laying hen diets has been shown to have a negative effect on productivity due to gut viscosity (Iji, 1999). However, previous

researchers have reported that supplementing barley-based diets with β-glucanase significantly improved egg production (Peterson and Sauter, 1968) and FE (Al Bustany and Elwinger, 1988). The short duration of our trial could attribute to the lack of response of the hens to the different rations. Their metabolic systems and body stores may not have been challenged during this trial and therefore performance was not affected by diet.

In summary, this study supports the potential for fortifying eggs with folic acid by the supplementation of crystalline folic acid to laying hen diets at 4 mg/ kg diet irrespective of cereal. Overall, performance was not affected by folate supplementation, dietary ingredient or enzyme supplementation. The influence of dietary ingredients on gut viscosity may have negatively impacted egg folate content, however further research is necessary to confirm this. A corn- or barley plus β-glucanase- based diet is optimal for maximal egg folate content.

6.0 General Discussion

The importance of dietary folic acid supplementation in reducing the risk of delivering a baby with a neural tube defect (Czeizel and Dudas, 1992) and the occurrence of cardiovascular disease in adults (Boushey et al., 1995) has resulted in an increased awareness by consumers to ensure an adequate intake of folic acid. The supplementation of laying hen diets with folic acid leads to the production of folate-enriched eggs (House et al., 2002; Sherwood et al., 1993). This not only improves the nutritional value of the egg but offers a way to increase folate intake by humans. House et al., (2002) reported that one large folate enriched egg contains 12.5% of the recommended dietary allowance (RDA) for adult human (RDA=400 μg). The folate in eggs is also ≥100% bioavailable compared to crystalline folic acid using a rat bioassay (House et al., 2003). Furthermore, 5-MTHF, the form of folate found in eggs, does not mask the symptoms of cobalamin deficiency associated with the crystalline folic acid form that is used to enrich cereal products (Institute of Medicine, 1998). The combination of these attributes positions the egg as an excellent alternative source of folate for consumers.

Based on the results of Manuscript One and that of House et al., (2002), it is possible to increase the folate content of eggs by two- to four- fold. Understanding the factors that influence the level of folate in the egg is critical for ensuring the delivery of a product to consumers that has a consistent level of this vital nutrient. The level of folate in the eggs is directly related to plasma folate concentrations, both of which appear to be governed by a saturable process.

Maximal egg folate content was achieved when dietary folate concentrations exceeded the NRC (1994) recommendation of 0.25 mg/kg by 8-fold or higher. House et al., (2002) observed a significant increase in egg folate content above a plateau value when diets were supplemented with 32 mg/kg diet, the highest level tested. However, results from our research did not find evidence of this biphasic response, as increasing folate levels up to 128 mg folic acid/ kg diet did not lead to an increase in egg folate content over and above those achieved at plateau.

As described in Manuscript One, an increase in egg folate level was observed when crystalline folic acid was added from 0 to 2 mg/ kg to the laying hen ration, after which egg folate level appears to reach a plateau. Plasma folate concentrations also became saturated with increasing dietary folate supplementation, at 16 mg folic acid/ kg diet. However the efficiency of folic acid incorporation into the egg yolk greatly diminished to less than 5% when supplementing in excess of 4 mg folic acid/ kg diet. Based on the combination of these findings, further trials evaluated the supplementation of rations with 0 or 4 mg folic acid/ kg diet over a longer production period.

When comparing two distinct strains of laying hens, Hyline W98 and W36, consuming barley-based diets enriched with 4 mg folic acid/ kg, total egg folate content was not significantly different (48.1 ± 9.67 vs. 45.7 ± 7.44 μg/ egg respectively). Similarly, the Bovan White laying hen strain supplemented with 4 mg folic acid /kg (Manuscript Three) produced eggs with 48.6 ± 11.59 μg folic acid/ egg. This suggests that other commercial strains of laying hens would probably respond similarly to folic acid supplementation at 4 mg/ kg.

In order to produce a consistent folate enriched egg, production and age effects were also evaluated. As reported in Manuscript Two, overall hen age and rate of lay did not affect total egg folate content. After period one of the production cycle egg folate content remained consistent, 46.9 μg folic acid/ egg ± 3.90; CV ~6.7% irrespective of strain of bird. Generally, as egg production decreased with the age of the hen, egg folate content increased (although not significant); this could be attributed to increased amount of time to form the yolk, larger yolk size and thus greater folic acid content. Therefore developing the standards for producing folate enriched eggs, it is recommended that the hen consume a folate enriched diet for 30 days prior to marketing the eggs as "folate enriched". This will ensure a consistent amount of folic acid in the egg throughout the production and marketing of these eggs.

It is not believed that the influence of environmental temperatures on feed consumption would significantly affect the folic acid content of eggs. As described in Manuscript Two, the decrease in feed consumption in July and August attributed to the hot weather, did not significantly decrease total egg folate content. It is also speculated that the increase in feed consumption in January and February attributed to the cold weather, would not significantly increase the total egg folate content. Marketers could be confident in supplying an egg with consistent folate levels year around.

However, dietary ingredients appear to affect total egg folate content. The absorption of the folic acid within the small intestine could be a limiting factor in the deposition of folic acid in the egg. Feed that is higher in non-starch polysaccharides

(NSPs) is poorly digested by the laying hen because they do not possess the enzymes required to break down the cell wall of the grain. NSPs result in high intestinal viscosity, decreased nutrient intake, digestibility and absorption (Classen and Bedford, 1992). As reported in Manuscript Three, a corn-based diet resulted in eggs with significantly higher total folate content than the wheat-based diet. However, due to the short duration of this trial (two week adaptation and seven day collection period), a significant difference in total egg folate content was not observed when the barley-based ration was supplemented with β-glucanase. The application of β-glucanase to wheat- and barley-based diets has improved bird performance and increased nutrient digestibility in numerous studies (Petterson et al., 1990; Friesen et al., 1992; Marquardt et al., 1994).

We must also consider the benefits of folic acid beyond the production of a folate enriched egg. As illustrated in Manuscript One, hens receiving additional folic acid supplementation had significantly lower plasma HCY levels. It has been well documented that low folic acid levels lead to high concentrations of plasma HCY, due to the role of this B-vitamin in HCY remethylation to methionine (House et al., 1999). High HCY levels have been linked to cardiovascular disease in humans (Boushey et al., 1995), suggesting folic acid depletion may also cause vascular abnormalities in the hens.

The basal diets contained 1.45 mg folic acid/ kg diet. This is 6 times the recommended NRC (1994) requirement for folic acid of 0.25 mg/kg. This could explain the overall very low mortality throughout the production cycle (2.40%), including the birds consuming the basal diet. However the poultry industry may

further improve health and welfare of the hens and that of the consumer, through the production of a folate-enriched egg. The Hyline W98 hen selected for large egg mass benefited from increased dietary folic acid through reduction in its plasma HCY concentration and increased egg production. This suggests that the industry needs to reassess the folic acid requirements for all laying hen strains.

Overall, by increasing the folate content of eggs, the egg could be marketed as an excellent dietary source of folate. This would provide the public with the option of including eggs as part of a nutritional breakfast to meet the necessary dietary requirements of folic acid instead of consuming crystalline formulated vitamin pills.

7.0 Summary and Conclusions

The overall objective of this thesis was to examine some factors affecting the production of folate-enriched eggs. The research assessed the optimal dietary folic acid level required for maximal egg folate deposition, potential differences due to strain, measures of folate status in hens (including plasma folate and homocysteine concentrations), production factors, such as level of production, age of flock and dietary factors that were likely to influence egg folate content. The following conclusions can be drawn:

1. The optimal level of dietary folate supplementation for maximal egg folate deposition (3-fold increase) of a barley-based ration is at a level in excess of 2 mg/ kg diet but not more than 4 mg/ kg.
2. Overall, performance is not affected by folate supplementation.
3. There are apparent strain differences for maximal egg folate content. The Hyline W98 produce larger eggs, with larger yolks and higher egg folate content and consume more feed than the Hyline W36 at the beginning of the production cycle. However, the total egg folate content of the Hyline W98, W36 and the Bovan White laying hens are similar over the production cycle, suggesting no strain difference and that all other current strains of commercial laying hens would have similar total egg folate levels.
4. The plasma folate concentration of the hens increase with increasing dietary folic acid supplementation up to 16 mg/ kg diet with no further increase at higher inclusion levels.

5. There is an inverse relationship between laying hen plasma folate concentration and plasma homocysteine concentration.
6. A strain of hen selected for large egg mass benefits from increased dietary folic acid supplementation through a reduction in plasma homocysteine concentration and increased egg production.
7. Overall, hen age and rate of lay did not affect total egg folate content. There appears to be a significant increase in egg folate content after 28 days of feeding, after which egg folate content is consistent for the remainder of the production cycle and is not affected by rate of lay or age of the hen.
8. Egg folate level does not impact interior egg or shell quality.
9. Corn-based diets yield eggs with significantly greater folate content than wheat-based diets. The influence of dietary ingredients on gut viscosity may have negatively impacted egg folate content.

8.0 Future Work

In order to progress towards best management practices for optimizing egg folate content further research is required. It is recommended that further research investigate the differences in diets supplemented with 0, 2 or 4 mg folic acid/ kg diet, to accurately reflect current commercial practices of feeding 1.5 to 2 mg/ kg diet.

Since the level of folate in the egg is directly related to the plasma folate concentrations and both appear to be governed by a saturable process, documentation of the mechanisms of folate deposition in eggs, factors regulating folate absorption and metabolism in the laying hen need to be identified. Further investigation into the cause of plasma folate saturation is also crucial, in order to identify another limitation of total egg folate content.

Cereal grains including barley and rye are the main energy sources in poultry diets. Due to the fact that increased gut viscosity associated with barley and rye, results in decreased absorption and digestion of nutrients, further long term studies are warranted to evaluate the effect of enzyme supplementation on folic acid deposition in the egg. Further research could provide new options for effective utilization of cereal grains when supplemented with enzymes and improved efficiency of folic acid and nutrient deposition in eggs.

To verify the consistency of folic acid deposition throughout the production cycle and the affects of rate of lay and age of the hen, another production trial is recommended. Further research is required to document the homocysteine levels in blood plasma of hens throughout the production cycle, to determine if homocysteine levels are a contributing factor to the mortality of the hens. This research is essential

in order to recommend an increase in the folic acid content in all commercial laying hen rations, ultimately fortifying all eggs with folic acid. This information has great ramifications for the poultry industry, through improved performance, health and welfare of the hens and the consumers.

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10.0 Appendix

Solution Preparation

Ascorbate Buffer (pH 7.8) (Buffer A)

1. Add 12.11 g trizma base to ~ 800 mL of deionized water and stir.
2. Add 20.0 g ascorbic acid to the trizma base solution.
3. Adjust pH to 7.8 and bring to a final volume of 1 L using deionized water.
4. Make final slight pH adjustments.

0.03 M Potassium Phosphate Buffer (pH 2.2) (Buffer B)

1. Add 4.08 g potassium phosphate monobasic (KH_2PO_4) to ~ 900 mL of deionized water and stir.
2. Adjust pH to 2.2 using phosphoric acid (85%).
3. Bring to a final volume of 1 L using deionized water
4. Filter.

2M Potassium Borate Buffer, pH 9.5 (Solution A)

1. Dissolve 112.22 g potassium hydroxide (KOH) in ~ 900 mL of deionized water. Bring to a final volume of 1 L using deionized water.
2. Add 123.66 g boric acid and 300 mL of KOH solution to ~ 900 mL of deionized water.
3. Dissolve the boric acid with constant stirring and low-medium heat.
4. Cool the solution only until it can be handled and bring the boric acid to a final volume of 1 L. Note that since 300 mL of KOH was added to the boric solution, the final combined boric acid . KOH volume should be 1.3 L.
5. Add KOH until pH 9.5 is reached.
6. Filter

2M Potassium Borate Buffer (pH 10.5, containing 5mM ethylenediaminetetracetic acid (EDTA) (Solution B)

1. Using Solution A, continue adding KOH until a pH of 10.5 is reached.
2. Calculate the amount of EDTA that must be added to make a 5mM EDTA solution (5mM EDTA = 1.46 g/L).

0.1 M Potassium Boarte Buffer (pH 9.5, containing 2mM EDTA) (Solution C)

1. Make a 1:20 diluation od Solation A using deionized water (50 mL of Solution A + 950 mL deionized water).
2. Calculate the amount of EDTA that must be added to make 2 mM EDTA solution (0.585 g/L).
3. Filter.

Tris (2-Carboxyethyl)- Phosphine Hydrochloride (TCEP) (Reducing Agent)

1. Make a 10% TCEP solution by weighing 100 mg of TCEP directly into a microcentrifuge tube and adding 1 mL of deionized water. A 1% TCEP solution may be made using 10 mL of deionized water.
2. Vortex.

0.6 M Perchloric Acid

1. Add 25.75 mL of perchloric acid to ~ 400 mL of deionized water and bring to a final volume of 500 mL using deionized water.

7-Fluro-2-Oxa-1,3-Dizole-4-Sulfonic Acid (SBDF)

1. Measure 1 mg of SBDF into a glass test tube.
2. Add 1 mL of Solution A.
3. Vortex.
4. Note: warming the solution may be necessary to dissolve the SBDF.

0.1M Sodium Acetate Buffer (pH 5.5, containing 2 % methanol) (Buffer C)

1. Dissolve 13.068 g sodium acetate trihydrate in ~ 900 mL deionized water. Bring solution to a final volume of 1 L using deionized water to make a 0.1 M sodium acetate solution.
2. Add 5.75 mL 0.1 M glacial acetic acid to ~ 900 mL deionized water. Bring to a final volume of 1 L using deionized water to make a 0.1 M glacial acetic acid solution.
3. Slowly add the 0.1 M glacial acetic acid solution to the entire volume of 0.1 M sodium acetate soltion until a pH of 5.5 is reached.
4. Measure the amount of the prepared pHed soluntion in a graduated cyclinder. Calculate and add the appropriate amount of methanol to achieve a 2 % methanol solution.
5. Filter.