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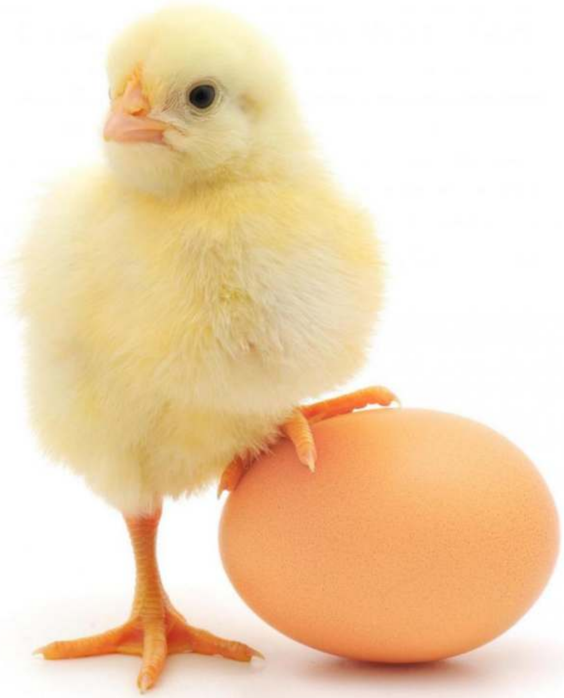

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ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorijps@gmail.com

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Evaluation of Dietary Methionine, Folic Acid and Cyanocobalamin (B_{12}) and Their Interactions in Laying Hen Performance

O.M. El - Husseiny, A.Z. Soliman, I.I. Omara and H.M.R. El - Sherif
Department of Animal Production, Faculty of Agriculture, Cairo University, Cairo, Egypt

Abstract: An experiment conducted to examine the effect of methionine (M), folic acid (F) and vitamin B_{12} and their interactions on Bovans White laying hen performance, egg quality, nutrient digestabilities and relative economic efficiency (REE) from 28 to 43 weeks of age. The experiment was conducted in a 3 x 3 x 2 factorial arrangement of treatments, three levels of M (0.40, 0.45 and 0.50%), three levels of F (6.0, 9.0 and 12.0 mg/kg) and two levels of B_{12} (0.01 and 0.02 mg/kg) were used. 0.45% M, 0.50 mg F/kg and 0.01 mg B_{12}/kg considered as a control. The results indicated that layers fed high level of M gave the best egg production (EP), the least feed consumption (FC) compared with either medium or low M levels with no significant differences. Layers fed high level of M recorded high egg weight (EW) and improved feed conversion ratio (FCR) compared with either medium or low M levels with significant differences ($p \leq 0.05$). The interactions of M x F x B_{12} had significant effect on egg shell percentage (ESP), egg content percentage (ECP) and blood hemoglobin (BH), with no significant effect on EP, FC, FCR and egg shell thickness (ST). The interaction of high level of M with F and B_{12} supplementation gave the best performance and REE compared with either medium or low M levels. It could be concluded that feeding diet at 0.50% M with supplemented F and B_{12} (12.0 and 0.02 mg/kg, respectively) gave the best performance and REE.

Key words: Methionine, folic acid, B_{12}, laying hens

Introduction

Methionine is the first limiting amino acid in most poultry diets and is crucial to the production of meat, eggs and the synthesis of enzymes and hormones. Hence, a deficiency of M is often a roadblock to growth and egg production (Liu *et al*., 2004b). It was concluded that M had positive effect on laying hens performance (Liu *et al*., 2004 a, b and Abd-Elsamee, 2005). In poultry feeding M supplementation is one of the most expensive items, especially with corn-soybean diets. Many attempts has been made to reduce this cost of poultry feed by using cheaper sources such as inorganic sulphates or betafin to replace the expensive commercial DL-M. In its role as a methyl donor (transmethylation) M is converted to homocysteine (HCY), which lies at the crossroads of sulfur amino acid metabolism (Fig. 1). Formation of cysteine may occur if homocysteine proceeds through the irreversible transsulfuration pathway; alternatively (HCY) may be converted back to M after addition of a methyl group by folate-vitamin B_{12}-dependent M synthase or betaine-(HCY) methyltransferase (Pillai *et al*., 2006). Folate is a collective term for a group of different water compounds with a pteroylglutamic acid backbone but differing oxidation states (i.e., F, 5-methyltetra-hydrofolate) whose primary function includes one-carbon transfer reactions (Selhub and Rosenberg, 1996). Examples of one carbon transfer reactions include the remethylation of homocysteine, glycine-serine interconversion and purine synthesis (Selhub and Rosenberg, 1996). Hebert *et al*. (2005) reported that no significant differences in hen day production due to crystalline F supplementation when laying hens fed diets supplemented with graded levels of crystalline F (0, 2, 4, 8, 16, 32, 64 and 132 mg/kg diet).

Vitamin B_{12} is one of eight water-soluble vitamins. Birds synthesize vitamin B_{12} using cobalt inside the ceca, but the levels are below the requirements and it must be supplemented (McDonald *et al*., 1975). To M synthase, B_{12} is essential for transfer of a methyl group from the methylated form of F (5-methyl-THF) to homocysteine for regeneration of methionine and tetrahydrofolate (THF) (Bassler, 1997). Kato *et al*. (2002) found that egg weight increased due to B_{12} supplementation while no effect of B_{12} supplementation on other parameters of performance when Lohmann laying hens on the second cycle of production fed diets supplemented with 2 levels of B_{12} (0, 0.01 mg/kg diet).

Therefore, this experiment aimed to study the effect of M, F and vitamin B_{12} and their interactions on the performance, egg quality and REE of Bovans White laying hens.

Materials and Methods

Experimental birds and management: A total number of 1824 Bovans White laying hens, 28 weeks of age was used. Hens were randomly divided into 19 groups of 96 hens each, four replicates of 24 hens each. Hens were kept in cleaned and famigented cages of wire floored batteries in closed system house. Feed and water were offered *ad - libitum* all over the experimental period (16

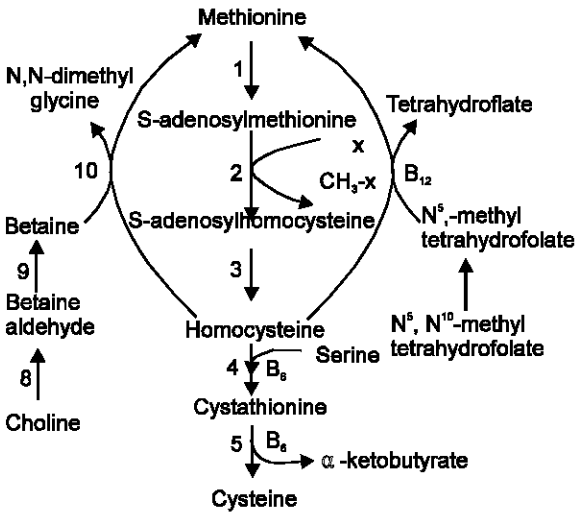

Fig. 1: Metabolism of sulfur amino acids, choline and betaine. Numerals indicate the following enzymes: 1) methionine adenosyltransferase, 2) various enzymes, 3) S-adenosylhomocysteine hydrolase, 4) cystathionine α-synthase, 5) cystathionine γ-lyase dehydrogenase, 9) betaine aldehyde dehydrogenase and 10) betaine-homocysteine methyltransferase, 6) N5,N10-methylenetetrahydrofolate reductase, 7) methionine synthase, 8) choline (Pillai *et al.*, 2006).

Table 1: Composition and calculated analysis of basal diet

Ingredients	%
Corn yellow	63.06
Soybean meal	18.50
Concentrate	10.00
Limestone	7.50
Bone meal	0.50
Vit and min. Premix *	0.10
Salt (NaCl)	0.25
D.L methionine	0.09
Total	100.0
Calculated analysis	
ME (kcal/kg)	2784
CP%	18.58
Total methionine%	0.36
Lysine%	1.00
Calcium	3.64
Available phosphorus	0.47
Total Folic acid mg/kg	0.50
Total B12 mg / kg	0.048
Ether extract	3.07
Crude fiber	2.84

*Vitamin and mineral at 0.1% of the diet supplies the following per 1.5 kg of the diet containing: Vit. A 12000000 I.U, vit.. D_3 3500000 I.U, vit. E 20000 mg, vit. 3000 mg, vit B_1 3000 mg, vit B_2 8000 mg, vit. B_6 3000 mg, vit. B_{12} 15 mg, calcium biotin 50 mg, manganese 80 gm, zinc 75 gm, iron 40 gm, copper 10 gm, iodine 2 gm, selenium 0.3 gm, cobalt 0.25 gm, choline chloride 600 gm and carrier ($CaCo_3$) to 1.5 kg. ** According to N.R.C. (1994).

weeks) from 28 to 43 weeks of age, with a total of 16 hours light per day regimen.

Experimental design: The experiment was conducted in a 3 x 3 x 2 factorial design. Three levels of M (0.04, 0.09 and 0.14%) added to the basal diet that contained 0.36% M, being 0.40, 0.45 and 0.50% M, three levels of F (5.5, 8.5 and 11.5 mg/kg) added to basal diet that contained 0.50 mg F/kg, being 6.0, 9.0 and 12.0 mg/kg and two levels of B_{12} (0.0052 and 0.0152 mg/kg) added to the basal diet that contained 0.0048 mg B_{12}/kg, being (0.01 and 0.02 mg/kg).

Experimental diets: The basal diet and its chemical composition are presented in Table 1. The experimental diets were formulated to contain three levels of M 0.40% (low M), 0.45% (medium M) and 0.50% (high M). Each M level was supplemented with three levels of F (6.0 or 9.0 or 12.0 mg/kg diet), each F level was supplemented with two levels of B_{12} either 0.01 or 0.02 mg/kg diet. M (DL-M 99%), F (96%) and B_{12} (1%) were purchased from Egypt for Feed Additives Company (EFAC). Nineteen experimental diets were formulated using linear programing to be isocaloric (2784 K.cal ME/kg) and isonitrogenous (18.58% CP). The experimental diets were formulated to meet the nutrient requirements according to the recommended allowances of the Bovans White breed manual, where 0.45% M, 0.50 mg F/kg and 0.01 mg B_{12} /kg which are considered as a control.

Measurements: All birds of each treatments were weighed at the beginning (initial live body weight) and at the end of experimental period (final live body weight) to calculate live body weight gain (LBWG). The daily feed consumed per hen and hen-day EP percentage were calculated every four wks. intervals during the experimental period (16 weeks). Eggs were collected and weighed every four wks. during the experimental periods. Records of EP and EW were used to calculate egg mass (g/hen/day), egg mass and FC were used to calculate the amount of feed (kg.) which was required to produce one kg. of eggs per hen or to calculate FCR during specific period.

ST was determined using a dial pipe gauge. Haugh units (HU) were calculated based upon the height of albumen determined by a micrometer and EW according to Eisen *et al.* (1962). Yolk index (YI) = (yolk height / diameter) x 10 according to Funk (1948). Dry shell weighed to the nearest 0.10 g. and ESP = egg shell weight / EW ×100. Egg content weight were calculated by the difference between EW and egg shell and ECP = egg content weight / EW × 100. Serum total immunoglobulin tetre (STIT) were also determined

Table 2: Effect of experimental treatments on body weight gain, egg production, egg weight, feed intake, feed conversion ratio and economic efficiency

Experimental treatments	Body weight gain(g)	Egg production (%)	Egg weight (g)	Feed intake (g/hen/d)	Feed conversion (g feed/gegg)	Relative economic efficiency
Methionine (M) effect:						
0.40%.	103^{b}	87.2	60.6^{ab}	107	2.02^{a}	101
0.45%.	156^{a}	87.6	60.1^{b}	106	2.01^{a}	102
0.50%.	100^{b}	89.7	61.4^{a}	105	1.91^{b}	112
P value	<0.0001	0.25	0.002	0.06	0.01	
Folic acid (F) effect:						
6.0 mg/kg.	133^{a}	87.7	60.1^{b}	106	2.02	103
9.0 mg/kg.	123^{b}	88.6	60.9^{a}	106	1.95	106
12.0 mg/kg.	103^{c}	88.3	60.7^{ab}	105	1.96	107
P value	<0.0001	0.84	0.004	0.51	0.34	
Cyanocobalamin (B_{12}) effect:						
0.01 mg/kg.	131^{a}	88.1	60.3^{b}	106	1.99	105
0.02 mg/kg.	108^{b}	88.3	60.5^{a}	106	1.96	105
P value	<0.0001	0.89	0.03	0.86	0.55	
M. x F. x B_{12} effect:						
T0 (0.45%+0.50 mg/kg+0.01 mg/kg).	203^{b}	87.4	62.0^{ab}	107	1.97	100
T1 (0.40%+6.0 mg/kg+0.01 mg/kg).	182^{c}	83.3	58.6^{f}	108	2.21	87
T2 (0.40%+6.0 mg/kg+0.02 mg/kg).	56^{ij}	86.2	61.0^{abcd}	107	2.03	97
T3 (0.40%+9.0 mg/kg+0.01 mg/kg).	114^{f}	88.2	59.7^{def}	105	1.99	107
T4 (0.40%+9.0 mg/kg+0.02 mg/kg)	87^{gh}	92.2	61.1^{abcd}	107	1.90	113
T5 (0.40%+12.0 mg/kg+0.01 mg/kg).	103^{fg}	88.7	60.8^{bcd}	106	1.97	106
T6 (0.40%+12.0 mg/kg+0.02 mg/kg).	73^{h}	84.8	62.1^{ab}	106	2.01	94
T7 (0.45%+6.0 mg/kg+0.01 mg/kg).	138^{e}	92.7	59.7^{def}	107	1.93	114
T8 (0.45%+6.0 mg/kg+0.02 mg/kg).	170^{cd}	88.2	59.1^{ef}	108	2.07	100
T9 (0.45%+9.0 mg/kg+0.01 mg/kg).	257^{a}	87.6	60.3^{cde}	107	2.03	101
T10 (0.45%+9.0 mg/kg+0.02 mg/kg).	72^{hi}	84.9	60.9^{abcd}	107	2.01	93
T11 (0.45%+12.0 mg/kg+0.01 mg/kg).	140^{e}	85.1	60.6^{bcd}	104	2.02	100
T12 (0.45%+12.0 mg/kg+0.02 mg/kg).	160^{d}	87.1	60.1^{cde}	104	1.99	106
T13 (0.50%+6.0 mg/kg+0.01 mg/kg).	113^{f}	85.8	61.3^{abc}	106	2.02	99
T14 (0.50%+6.0 mg/kg+0.02 mg/kg).	140^{e}	89.8	60.8^{bcd}	102	1.87	119
T15 (0.50%+9.0 mg/kg+0.01 mg/kg).	53^{j}	89.4	61.3^{abc}	102	1.86	117
T16 (0.50%+9.0 mg/kg+0.02 mg/kg).	153^{de}	89.0	62.3^{a}	106	1.91	106
T17 (0.50%+12.0 mg/kg+0.01 mg/kg).	79^{h}	92.0	60.9^{abcd}	107	1.91	113
T18 (0.50%+12.0 mg/kg+0.02 mg/kg).	60^{ij}	92.2	59.9^{cdef}	104	1.88	120
P value	<0.0001	0.48	<0.0001	0.06	0.17	

^{a,b,c},.... etc. means in same column, within each factor with different superscripts are significantly ($P \leq 0.05$) different.

according to Van der Zipp *et al.* (1983).Blood haemoglobin was measured according to Henry *et al.* (1974). EE of egg production was calculated from the input-output analysis which was calculated according to the price of the experimental diets and egg produced during the entire experimental period. The values of economical efficiency were calculated as the net revenue per unit of feed cost. Prices of the supplements (M, F and B_{12}) were taken in the consideration.

At the end of the experimental period, 43 weeks of age, a total number of 228 hens, 12 from each treatment were randomly taken for digestion trials to estimate the nutrient digestibility. Feeds and fresh water were offered *ad-libitum*, excreta was collected quantitively every 24 hours, during a three days collection period. Proximate analysis of the feed and dried excreta was done following the methods of A.O.A.C. (1990). Fecal nitrogen was determined according to Jakobsen *et al.* (1960). The data pooled through the experiment were proceed by General Linear Model procedures (GLM) described in SAS User's Guide (SAS, Institute, 2004).

Results and Discussion

Laying hen performance

Live body weight gain: Significant differences ($p \leq 0.05$) in LBWG among hens fed the three different M levels, while, hens receiving 0.45% M gained the best being 156 g vs. 0.40 and 0.50% M that gained 103 and 100 g, respectively. These results may be attributed to reduce EP and increase FI with hens fed medium level of M, while, hens fed 0.50% and 0.40 M gained similar LBWG because of decreasing FI and increasing EP reduced LBWG for 0.50% M compared with hens fed 0.40% M which consumed more FI with low level of M. Significant differences ($p \leq 0.05$) among LBWG due to F and B_{12} levels where, LBWG decreased with increasing the levels of F and B_{12} (Table 2). A significant difference ($p \leq 0.05$) was observed in the LBWG due to M × F × B_{12}

Table 3: Effect of experimental treatments on nutrients digestibility (%)

Experimental treatments	Digestion coefficient %					
	Dry matter	Crude protein	Ether extract	Crude fiber	Nitrogen free extract	Organic matter
Methionine (M) effect:						
0.40%.	73.6	86.9^{b}	80.8	26.0^{c}	79.9	79.5
0.45%.	74.0	86.6^{b}	80.8	27.8^{b}	79.2	78.8
0.50%.	73.9	88.1^{a}	81.4	28.2^{a}	79.1	78.8
P value	0.06	0.002	0.75	<.0001	0.24	0.42
Folic acid (F) effect:						
6.0 mg/kg.	73.8	87.8^{a}	81.4^{ab}	28.2^{a}	79.1	79.6
9.0 mg/kg.	73.8	87.0^{b}	79.2^{b}	26.4b	79.5	78.4
12.0 mg/kg.	74.0	86.8^{b}	82.5^{a}	28.4^{a}	79.7	79.1
P value	0.44	0.04	0.01	<.0001	0.47	0.18
Cyanocobalamin (B_{12}) effect:						
0.01 mg/kg.	73.9	86.8^{b}	81.6	28.0^{a}	79.5	79.1
0.02 mg/kg.	73.8	87.5^{a}	80.4	27.4^{b}	79.3	78.7
P value	0.81	0.04	0.11	<.0001	0.76	0.51
M. x F. x B_{12} effect:						
T0 (0.45%+0.50 mg/kg+0.01 mg/kg).	73.6	86.9^{ab}	80.8^{bcdef}	24.9^{efg}	79.4^{bcd}	78.2
T1 (0.40%+6.0 mg/kg+0.01 mg/kg).	73.5	88.3^{ab}	78.9^{cdef}	29.3^{abcd}	77.5^{d}	81.7
T2 (0.40%+6.0 mg/kg+0.02 mg/kg).	73.5	86.1^{bc}	76.2^{fg}	27.9^{cde}	81.4^{ab}	79.1
T3 (0.40%+9.0 mg/kg+0.01 mg/kg).	73.4	86.8^{ab}	83.3^{abcde}	25.1^{efg}	81.2^{abc}	79.2
T4 (0.40%+9.0 mg/kg+0.02 mg/kg)	73.6	87.7^{ab}	76.7^{efg}	23.4^{h}	81.3^{abc}	79.2
T5 (0.40%+12.0 mg/kg+0.01 mg/kg).	73.7	86.2^{bc}	85.9^{ab}	27.2^{cdef}	78.4^{cd}	77.9
T6 (0.40%+12.0 mg/kg+0.02 mg/kg).	74.1	86.1^{bc}	83.8^{abcd}	29.5^{abc}	79.5^{bcd}	79.9
T7 (0.45%+6.0 mg/kg+0.01 mg/kg).	73.8	87.8^{ab}	84.4^{abc}	30.7^{a}	78.6^{bcd}	79.7
T8 (0.45%+6.0 mg/kg+0.02 mg/kg).	73.7	88.4^{ab}	89.2^{a}	27.6^{cde}	79.6^{abcd}	79.8
T9 (0.45%+9.0 mg/kg+0.01 mg/kg).	74.0	84.4^{dc}	75.4^{fg}	24.1^{gh}	78.7^{bcd}	77.6
T10 (0.45%+9.0 mg/kg+0.02 mg/kg).	73.8	86.3^{abc}	76.7^{efg}	29.2^{abcd}	77.5^{d}	77.5
T11 (0.45%+12.0 mg/kg+0.01 mg/kg).	74.9	83.7^{d}	79.1^{cdef}	30.7^{a}	82.3^{a}	80.1
T12 (0.45%+12.0 mg/kg+0.02 mg/kg).	73.9	88.7^{a}	79.8^{bcdef}	24.5^{fgh}	78.8^{bcd}	78.2
T13 (0.50%+6.0 mg/kg+0.01 mg/kg).	73.9	87.9^{ab}	77.9^{cdef}	24.7^{fgh}	79.4^{bcd}	78.7
T14 (0.50%+6.0 mg/kg+0.02 mg/kg).	74.4	88.3^{ab}	81.5^{bcdef}	29.3^{abcd}	77.9^{d}	78.3
T15 (0.50%+9.0 mg/kg+0.01 mg/kg).	74.0	88.3^{ab}	80.8^{bcdef}	29.6^{abc}	79.2^{bcd}	78.2
T16 (0.50%+9.0 mg/kg+0.02 mg/kg).	73.8	88.1^{ab}	82.1^{bcdef}	27.2^{cdef}	78.9^{bcd}	78.8
T17 (0.50%+12.0 mg/kg+0.01 mg/kg).	73.7	87.7^{ab}	88.9^{a}	30.2^{ab}	79.8^{abcd}	79.5
T18 (0.50%+12.0 mg/kg+0.02 mg/kg).	73.7	88.2^{ab}	77.2^{def}	28.4^{bcd}	79.2^{bcd}	78.9
P value	0.14	0.0003	<.0001	0.001	0.01	0.50

^{a,b,c,}.... etc. means in same column, within each factor with different superscripts are significantly ($P \leq 0.05$) different.

interactions. The average values of LBWG ranged from 53 to 257 g for T15 and T9 respectively, while, the highly significant ($p \leq 0.05$) differences in LBWG values were noticed between the control treatment and the other experimental treatments. (Table 2). These results supported the findings of William *et al.* (2005), Liu *et al.* (2005) who noticed that hen weight gain was affected by M levels. Hebert *et al.* (2004) and El-Husseiny *et al.* (2005) concluded that no significant differences due to folic acid levels on LBWG were observed.

Egg production: EP increased with increasing dietary M levels with no significant differences ($p>0.05$) among M levels in EP. No significant differences in EP due to folic acid and B_{12} levels (Table 2). The differences in EP due to M × F × B_{12} interactions were not significant ($p \leq 0.05$). Supplementation of 9.0 mg F/kg and 0.02 mg B_{12}/kg to 0.40% M (T4) improved EP compared to the other treatments containing 0.40% M. These results may be attributed to the supplementation of vitamins (F and B_{12}) to low M diet (0.40 %) that improved EP percentage.

No significant differences ($p \leq 0.05$) from adding supplemental M to the basal diet (0.36% M), indicating the low sensitivity of laying hens to M supplementation (0.04, 0.09 and 0.14% M). The low sensitivity of laying hens to M supplementation did not clear any significant differences among treatments in EP, although, increasing EP with the diets containing high level of M with F and B_{12} supplementation compared with either medium or low level of M. Low level of M gave EP closed to medium level confirm the reaction of remethylation where (HCY) may be converted back to M after addition of a methyl group by folate-vitamin B_{12}-dependent M synthase. No significant differences between control diet (T0) and the other treatments which are containing different levels of M, F and B_{12}. These results agreed with Novak *et al.* (2004) and Bateman *et al.* (2005) who concluded that no significant differences in EP with

Table 4: Effect of experimental treatments on egg shell thickness, egg contents, egg contents percentage, egg shell weight, egg shell weight percentage, Haugh units immunoglobulin titer and hemoglobin

Experimental treatments	Egg shell thickness (mm)	Egg shell Percent (%)	Egg contents Percent	Haugh Units (%)	Yolk index (%)	Immuno-globulin titer	Heamo-glubin (g/dl)
Methionine effect:							
0.40%.	0.403	9.4	90.6	79.7	40.2[a]	7.3	21.3[b]
0.45%.	0.399	9.5	90.5	78.6	39.3[b]	6.5	24.7[a]
0.50%.	0.396	9.5	90.5	78.3	38.8[b]	7.3	20.8[c]
P value	0.19	0.17	0.13	0.06	0.001	0.09	<0.0001
Folic acid effect:							
6.0 mg/kg.	0.400	9.5	90.5	78.1	39.2	7.3	20.4[c]
9.0 mg/kg.	0.400	9.4	90.6	79.1	39.7	6.8	21.8[b]
12.0 mg/kg.	0.397	9.5	90.5	79.3	39.4	7.3	24.5[a]
P value	0.71	0.24	0.23	0.80	0.53	0.09	<0.0001
Cyanocobalamin (B_{12}) effect:							
0.01 mg/kg.	0.399	9.5	90.5	79.1	39.5	7.4[a]	21.6[b]
0.02 mg/kg.	0.399	9.4	90.6	78.6	39.3	6.9[b]	22.9[a]
P value	0.94	0.10	0.10	0.26	0.76	0.01	<0.0001
M. x F. x B_{12} effect:							
T0 (0.45%+0.50 mg/kg+0.01 mg/kg).	0.407	9.6[ab]	90.4[bcd]	79.2	41.2[a]	6.0[b]	19.9[cde]
T1 (0.40%+6.0 mg/kg+0.01 mg/kg).	0.412	9.7[a]	90.3[cd]	79.3	40.0[abcd]	8.0[a]	20.4[bcde]
T2 (0.40%+6.0 mg/kg+0.02 mg/kg).	0.411	9.5[abc]	90.5[bcd]	78.0	39.9[abcd]	6.0[b]	25.3[bcd]
T3 (0.40%+9.0 mg/kg+0.01 mg/kg).	0.390	9.3[bc]	90.7[b]	84.1	40.3[abc]	8.0[a]	19.7[cde]
T4 (0.40%+9.0 mg/kg+0.02 mg/kg)	0.385	8.8[d]	91.2[a]	78.9	40.5[ab]	6.0[b]	20.0[cde]
T5 (0.40%+12.0 mg/kg+0.01 mg/kg).	0.398	9.5[abc]	90.5[bcd]	78.2	39.8[abcd]	8.0[a]	19.9[cde]
T6 (0.40%+12.0 mg/kg+0.02 mg/kg).	0.402	9.6[ab]	90.4[bcd]	79.5	40.4[ab]	8.0[a]	22.5[bcde]
T7 (0.45%+6.0 mg/kg+0.01 mg/kg).	0.399	9.5[abc]	90.6[bc]	79.6	40.3[abc]	7.0[ab]	19.8[cde]
T8 (0.45%+6.0 mg/kg+0.02 mg/kg).	0.393	9.5[abc]	90.5[bcd]	81.0	40.3[abc]	7.0[ab]	21.4[bcde]
T9 (0.45%+9.0 mg/kg+0.01 mg/kg).	0.406	9.6[ab]	90.4[bcd]	77.3	40.1[abcd]	6.0[b]	22.3[bcde]
T10 (0.45%+9.0 mg/kg+0.02 mg/kg).	0.401	9.6[ab]	90.4[bcd]	76.6	39.1[abcde]	7.0[ab]	20.4[bcde]
T11 (0.45%+12.0 mg/kg+0.01 mg/kg).	0.395	9.4[abc]	90.6[bc]	78.1	38.0[cde]	7.0[ab]	27.4[b]
T12 (0.45%+12.0 mg/kg+0.02 mg/kg).	0.391	9.4[abc]	90.6[bc]	79.3	37.9[de]	6.0[b]	36.7[a]
T13 (0.50%+6.0 mg/kg+0.01 mg/kg).	0.390	9.3[bc]	90.7[b]	75.1	37.6[e]	8.0[a]	18.5[de]
T14 (0.50%+6.0 mg/kg+0.02 mg/kg).	0.393	9.4[abc]	90.6[bc]	75.8	37.1[e]	7.0[ab]	17.2[e]
T15 (0.50%+9.0 mg/kg+0.01 mg/kg).	0.399	9.6[ab]	90.4[bcd]	80.3	38.8[bcde]	7.0[ab]	26.3[bc]
T16 (0.50%+9.0 mg/kg+0.02 mg/kg).	0.398	9.6[ab]	90.4[bcd]	77.6	39.1[abcde]	7.0[ab]	22.1[bcde]
T17 (0.50%+12.0 mg/kg+0.01 mg/kg).	0.396	9.7[a]	90.3[cd]	80.3	40.2[abcd]	7.0[ab]	19.7[cde]
T18 (0.50%+12.0 mg/kg+0.02 mg/kg).	0.397	9.5[abc]	90.5[bcd]	80.5	39.8[abcd]	8.0[a]	20.9[bcde]
P value	0.71	0.01	0.01	0.07	0.002	0.001	<0.0001

[a,b,c,]…. etc. means in same column, within each factor with different superscripts are significantly ($P \leq 0.05$) different

supplemented M to the basal diet. Keshavarz (2003) and Hebert *et al.* (2005) found that there was no significant difference in egg production rate due to F supplementation. Kato *et al.* (2002) found that there was no effect on egg production due to B_{12} supplementation.

Egg weight: The differences in EWs were significant due to either M or F levels (Table, 2). However, no significant differences between high level of M (0.50%) and low level of M (0.40%) on EW (61.4 vs. 60.6). The same was observed with high and low F levels (60.7 and 60.9 g, respectively). More over, a significant difference in EW due to B_{12} levels (60.5 and 60.3 g, respectively) (Table, 2). Besides, the effect of M × F × B_{12} interaction on EW was significant ($p \leq 0.05$). The highest EW was recorded for T16 being 62.3 g and the lowest value of EW was recorded for T1 being 58.6 g. Obviously, the increasing in EW due to M supplementation may be due to the effect of M on increasing edible part of the egg and the relation among M × F × B_{12} gave an effect on EW especially, between M and F. These results were confirmed by Bateman *et al.* (2005), Liu *et al.* (2005), William *et al.* (2005) who reported that EW affected positively with increasing M level. Results obtained herein disagreed with those obtained by Amaefule *et al.* (2004) and Novak *et al.* (2004) who reported that no effect of M supplementation on EW. House *et al.* (2002) concluded that there was a difference in EW among F levels. Keshavarz (2003) found that reducing dietary F resulted in reducing EW. More over, Kato *et al.* (2002) found that EW increased due to B_{12} supplementation.

Feed consumption and conversion: No significant effect on the amount of feed consumed (g/hen/day) due to M, F and B_{12} levels and the results showed that FC decreased with increasing either M or F. Statistical analysis showed no significant differences in daily FC between the birds fed control diet and those fed the other diets. These results are in agreement with Abd-Elsamee (2005) Bateman *et al.* (2005) who reported that FC was not significantly affected by supplemented M. Hebert *et al.* (2005) found that no significant differences in FC due to F supplementation. Kato *et al.* (2002) found that there was no effect on FC due to B_{12} supplementation.

A significant difference ($p \leq 0.05$) of M levels on FCR was noticed, where, the FCR improved with increasing M level, being the best at 0.50% M level. No significant differences in FCR due to supplemental F and B_{12}. Obviously, FCR improved by increasing M, F and B_{12} levels. No significant differences were observed in the FCR due to M × F × B_{12} interactions. The differences of FCR may be attributed to different amounts of feed consumed and EM. These results are in agreement with those obtained by Liu *et al.* (2004 a,b), Abd-Elsamee (2005) and Bateman *et al.* (2005) who reported that FCR was improved by supplemental M. Hebert *et al.* (2005) indicated that no significant differences in FCR due to F supplementation. Kato *et al.* (2002) found that there was no effect on FCR due to B_{12} supplementation.

Relative economic efficiency: REE increased with increasing the levels of M, F and B_{12}. REE values varied 87-120%. The lowest values were listed for T1. Meanwhile, the highest values were listed for T18. The interaction between 0.40% M with F and B_{12} gave a slightly increased in REE in most treatments than diets including 0.45% M. Generally, it is recommended that the economic study was affected by different M, F and B_{12} levels, where increasing the dietary previous nutrients increased REE.

Digestibility coefficient: The differences among M levels were not significant in all nutrient digestibilities except for crude protein (CP) and crude fiber (CF) ($p \leq 0.05$) that improved with high M level compared with the other levels. Significant differences ($p \leq 0.05$) in CP, ether extract (EE) and CF due to F levels. 0.02 mg B_{12}/kg was significantly ($p \leq 0.05$) improved CP digestibility compared with 0.01 mg B_{12}/kg, while, 0.01 mg B_{12}/kg significantly improved CF digestibility compared with 0.02 mg B_{12}/kg (Table, 3). The effect of interactions of M, F and B_{12} were significant on all nutrient digestibilities except for dry matter (DM) and organic matter (OM) digestibilities. The data indicated that increase M level gave some improve in digestibilites of nutrients compared with other levels and addition of F and B_{12} to high M level slightly improved the digestibility of nutrients.

Energy utilization: Equations prediction of nutrients intake/egg energy ratio were calculated by different ways (Table 6).

Nutrient intake: Nutrients (M, F and B_{12}) intake were increased significantly ($P \leq 0.05$) with increasing the level of these nutrients (Table 5). The value of R^2 was high with equations predicted the nutrient intake/ egg energy ratio from nutrient intake (Table 6).

Nutrient intake/ feed energy ratio: Nutrients intake/ feed energy ratio were increased significantly ($P \leq 0.05$) with increasing the level of these nutrient (Table 5). The value of R^2 was high with equations predicted the nutrients intake/egg energy ratio from nutrient intake/feed energy ratio (Table 6).

Egg quality

Egg shell thickness: No significant effect due to M, F and B_{12} levels on ST including shell membrane, however, slightly improvement with low levels of either M or F (Table 4). The interaction of M × F × B_{12} on ST were not significant. Moreover, the differences between the control diet and the other experimental diets were not significant on ST. Amaefule *et al.* (2004) and El-Husseiny *et al.* (2005) concluded that no significant differences due to M levels on ST. Hebert *et al.* (2004) noticed that no significant differences in ST due to F supplementation. Although, Kato *et al.* (2002) observed that ST were higher for birds fed diet without B_{12} supplementation.

Egg shell percentage: No significant differences in ESP due to M, F and B_{12} levels with significant differences ($p \leq 0.05$) due to their interaction on ESP (Table 4). T1 and T17 recorded the highest values of ESP (9.7 %) vs. T4 that gave the least values (8.8%). Therefore, significant differences in ESP between the control treatment and T4 only. The percentage of egg shell was not affected by any of M, F and B_{12} levels, however, it was affected only by the interaction among M × F × B_{12} may be due to variation in EW. These findings agree with those reported by Amaefule *et al.* (2004) showed that supplemental M did not significantly influence egg shell weight. Novak *et al.* (2004) reported that egg quality (wet and dry shell percentages) was not affected by dietary M. Although, Kato *et al.* (2002) noticed smaller ESP with B_{12} supplementation.

Egg contents percentage: No significant differences in ECP among M, F and B_{12} levels were observed with significant differences ($p \leq 0.05$) in ECP due to M × F × B_{12} interactions (Table 4). T4 recorded the highest value of ECP (91.2%), while, T1 and T17 gave the least value ECP (90.3%). The percentage of EC was not affected by any of M, F and B_{12} levels, while, it was affected only by the interaction among M × F × B_{12}, this may be due to variation in EW. In this regard, Harms and Russell (2003) Novak *et al.* (2004) concluded that egg components (albumen % and yolk %) were affected by

Table 5: Efficiency of energy utilization

Experimental treatments	Feed energy (Kcal/g)[1]	Egg energy (Kcal/g)[2]	Nutrient intake M (mg/ hen/d)	Nutrient intake F (mg/ hen/d)	Nutrient intake B_{12}(µg/ hen/d)	(A)[3] M (mg)	(A)[3] F (mg)	(A)[3] B_{12} (µg)	(B)[4] M (mg)	(B)[4] F (mg)	(B)[4] B_{12} (µg)
M. effect:*											
0.40%.	2.78	1.57	426^{c}	9.6	16.0	11.1^{c}	0.250	0.418	12.7^{c}	0.283	0.473
0.45%.	2.78	1.57	478^{b}	9.5	15.9	12.5^{b}	0.250	0.418	14.3^{b}	0.285	0.477
0.50%.	2.78	1.57	524^{a}	9.4	15.7	14.0^{a}	0.250	0.447	15.0^{a}	0.269	0.449
P value			<0.0001	0.17	0.08	<0.0001	1.00	1.00	<0.0001	0.43	0.59
F. effect:*											
6.0 mg/kg.	2.78	1.57	478	6.4|^{c}	15.9	12.5	0.167^{c}	0.418	14.2^{a}	0.191^{c}	0.474
9.0 mg/kg.	2.78	1.57	476	9.5^{b}	15.9	12.5	0.250^{b}	0.419	13.9^{b}	0.277^{b}	0.463
12.0 mg/kg.	2.78	1.57	474	12.6^{a}	15.8	12.5	0.334^{a}	0.418	13.9^{b}	0.370^{a}	0.463
P value			0.60	<0.0001	0.41	1.00	<0.0001	1.00	0.01	<0.0001	0.69
B_{12} effect:*											
0.01 mg/kg.	2.78	1.57	476	9.5	10.6^{b}	12.5	0.250	0.278	14.1^{a}	0.280	0.313
0.02 mg/kg.	2.78	1.57	475	9.5	21.1^{a}	12.5	0.250	0.558	13.9^{b}	0.278	0.620
P value			0.91	0.76	<0.0001	1.00	1.00	<0.0001	0.01	0.60	<0.0001
M.xF.xB_{12} effect:**											
T0	2.78	1.55	482de	0.5^{e}	10.7^{d}	12.5^{b}	0.013^{d}	0.278^{b}	13.8^{bcd}	0.014^{e}	0.305^{d}
T1	2.78	1.57	432^{f}	6.5^{d}	10.8^{d}	11.1^{c}	0.168^{c}	0.278^{b}	13.9^{bcd}	0.209^{e}	0.347^{d}
T2	2.78	1.58	427^{f}	6.4^{d}	21.4^{ab}	11.1^{c}	0.167^{c}	0.557^{a}	12.8^{cd}	0.192^{cd}	0.641^{ab}
T3	2.78	1.56	418^{f}	9.5^{bc}	10.5^{d}	11.1^{c}	0.252^{b}	0.278^{b}	12.4^{cd}	0.282^{b}	0.311^{d}
T4	2.78	1.56	429^{f}	9.6^{b}	21.5^{ab}	11.2^{c}	0.250^{b}	0.559^{a}	11.9^{d}	0.267^{b}	0.597^{abc}
T5	2.78	1.56	425^{f}	12.7^{a}	10.6^{d}	11.2^{c}	0.334^{a}	0.278^{b}	12.3^{cd}	0.368^{a}	0.307^{d}
T6	2.78	1.58	423^{f}	12.7^{a}	21.2^{abc}	11.1^{c}	0.334^{a}	0.557^{a}	12.7^{cd}	0.380^{a}	0.634^{abc}
T7	2.78	1.57	483de	6.4^{d}	10.7^{d}	12.6^{b}	0.167^{c}	0.278^{b}	13.7^{bcd}	0.181^{cd}	0.303^{d}
T8	2.78	1.56	484de	6.5^{d}	21.6^{a}	12.5^{b}	0.168^{c}	0.557^{a}	14.5^{ab}	0.194^{cd}	0.646^{ab}
T9	2.78	1.56	482de	9.6^{b}	10.7^{d}	12.5^{b}	0.250^{b}	0.278^{b}	14.2^{abc}	0.284^{b}	0.316^{d}
T10	2.78	1.56	482de	9.6^{b}	21.4^{ab}	12.5^{b}	0.250^{b}	0.557^{a}	14.6^{ab}	0.290^{b}	0.647^{a}
T11	2.78	1.58	467^{e}	12.5^{a}	10.4^{d}	12.5^{b}	0.335^{a}	0.278^{b}	14.3^{abc}	0.382^{a}	0.318^{d}
T12	2.78	1.58	472de	12.5^{a}	20.8^{abc}	12.6^{b}	0.335^{a}	0.557^{a}	14.2^{abc}	0.377^{a}	0.627^{abc}
T13	2.78	1.57	529^{abc}	6.4^{d}	10.6^{d}	13.9^{a}	0.168^{c}	0.278^{b}	15.8^{a}	0.191^{cd}	0.316^{d}
T14	2.78	1.56	510^{c}	6.1^{d}	20.4^{c}	13.9^{a}	0.166^{c}	0.557^{a}	14.5^{ab}	0.174^{cd}	0.582^{c}
T15	2.78	1.58	513^{bc}	9.2^{c}	10.3^{d}	14.0^{a}	0.251^{b}	0.281^{b}	14.8^{ab}	0.265^{b}	0.296^{d}
T16	2.78	1.57	531^{ab}	9.5^{bc}	21.3^{ab}	13.9^{a}	0.250^{b}	0.559^{a}	15.0^{ab}	0.269^{b}	0.604^{abc}
T17	2.78	1.56	538^{a}	12.8^{a}	10.7^{d}	14.0^{a}	0.333^{a}	0.278^{b}	15.0^{ab}	0.357^{a}	0.298^{d}
T18	2.78	1.56	521^{abc}	12.5^{a}	20.9^{bc}	13.9^{a}	0.335^{a}	0.559^{a}	14.7^{ab}	0.353^{a}	0.591^{bc}
P value			<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

^{a,b,c},.... etc. means in same column, within each factor with different superscripts are significantly (P =0.05) different. [1]Feed energy kcal/ g calculated according to NRC (1994). [2]Egg energy Kcal/g calculated according to Merrill and Watt (1973). [3]Nutrient intake/ feed energy ratio (nutrient intake/ (feed energy per g * feed intake)). 4Nutrient intake/ egg energy (nutrient intake/ (egg energy per g * egg mass)). * M means methionine, F means Folic acid and B12 means cyanocobalamin. ** T0 (0.45%+0.50 mg/kg+0.01 mg/kg), T1 (0.40%+6.0 mg/kg+0.01 mg/kg), T2 (0.40%+6.0 mg/kg+0.02 mg/kg), T3 (0.40%+9.0 mg/kg+0.01 mg/kg), T4 (0.40%+9.0 mg/kg+0.02 mg/kg), T5 (0.40%+12.0 mg/kg+0.01 mg/kg), T6 (0.40%+12.0 mg/kg+0.02 mg/kg), T7 (0.45%+6.0 mg/kg+0.01 mg/kg), T8 (0.45%+6.0 mg/kg+0.02 mg/kg), T9 (0.45%+9.0 mg/kg+0.01 mg/kg), T10 (0.45%+9.0 mg/kg+0.02 mg/kg), T11 (0.45%+12.0 mg/kg+0.01 mg/kg), T12 (0.45%+12.0 mg/kg+0.02 mg/kg), T13 (0.50%+6.0 mg/kg+0.01 mg/kg), T14 (0.50%+6.0 mg/kg+0.02 mg/kg), T15 (0.50%+9.0 mg/kg+0.01 mg/kg), T16 (0.50%+9.0 mg/kg+0.02 mg/kg), T17 (0.50%+12.0 mg/kg+0.01 mg/kg) and T18 (0.50%+12.0 mg/kg+0.02 mg/kg).

dietary M levels. Hebert *et al.* (2004) and Hebert *et al.* (2005) reported that no significant differences in yolk weight due to F supplementation.

Haugh units: No significant differences of M, F and B_{12} levels on HU, while, it could be detected some improvement in HU with the low level (0.40%) compared with the other levels of M (0.45 and 0.50%) (Table 4). No significant differences in HU due to M × F × B_{12} interaction. Moreover, statistical analysis for HU values showed that no significant differences between control treatment and the other different experimental treatments. These results are in accordance with those reported by Amaefule *et al.* (2004) and Novak *et al.* (2004) indicated that HU were not affected by M level. El-Husseiny *et al.* (2005) and Hebert *et al.*, 2004) concluded that no significant differences due to F levels on HU. Kato *et al.* (2002) reported that there was no effect on HU values due to B_{12} supplementation.

Yolk index: Significant differences ($p \leq 0.05$) in YI between M levels, where YI increased with decreasing M level, while, no significant differences were noticed among F and B_{12} levels in YI (Table 4). Significant differences ($p \leq 0.05$) among treatments in YI were observed.

Blood parameters

Serum total immunoglobulin titres: No significant differences of M, F levels on STIT, while, a significant difference ($p \leq 0.05$) in STIT due to B_{12} supplementation (Table 4). Moreover, significant differences due to M × F

Table 6: Prediction of nutrient intake/ egg energy ratio by either nutrient intake or nutrient intake/ feed energy ratio

Y	NI* / egg energy ratio		
X	Methionine (mg)	Folic acid (mg)	B_{12} (µg)
NI*	Y= 2.6825+0.0237x	Y= -0.0028+0.029x	Y= -0.0036+0.0291x
R^2	0.8171	0.9892	0.9889
NI* / feed energy ratio	Y = 3.7285+0.8148x	Y = 0.0053+1.0898x	Y =0.0056+1.0992x
R^2	0.7776	0.9876	0.9833

* NI = nutrient intake

× B_{12} interactions on STIT, where, the highest value of STIT being 8.0 was recorded for T1, T3, T5, T6, T13 and T18 while, the lowest value being 6.0 for T2, T4, T9, T12, T0. Balnave (2000) reported that decreased STIT go parallel with increasing dietary M concentration.

Blood haemoglobin: Significant differences ($p \leq 0.05$) were detected of M, F and B_{12} levels on BH. BH increased with increasing either level of F or B_{12} that confirm the relation between F and B_{12} and BH (anemia). Medium level of M (0.45%) recorded the highest value of BH (24.7 g/dl) compared with either low or high levels that recorded 21.3 and 20.8 g/dl, respectively. Significant differences ($p \leq 0.05$) were noticed between treatments in BH where, the highest value was recorded for T12 (36.7 g/dl) and the least was recorded for T14 (17.2 g/dl). Significant differences among each of T12, T11 and T0 in BH (Table 4).

Conclusion: It may be concluded that the high level of M (0.50%) with F (12.0 mg/kg) and B_{12} (0.02 mg/kg) supplementation gave the best laying hen performance and REE compared with either medium or low level of M with F and B_{12} supplementation. It is possible to reduce the level of M from 0.45 down to 0.40% with adding vitamins F and B_{12} up to 12.0 and 0.02 mg/kg, respectively without any adverse effect on laying hen performance, REE and to improve the egg quality.

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