Choline and methionine supplementation in layer hens fed flaxseed: effects on hen production performance, egg fatty acid composition, tocopherol content, and oxidative stability

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ABSTRACT Choline is an essential nutrient in laying hen diets and is needed for the formation of phosphatidylcholine (**PC**), that serves as a rich source of long chain (≥20 C) n-3 fatty acids (**FA**) in eggs. Methionine (**Met**) is the first limiting amino acid in layer hen diets and serves as a lipotropic agent with antioxidant properties. The objectives of the current study is based on the hypothesis that choline and Met supplementation will enhance egg PC and n-3 FA status, lipid stability, and production indices in layer hens fed flaxseed. Ninety-six, 40-wk-old laying hens (W-36 White Leghorns) were randomly allocated to 4 treatment groups, with 6 replicates containing four hens per cage. Hens were fed corn-soybean meal-based diet containing 0% flaxseed (**Control**), 15/100 g flaxseed (**Flax**), Flax+50% more methionine requirement for W-36 White Leghorns (**Flax+Met**), or Flax+0.15g/100g choline chloride (**Cho**) (**Flax+Cho**). All experimental diets were isocaloric and isonitrogenous and fed for a period of 120 d. Egg production and egg mass (g/hen/d) was higher for Flax+Met and Flax +Cho when compared to Flax and Control ($P < 0.05$). Egg weight was greater ($P < 0.05$) among hens fed the Control and Flax+Cho diets compared to Flax diet. Feeding flaxseed to hens led to over 6-fold increase in total n-3 FA. Choline supplementation increased egg α-tocopherol content ($P < 0.05$) while reducing lipid oxidation products measured as thiobarituric acid reactive substances in egg yolk ($P < 0.05$). Neither Met nor Cho had any impact on docosahexaenoic (22:6 n-3) acid concentration in eggs from hens fed flaxseed. However, addition of Met and Cho to layer diets increased docosapentaenoic acid (22:5 n-3) levels in eggs from hens fed flaxseed ($P < 0.05$). The PC content was lower in Control and Flax+Met ($P < 0.05$) when compared to Flax+Cho group. No difference was found in total lipid or phosphatidylethanolamine content of eggs ($P > 0.05$). The results from the current study suggest that n-3 FA content of egg yolk can be greatly increased by feeding flaxseed but reduced egg production. However, dietary Met and Cho can improve production performance in hens fed flaxseed-containing diets. Addition of Cho to flaxseed increased in egg weight, yolk α-tocopherol levels, PC content and oxidative stability of eggs when compared to hens fed flaxseed. Met and choline could be used in flaxseed (>15%) to increase egg production and egg mas.

Key words: choline, methionine, hen, eggs, flaxseed, tocopherol

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INTRODUCTION

Flaxseed (*Linum usitatissimum L.*) is an oilseed that contains 34 to 35% oil. The ME value of flaxseed is 3,957 kcal/kg and the oil is rich in α-linolenic acid (**ALA**, 18:3 n-3) (40−50% of total fatty acids [**FA**]), which serves as the precursor of long chain (≥20 C) n-3 FA. Due to these nutritional values, flaxseed is the most common feed ingredient used in poultry diets for production of value-added n-3 FA-rich eggs and meat (Cherian, 2017). Moreover, flaxseed has several other phenolic compounds, including flavonoids and lignans that have significant antioxidant, anti-inflammatory and therapeutic effects (Oomah, 2001; Davis et al., 2016).

However, feeding flaxseed beyond 10% inclusion level to layers includes reduction in production performance and egg oxidative stability aspects (Scheideler and Froning, 1996; Cherian, 2017). The negative impacts on egg production were associated with antinutritional factors in the flaxseed impairing digestion and absorption of energy yielding nutrients ultimately decreasing dietary AMEn (Gonzalez-Esquerra and Leeson, 2001;

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Rodriguez et al., 2001). In this context, recent studies from our laboratory were aimed at using different strategies in enhancing the production efficacy and egg lipid oxidative stability in layer hens fed flaxseed (Westbrook and Cherian, 2019; Beheshti Moghadam et al., 2020a).

Choline is considered a member of the B complex vitamin group and is an essential nutrient in laying hen diets that is needed for the formation of phosphatidylcholine (**PC**), the main constituent of very low density lipoprotein (**VLDL**). Choline and PC are required for VLDL secretion and FA transport from hepatic tissue to eggs through blood. Choline supplementation in layer hen diets has been reported to increase choline and PC content in egg yolk (Tsiagbe et al., 1988). Recent research from our laboratory in supplementing choline in flaxseed or microalgae (rich in long chain 20C n-3 FA)-containing diets led to a significant enhancement in oxidative stability and antioxidant status in hen liver and eggs (Aziza et al., 2019; Yonke and Cherian, 2019).

Methionine (**Met**) is an essential sulfur-containing amino acid and is the first limiting amino acid in hens fed corn-soybean based diets. Methionine acts as a lipotropic agent and is the precursor of glutathione, protecting cells against oxidative stress (Li et al., 2007). Methionine supplementation in broilers fed n-3 FA-rich diets led to significant increase in n-3 FA content in muscle tissue while enhancing lipid oxidative stability (Beheshti Moghadam et al., 2017; Khan et al., 2021). It is hypothesized that supplementing Met and Cho to hen diets containing flaxseed at 15% level would improve egg phospholipid and n-3 FA composition and lipid oxidative stability. The objectives of the current study were to investigate the effect of Cho and Met on hen production performance, egg quality aspects, FA composition, phospholipid composition, tocopherol (**Toc**) content, and oxidative status of laying hens fed diets containing ALA-rich flaxseed.

MATERIALS AND METHODS

Ethics Approval

An institutional animal care and use committee approved all experimental protocols to ensure adherence to Animal Care Guidelines. (IACUC approval number # 4931).

Birds, Diet, and Housing

A total of ninety six, 40-wk-old laying hens (W-36 commercial White Leghorns) were kept in individual cages (46 cm x 53 cm x 58 cm) (length × width × height) and were randomly allocated to 4 treatment groups (24 cages per each treatment). Four adjacently located cages were considered as one replicate, and 6 replications were made for each of the dietary treatments. The hens were fed corn-soybean meal-based diet containing 0% flaxseed (Control), 15% flaxseed (**Flax**), Flax+ 50% more W-36 White Leghorn's Met requirement (**Flax+Met**), and Flax +0.15 g/100g choline chloride (**Flax+Cho**). All the diets were isocaloric and isonitrogenous. The ingredient content and analyzed nutrient composition of the diets are shown in Table 1. Gross energy and crude protein content of the experimental diets were analyzed at the Center of Excellence for Poultry Science Central Analytical Laboratory of the University of Arkansas (Fayetteville, AR). The laying hens did not receive any vaccines or drugs during the entire experimental period. The birds were maintained on a 16L: 8D photoperiod in an environmentally controlled facility under standard conditions of temperature and ventilation as per University Poultry Farm standard operating procedures. The diets were prepared biweekly and kept in a cold room (4°C) in air-tight containers. Hens were fed the experimental diets for a period of 120 d. Water and feed were provided ad libitum.

Table 1. Ingredient content and calculated analysis of experimental diets.

	Dietary treatment[1]			
Ingredients (g/100 g)	Control	Flax	Flax+Met	Flax+Cho
Corn	60.70	53.27	53.60	52.80
Soybean meal	22.73	17.95	17.43	18.02
Flaxseed	0.00	15.00	15.00	15.00
Common salt	0.39	0.37	0.37	0.37
Vitamin-mineral premix[2]	0.23	0.23	0.23	0.23
Limestone	10.80	10.86	10.86	10.86
Monocalcium phosphate	1.86	1.58	1.58	1.58
DL-methionine	0.13	0.12	0.51	0.12
Vegetable oil	3.14	0.59	0.40	0.76
Choline	0.00	0.00	0.00	0.15
Calculated composition				
ME, kcal/kg	2,900	2,900	2,900	2,900
CP, (g/100 g)	16.50	16.50	16.50	16.50
Ca, (g/100 g)	4.48	4.48	4.48	4.48
Available P	0.49	0.49	0.49	0.49
Lysine (g/100 g)	0.81	0.78	0.78	0.78
Analyzed values				
Gross energy, kcal/kg	3,640	3.663	3.722	3,688
CP (%)	16.80	16.50	16.80	16.40
Fatty acids (%)				
Palmitic acid	12.17	7.33	7.21	7.16
Palmitoleic acid	0.84	0.43	0.43	0.50
Stearic acid	4.35	2.54	2.54	2.34
Oleic acid	24.63	22.04	23.11	24.92
Linoleic acid	54.84	29.29	28.13	27.27
α-Linolenic acid	2.27	37.90	38.58	37.72

[1]Control, Flax, Flax+Met, and Flax+Cho, represent corn-soybean meal basal diet containing 0% flaxseed, (Control), 15 g/100 g flaxseed (Flax), Flax+ 50% more Met (Flax+Met), and Flax+0.15/100 g choline chloride (Flax+Cho), respectively.

[2]Supplied per kilogram of feed: vitamin A, 12,500 IU; vitamin D3, 4,000 IU; vitamin E, 25 IU; vitamin B12, 0.014 mg; riboflavin, 8 mg; pantothenic acid, 12 mg; niacin, 40 mg; menadione, 2.5 mg; choline, 500 mg; thiamine, 1.75 mg; folic acid, 0.75 mg; pyridoxine, 2 mg; d-biotin, 0.15 mg; ethoxyquin, 2.5 μg; Mn, 90.4 mg; Zn, 92.4 mg; Se, 0.264 mg. The values reported for feed fatty acid analyses are mean of two samples, n = 2.

Hen Production Parameters and Egg Physical Quality Characteristics

Total feed consumption (biweekly) and daily egg production was monitored during the trial. Egg production

is expressed as average hen-day production, calculated from the total eggs divided by the total number of days and hens. For egg quality characteristics and lipid analytical aspects, 24 eggs (4 eggs from each replicate) from each treatment were collected every four weeks (44, 48, 52, and 56 wk of hen age) on the experimental diet. The eggs were weighed, and yolks were separated using an egg separator and were rolled on wet paper to remove any albumen and were then weighed. Albumen height was recorded. Haugh unit (**HU**) ([Haugh, 1937) (AMES, S-6428, Framingham, MA) was determined. The HUs were calculated by the formula HU = 100 log (H + 7.57 − 1.7 W0.37), where H is the average albumen height (mm) and W is the weight of the egg (g). Egg shells were rinsed with distilled water and dried in an oven before weighing and measurement of thickness twice on opposite sides of the midline with a digital micrometer (iGaging, Digital Caliper Gage, San Clemente, CA). Yolk color was determined by comparing yolk color to the Roche color fan (Basel, Switzerland). Albumen weight was calculated by subtracting yolk and shell weight from total egg weight and yolk:albumen ratio was determined. Yolk height and width were measured using an electronic caliper (Calipro 150 mm Digital Caliper) and yolk index was calculated as follows, yolk index = yolk height (mm)÷yolk width (mm).

Sample Analysis

Total Lipid and Phospholipid Analysis Eggs collected at 48 wk of hen age were analyzed for total lipids, PC, and PE contents estimation. The PE and PC contents of egg yolk were determined as per Chen and Kou (1982), with modifications described in Balazs et al. (1996). Phospholipid classes were separated and quantified using a Shimadzu LC-2010 HT high performance liquid chromatograph with an LC-2010 AHT High Speed Autosampler and a Shimadzu RF-535 fluorescence detector (Shimadzu, Columbia, MD). A Cosmosil 5SL-II, 250 × 4.6 mm packed column and guard column were used (Nacalai Tesque Inc., Japan). The mobile phase was acetonitrile:methanol: phosphoric acid (100:30:0.05 vol/vol/vol), oven temperature set at 40°C, the flow rate 1 mL/min., and wavelength 205 nm. Twenty five mL of lipid extract was evaporated under nitrogen gas and resuspended in 800 μL of methanol. Samples were passed through 0.45 μm nylon membrane syringe filters and 10 μL injected into the chromatograph for analysis. Standard curves were generated with a polar lipid mixture (1127, Matreya, Inc., PA).

FA Analysis Eggs collected at 48 and 56 wk of hen age were analyzed for FA analysis. Four egg yolks from each treatment replicate collected for egg quality were pooled and homogenate aliquot (~5 g) was taken for total lipid extraction and FA analyses. Total lipids were extracted from egg yolk using chloroform: methanol (2:1) Folch et al. (1957) and total lipids were determined gravimetrically. FA methyl esters were prepared from lipid extracts using boron trifluoride methanol as the derivatizing agent. FA methyl esters were analyzed using an HP 6890 gas chromatograph (Hewlett-Packard Co., Wilmington, DE) equipped with an autosampler, flame ionization detector, and SP-2330 fused silica capillary column (30 mm × 0.25 mm i.d). Conditions of the gas chromatograph were as previously reported (Apperson and Cherian, 2017). FA methyl esters were identified and quantified by comparison using authentic internal or external standards as reported earlier (Yonke and Cherian, 2019).

Thiobarbituric Acid Reactive Substances Lipid peroxidation in egg yolks was evaluated by estimating malondialdehyde (**MDA**) concentration, a thiobarbituric acid reactive substance (**TBARS**). Egg samples collected at 48 wk of hen age were taken for MDA analysis. Yolk samples (2 g) were homogenized with 3.86% perchloric acid. Butylated hydroxytoluene (50 μL in 4.5% EtOH) was added to each sample during homogenization to control lipid oxidation. The homogenate was filtered and the filtrate was mixed with 20 mM TBA in distilled water and incubated (Cherian et al., 2002). Two duplicates of each sample were averaged for data analysis. Tetraethoxypropane was used as standard. Absorbance was determined at 531 nm. TBARS were expressed as mg MDA/g of egg yolk.

Tocopherols Tocopherols were extracted from egg yolk and feed samples by method described by Podda et al. (1996). Egg samples collected at 48 wk of hen age were analyzed for Toc analysis. Briefly, ~50 mg of egg yolk samples were mixed with 1.5 mL saturated potassium hydroxide, 5 mL water with 1% ascorbic acid and 10 mL ethanol and incubated at 70°C for 30 min, extracted with *n*-hexane, dried under nitrogen, resuspended in 1:1 ethanol:methanol, then injected into an HPLC system. A Shimadzu LC-10AD VP HPLC system was used with a Shimadzu SIL-10AD VP Auto Injector. A C18, 4.6 × 100 mm, 3 μM, isocratic 1 mL/min column was used, with 99% methanol as a mobile phase at a 1.0 mL/min flow rate. A Shimadzu Prominence UFLC (Shimadzu USA MFG, Inc., Columbia, MD) with fluorescence detection was used to quantify Vitamin E as α-Toc. Excitation and emission wavelengths were 295 and 325 nm, respectively. Resulting values were compared to a standard curve generated with α- (T3251) and γ- (T1782) tocopherol standards (Sigma-Aldrich Corp., St. Louis, MO) and are reported as μg/g.

Statistical Analysis

Variables measured multiple times during the study (hen production performances, egg quality, and FA composition) were analyzed as by two-way ANOVA. Diet and week were the main factors. The effects of diet on egg total lipids, PC, PE, MDA, and Toc content were analyzed by one-way ANOVA. Significant differences among treatment means were analyzed by Tukey's HSD test at $P \leq 0.05$ (Steel and Torrie, 1980). Cage was considered as an experimental unit for production performance and eggs collected from each replicate cage was

Table 2. Effect of methionine and choline supplementation on hen production performance and quality characteristics of eggs from hens fed flaxseed-based diets.

	Dietary treatments[1]					*P* value		
Egg variables	Control	Flax	Flax+Met	Flax+Cho	Pooled SEM	Diet	Week	Diet x Week
Egg weight, g	58.43[a]	57.35[b]	57.19[b]	58.32[a]	0.32	0.007	<0.0001	0.0004
Egg mass, g/hen/d	153.60[b]	141.53[c]	166.75[a]	167.5[a]	2.31	<0.0001	0.0002	0.002
Hen day egg production	65.10[b]	59.66[c]	71.89[a]	70.63[a]	0.98	<0.0001	0.0003	0.010
Feed consumption, g	100.48[b]	92.09[c]	110.03[a]	109.90[a]	2.89	<0.0001	0.489	0.980
Yolk weight, g	18.31	17.90	18.16	18.59	0.01	0.226	<0.0001	0.562
Shell weight, g	5.50	5.34	5.59	5.454	0.01	0.564	0.223	0.548
Shell thickness, mm	42.26[a]	39.78[c]	41.71[ab]	40.75[bc]	0.01	<0.0001	0.152	0.102
Yolk color	7.86	7.68	7.76	7.87	0.01	0.249	0.003	0.030
Yolk height	19.01[b]	19.67[a]	19.94[a]	19.64[a]	0.01	<0.0001	0.002	0.009
Yolk width	43.83[a]	42.74[b]	42.55[b]	43.01[b]	0.01	0.003	0.001	0.058
Albumen thickness	6.17	6.12	6.20	6.08	0.01	0.812	0.184	0.037
Albumen weight, g	35.73[a]	36.84[a]	33.25[b]	35.68[a]	0.02	0.009	0.440	0.334
Yolk (%)	30.72	29.77	30.26	31.14	0.02	0.356	0.044	0.677
Albumen (%)	166.94[a]	163.09[b]	167.57[a]	167.60[a]	0.03	0.042	0.018	0.523
Haugh unit	77.85	77.19	79.45	77.09	0.02	0.273	0.309	0.054
Yolk:albumen	0.51	0.48	0.51	0.52	0.002	0.176	0.016	0.636
Yolk index	0.43[c]	0.46[ab]	0.47[a]	0.46[b]	0.001	<0.0001	<0.0001	<0.0001

[a-c]Means within a row with no common superscripts differ significantly ($P \leq 0.05$).

[1]Control, Flax, Flax+Met, and Flax+Cho, represent corn-soybean meal basal diet containing 0% flaxseed, (Control), 15/100 g flaxseed (Flax), Flax+50% more Met (Flax+Met), and Flax+0.15/100 g choline chloride (Flax+Cho) respectively, n = 6.

considered as an experimental unit for all other analysis. Computations were done using the General Linear Models procedure of the SAS 9.4 (SAS Institute Inc, 2016). Mean values and SEM were reported.

RESULTS

Feed Analysis

The formulations and chemical composition of experimental diets are shown in Table 1. Inclusion of flaxseed increased the ALA content of feed from 2.27 to >37% of total FA in Flax, Flax+Met and Flax+Cho diets. The increase in ALA content in the flaxseed-based diets, led to a reduction in the concentrations of linoleic, stearic, palmitic acid, and palmitoleic acids compared to Control diet.

Hen Production Parameters and Egg Physical Quality Characteristics

Production performance and egg quality characteristics of eggs from hens fed experimental diets are shown in Table 2. Addition of Cho led to flaxseed diet increased in egg weight ($P < 0.05$) compared to Control ($P > 0.05$). Hen-day egg production and egg mass (g/hen/d) were higher for Flax+Met and Flax+Cho when compared to Flax and Control ($P < 0.05$). Inclusion of Met and Cho in flaxseed-based diets led to over 10% increase in hen-day egg production when compared to Flax ($P < 0.05$). Feed consumption was higher in Flax+Met and Flax+Cho diets compared to Control and Flax ($P < 0.05$). Egg yolk height was higher in Flax, Flax+Met and Flax+Cho than Control ($P < 0.05$) while egg yolk width was reduced in eggs from hens fed flaxseed-based diets ($P < 0.05$). Albumen as percent of egg weight was lowest in Flax when compared to the other 3 treatments ($P < 0.05$). No effect of diet on physical egg quality characteristics such as yolk weight, shell weight, yolk color, albumen thickness, albumen weight, HU, and yolk:albumen ratio was observed ($P < 0.0.05$). No mortality was noticed among the birds during the experimental period.

Egg Total Lipid and Phospholipid Composition

Egg total fat content was not different across treatments ($P > 0.05$; Table 3). Egg PC content was higher in Flax+Cho than Flax+Met ($P < 0.05$) and was not different from Control and Flax ($P > 0.05$). There was no effect of diet in egg PE, PC+PE or PC:PE was noticed among the treatments ($P > 0.05$).

The Egg FA Composition, Lipid Peroxidation Products, and Tocopherol Content

FA composition of eggs is shown in Table 4 (percentage of total FA methyl esters) and Figures 1 A and 1B (mg per egg). Egg palmitic acid was higher in Control than Flax and Flax+Cho ($P < 0.05$) and was not different from Flax+Met. There was no effect of diet on palmitoleic, stearic, oleic, and linoleic acid ($P > 0.05$). An increase in yolk ALA content was observed in hens fed flaxseed-containing diets compared to Control diet ($P < 0.05$). Total ALA and DHA was higher ($P < 0.05$) in flaxseed-containing diets (209−255 vs. 60 mg and 13.38 vs. 76−93 mg/egg) compared to Control (Figure 1B). Long chain n-3 polyunsaturated FA such as, EPA and DPA was higher in Flax+Met and Flax +Cho when compared to Flax ($P < 0.05$). The DHA content was not influenced by the supplementation of Met or Cho ($P > 0.05$). Over 2-fold reduction in arachidonic acid (20:4 n-6) was observed in eggs from hens fed flaxseed-containing diets ($P < 0.05$), although there

Table 3. Effect of methionine and choline supplementation on total lipids and phospholipid classes in egg yolk from hens fed flaxseed-based diets

	Dietary treatments[1]				
Egg lipids	Control	Flax	Flax+Met	Flax+Cho	*P* value
Total lipids (g/100 g)	28.10	27.11	27.75	26.73	0.201
Phosphatidylcholine (PC) (μg/mg)	47.51[b]	53.51[ab]	44.18[b]	57.06[a]	0.010
Phosphatidylethanolamine (PE) (μg/mg)	30.37	30.56	24.78	25.86	0.294
PC+PE	77.88	84.07	68.97	82.92	0.189
PC:PE	1.56	1.75	1.78	2.23	0.081

[ab]Means within a row with no common superscript differ significantly ($P \leq 0.05$).

[1]Control, Flax, Flax+Met, and Flax+Cho, represent corn-soybean meal basal diet containing 0% flaxseed, (Control), 15/100 g flaxseed (Flax), Flax+ 50% more Met (Flax+Met), and Flax+0.15/100 g choline chloride (Flax+Cho) respectively, n = 6.

was no effect of diet on linoleic acid content in eggs ($P > 0.05$). Supplementing Cho to flaxseed diet led to reduction in total saturated FA in eggs (>260 mg less) in eggs compared to Control diet ($P < 0.05$). However, no effect of diet on egg total monounsaturated FA content was observed ($P > 0.05$). Supplementing Met or Cho flaxseed diets had no effect on total n-6, n-3 FA, or n-6:n-3 FA ($P > 0.05$). Overall, total n-3 FA in eggs contributed over 337 to 363 mg in eggs from hens fed flaxseed and was not influenced by Met or Cho supplementation ($P > 0.05$). Choline supplementation led to a significant increase in

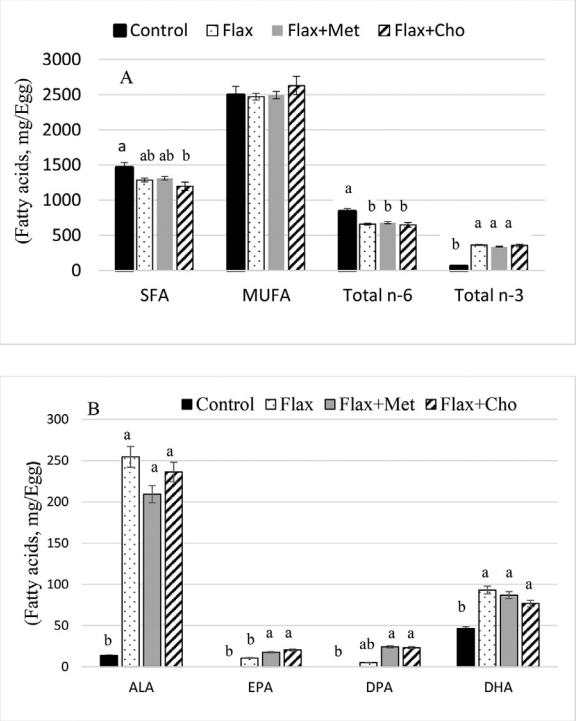

Figure 1. Effect of methionine and choline supplementation on fatty acid content of eggs from hens fed flaxseed-based diets**.** Control, Flax, Flax+Met, and Flax+Cho, represent corn-soybean meal basal diet containing 0% flaxseed, (Control), 15/100 g flaxseed (Flax), Flax+ 50% more Met (Flax+Met), and Flax+0.15/100 g choline chloride (Flax+Cho) respectively. n = 6. a−b with no common superscript on each bar cluster differ significantly ($P \leq 0.05$). Egg weight was 58.4, 57.3, 57.2, and 58.3 g for Control, Flax, Flax+Met, and Flax+Cho, respectively.

Table 4. Effect of methionine and choline supplementation on fatty acid composition of eggs from hens fed flaxseed-based diets.

	Dietary treatments[1]					*P* value		
Total fatty acids, (%)	Control	Flax	Flax+Met	Flax+Cho	Pooled SEM	Diet	Week	Diet × Week
14:0	0.14[bc]	0.01[c]	0.24[ab]	0.29[a]	0.01	0.001	0.078	0.004
16:0	24.14[a]	21.92[b]	22.74[ab]	22.05[b]	0.03	0.004	<0.0001	0.355
16:1	2.88	2.88	2.88	2.57	0.02	0.857	0.677	0.927
18:0	5.85	4.98	4.30	2.49	0.04	0.120	0.320	0.128
18:1	48.58	48.69	48.52	51.62	0.05	0.401	0.214	0.607
18:2 n-6	13.46	12.42	12.42	12.07	0.03	0.254	0.874	0.841
18:3 n-3	0.29[c]	5.34[a]	4.33[b]	4.90[ab]	0.02	<0.0001	<0.0001	0.092
20:1	0.02[b]	0.04[b]	0.16[a]	0.16[a]	0.01	0.0003	<0.0001	0.0003
20:2 n-6	1.06	0.19	0.30	0.14	0.03	0.514	0.358	0.470
20:3 n-6	0.00	0.00	0.06	0.06	0.01	0.197	0.033	0.197
20:4 n-6	2.58[a]	1.18[b]	1.34[b]	1.15[b]	0.01	<0.0001	<0.0001	0.014
20:5 n-3	0.00[c]	0.22[b]	0.36[ab]	0.42[a]	0.01	<0.0001	0.005	<0.0001
22:4 n-6	0.00	0.00	0.02	0.02	0.01	0.572	0.168	0.572
22:5 n-6	0.05	0.00	0.00	0.00	0.01	0.074	0.122	0.074
22:5 n-3	0.00[b]	0.10[b]	0.50[a]	0.48[a]	0.01	<0.0001	<0.0001	<0.0001
22:6 n-3	0.95[b]	1.93[a]	1.81[a]	1.57[a]	0.01	<0.0001	<0.0001	0.613
Total SFA	30.13[a]	26.99[ab]	27.28[ab]	24.83[b]	0.04	0.027	0.012	0.293
Total MUFA	51.47	51.62	51.57	54.35	0.05	0.419	0.174	0.523
Total n-6 FA	17.16[a]	13.79[b]	14.14[b]	13.44[b]	0.03	0.0002	0.979	0.751
Total n-3 FA	1.24[b]	7.59[a]	7.00[a]	7.38[a]	0.02	<0.0001	<0.0001	0.001
n-6:n-3	14.07[a]	1.83[b]	2.09[b]	1.88[b]	0.05	<0.0001	0.419	0.316
LC n-6 FA	3.69[a]	1.37[b]	1.72[b]	1.37[b]	0.03	0.0063	0.856	0.903
LCn-3 FA	0.95[b]	2.25[a]	2.67[a]	2.48[a]	0.01	<0.0001	0.671	0.009

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids.

Total SFA = 14:0 + 16:0 + 18:0 + 20.0); Total MUFA = 16:1 + 18:1 + 20:1); Total n-6 FA (18:2 n-6 + 20:4 n-6 + 22:4 n-6 + 22:5 n-6); Total n-3 FA (18:3 n-3 + 20:5 n-3 + 22:5 n-3 + 22:6 n-3); LC n-6 and LC n-3 FA = ≥20C n-6 or n-3 fatty acids.

[a-c]Means within a row with no common superscripts differ significantly ($P \leq 0.05$). N = 6.

[1]Control, Flax, Flax+Met, and Flax+Cho, represent corn-soybean meal basal diet containing 0% flaxseed, (Control), 15/100 g flaxseed (Flax), Flax+ 50% more Met (Flax+Met), and Flax+0.15/100 g choline chloride (Flax+Cho) respectively.

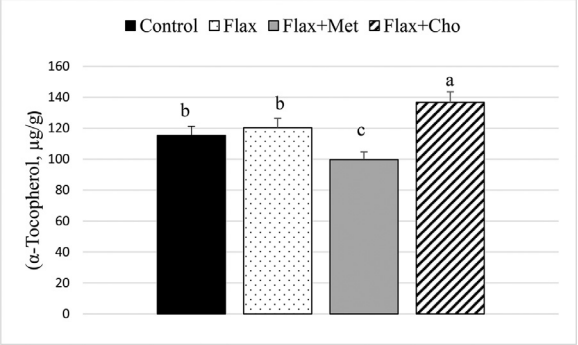

Figure 2. Effect of methionine and choline supplementation on tocopherol content of eggs from hens fed flaxseed-based diets. Control, Flax, Flax+Met, and Flax+Cho, represent corn-soybean meal basal diet containing 0% flaxseed, (Control), 15/100 g flaxseed (Flax), Flax+ 50% more Met (Flax+Met), and Flax+0.15 /100 g choline chloride (Flax+Cho) respectively. n = 6. a−c with no common superscript on each bar differ significantly ($P \leq 0.05$).

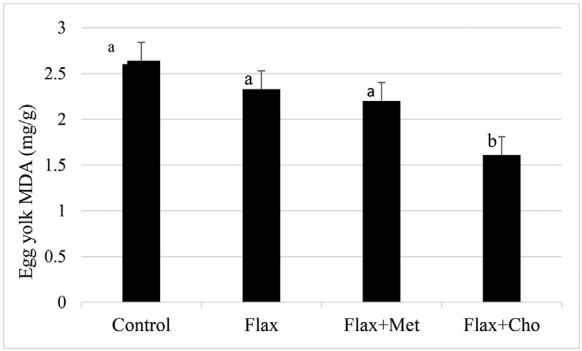

Figure 3. Effect of methionine and choline supplementation on lipid oxidation products measured as thiobarbituric acid reactive substances in eggs from hens fed flaxseed-based diets. Control, Flax, Flax +Met, and Flax+Cho, represent corn-soybean meal basal diet containing 0% flaxseed, (Control), 15/100 g flaxseed (Flax), Flax+ 50% more Met (Flax+Met), and Flax+0.15 /100 g choline chloride (Flax+Cho) respectively. n = 6. a−b with no common superscript on each bar differ significantly ($P \leq 0.05$). Abbreviation: MDA, malondialdehyde.

yolk α-Toc content when compared to Control, Flax, or Flax+Met treatments ($P < 0.05$; Figure 2). The oxidative stability of eggs based on TBARS revealed a significant effect of diet (Figure 3). Eggs from the Flax+Cho regime had lower TBARS than those from Control, Flax, or Flax+Met ($P < 0.05$).

DISCUSSION

Egg enrichment with n-3 FA offers an alternate route to meet the human need of n-3 FA (Fraeye et al., 2012; Beheshti Moghadam and Cherian, 2017). In the current study, supplementing Met and Cho led to significant improvement in hen day egg production and egg mass when flaxseed was added to the diet at 15% level. It should be mentioned that supplementing Met and Cho increased feed intake and thus the increase in availability of Met and Cho may have contributed to the improvement in egg production. Role of Met and Cho in layer diets in enhancing feed consumption, egg mass, egg yolk weight, and egg production has been reported previously (Parsons and Leeper, 1984; Uzu et al., 1993; Zhai et al., 2013). However, positive effects associated with Met and Cho supplementation in layer hens fed diet flaxseed-based diets are not reported. Met is the first limiting amino acid in hens fed corn-soybean meal based diets and as an essential amino acid, it plays a key role in protein synthesis. Methionine can also serve as a methyl donor affecting choline and PC synthesis, the major phospholipid in egg. In this context, a recent study by Yonke and Cherian (2019) provided the evidence that supplementing 0.1% Cho in layer hens fed diets containing microalgae increased hen day egg production. Choline is an essential nutrient in laying hen diets and as a methyl donor it is involved in phospholipid synthesis and lipid transport to the egg yolk. Cho or Met supplementation had minimal effects on other egg quality parameters investigated.

The FA profile of eggs from the current study support previous studies about feeding flaxseed to layer hens (Jiang et al., 1991; Westbrook and Cherian, 2019; Beheshti Moghadam et al., 2020a). Proportions of ALA accounted about 5% of total n-3 FA (>15-fold increase compared to Control) in hens fed Flax, Flax+Met and Flax+Cho diets, respectively. This is not surprising as flaxseed-based diets contained 38% ALA when compared to 2.23% in Control diet. The observation that long chain n-3 FA such as EPA, DPA, and DHA were significantly higher in eggs from hens fed Flax, Flax +Met, and Flax+Cho diets shows hens utilized available precursor ALA for EPA and DHA biosynthesis through activity of hepatic FA desaturases and elongases (Brenner, 1971). It is interesting to note that that supplementing Met and Cho increased DPA, although no effect on DHA was observed in Flax+Met and Flax+Cho eggs. The n-3 FA deposition in the egg is a consequence of dietary FA digestibility, in vivo metabolism and substrate availability. Notably a recent study from our laboratory showed that supplementing extra Met (>100% of Cobb requirement) increased CP and essential FA digestibility in broiler birds fed flaxseed (Beheshti Moghadam et al., 2020b). As an essential sulfur-containing amino acid, Met is the first limiting amino acid in poultry fed corn-soybean meal based diets. Similarly, Wang et al. (2017) reported a significant increase in egg yolk DHA of hens fed microalgae-based diets containing 1,000 mg of choline. Further, both Met and choline function as lipotropic agents and are associated with PC biosynthesis from PE (Ridgway and Vance, 1992) and lipid transport through VLDL into the egg yolk. In the current study, we observed a significant ($P < 0.05$) increase in PC content and a trend ($P = 0.08$) in the increase in PC:PE in the egg yolk of Flax+Cho when compared to Control diets. As PC, being the major phospholipid in eggs, extra availability of Cho may have contributed to the significant increase in egg weight and egg mass in Flax+Cho eggs. The increase in egg yolk n-3 FA led to a concomitant reduction in n-6:n-3 FA ratio in Flax, Flax+Met, and Flax+Cho eggs. These results are in agreement with our previously published results (Cherian and

Sim, 1991; Hayat et al., 2009). Simopoulos (2000) suggested that the optimal dietary n-6:n-3 FA is between 4 and 10. In the current study, n-6:n-3 FA of eggs from Flax, Flax+Met, and Flax+Cho eggs ranged from 1.83 to 2.09 when compared to 14.07 in hens fed the Control diet. Increasing the dietary intake of ALA and long chain n-3 FA remains a priority as they have been reported to have several health-promoting effects (Yashodhara et al., 2009; Jump et al., 2012). Overall, total n-3 FA were over 6-fold (60 vs. >356 mg/egg) higher in hens fed Flax, Flax+Met, and Flax+Cho when compared to Control diets (Figure 1). The typical American intake of ALA and long chain n-3 FA is 1.3 and 0.1 g/day and the recommended intake is 2.2 and 0.65 g/d (Kris-Etherton et al., 2003, 2009; Richter et al., 2017). Consuming a serving of 2 eggs from Flax, Flax +Met, and Flax+Cho will provide over 700 mg total n-3 FA and 250 mg of long chain n-3 FA compared to 120 mg total n-3 FA, and 92.0 mg long chain n-3 FA from the hens fed the Control diet.

Feeding layer hens with Cho supplemented diets increased egg yolk α-Toc content and oxidative stability of egg yolk as shown by the reduction in TBARS (Figures 2 and 3). This response to reduction in lipid peroxidation through choline supplementation may be through its role as a methyl donor, which may indirectly increase antioxidant capacity (Li et al., 2014). It has been reported that hydroxy amines in the side chains of choline inhibited lipid peroxidation providing an antioxidant function (Dong et al., 2019). As a methyl group donor, choline reduces NADPH that can be used to regenerate glutathione and ultimately vitamin E, which stabilizes peroxidized lipids. A similar decrease in TBARS along with an increase in Toc content was observed in egg yolk from hens fed microalgae-based diets supplemented with Cho (Yonke and Cherian, 2019). Notably, a similar increase in hepatic α-Toc content was observed in hens fed flaxseed-containing diets supplemented with 0.15% choline chloride (Aziza et al., 2019).

Overall, the results from the current study demonstrates that supplementing flaxseed leads to over 6-fold increase in n-3 FA content of eggs. and supplementing Met and Cho can improve egg production in hens fed flaxseed. Met and Cho had no effect on n-3 FA deposition in egg yolk. The positive effects of Met and Cho in hens fed flaxseed containing diets were associated increased feed intake, egg production and egg mass. Cho supplementation also increased Toc content, and oxidative stability in eggs from hens fed flaxseed.

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DISCLOSURES

The authors declare that there is no conflict of interests.

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