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Production of Omega-3 Fatty Acid Enriched Eggs Using Pearl Millet Grain, Low Levels of Flaxseed and Natural Pigments

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Abstract: We have previously reported that pearl millet (PM) could substitute corn and reduce the amount of flaxseed (8%, FS) needed to produce omega-3 enriched eggs in a 6 week trial, but reduced yolk pigmentation. In this experiment we evaluated egg fatty acid (FA) profile, yolk pigmentation, laying performance, and liver integrity in a 12 week experiment using PM-based diets with lower levels of FS (4, 6 and 8%) and natural pigments (PG, 0.1% and 0.2%) in a factorial arrangement of treatments (six cage replicates per treatment). Diets were formulated to be isocaloric and isonitrogenous and to meet or exceed NRC requirements. Egg number and egg mass produced were measured and recorded on a daily basis, whereas BW and feed consumption measurements were recorded every two weeks. At the end of each two week period, three eggs were collected from each cage to measure egg trait parameters and then yolks were separated, pooled and lyophilized for FA determination by Gas Chromatography. At the end of the experiment, all the hens were euthanized to determine liver integrity. Egg traits and flock performance parameters, were not different among treatments, except in week 8, 10 and 12, when birds fed PM-based diets including 8% FS produced smaller ($P < 0.05$) eggs than hens fed 4% FS. The inclusion of the PG at 0.1% restored yolk pigmentation to marketable levels (above 7 on the Roche® color fan scale). In summary, birds fed a diet containing PM as the sole grain source and 6% FS, consistently produced eggs with more than 350 mg/egg of n-3 FA, which is the lower standard to market eggs as "omega-3 enriched", whereas hens fed the diet containing 8% FS produced eggs with about 500 mg/kg of n-3 FA. Liver integrity was not affected by dietary treatment. Thus, PM based diets with levels as low as 6% of FS and low levels of natural PG (0.1%) can be used to produce n-3 FA enriched eggs, preserving egg quality and restoring yolk color, and maintaining hen health and productive performance.

Key words: Laying hens, pearl millet, flaxseed, natural pigment, omega eggs

Introduction

The n-3 and n-6 poly unsaturated fatty acids (PUFA) are essential in human diets. The alpha-linolenic acid (LNA, C 18:3 n-3), representative of the n-3 fatty acid (FA) family, is found in considerable quantities in some oil seeds, such as canola and flaxseed (FS). Linoleic acid (LA, C 18:2 n-6) is the most important FA of the n-6 series and is abundant in vegetable oils, such as sunflower, corn, soybeans and cottonseed oils (Dziezak, 1989). Recent studies have shown that n-3 FA may prevent cardiovascular diseases, diabetes, several auto-immune disorders and some types of cancer. They also play a significant role in neonatal growth (Simopoulos, 2000).

Eggs enriched with n-3 FA can be obtained by enriching layer feeds with flax and/or canola as these feedstuffs easily promote the incorporation of n-3 FA's into the yolk (Cherian and Sim, 1991; Van Elswyk, 1997b). Dietary FS is a rich source of LNA that has been shown to increase the n-3 FA content of eggs (Caston and Leeson, 1990; Jiang *et al*., 1991, 1992). However, long term supplementation with FS causes reproductive alterations, attributed to phytoestrogenic compounds (Van Elswyk *et al*., 1994; Aymond *et al*.,1994; Ahn *et al*., 1995) and is also associated with a high incidence of liver haemorrhages (Bean and Leeson, 2003). Furthermore, a fishy or fish-related flavour has been reported in the eggs from hens fed a diet with 15% inclusion of FS (Jiang *et al*., 1992). Pearl millet (PM) is high in oil content in comparison with other cereals, with an average fat content of more than 5%. According to Rooney (1978), LNA comprises about 4% of the total FA in PM's fat, giving it a higher content of n-3 FA than other cereal grains. PM supplies more n-3 FA for deposition into eggs than corn, but it is not sufficient to produce "omega-3 enriched eggs" (Collins *et al*., 1997). The use of PM in laying hens does not affect production parameters and egg flavour, however it causes reductions in yolk pigmentation compared with corn based feeds (Collins *et al*., 1997; Amini and Ruiz-Feria, 2007).

In a previous experiment, we found that hens fed a diet based on PM and 8% FS supplementation produced eggs with an n-3 FA content averaging 456 mg/egg during a six week experimental period, which is considerably higher than the 350 mg/egg required for

marketing eggs as "omega-3 enriched eggs" (Amini and Ruiz-Feria, 2007). This may open the possibility of lowering the FS content of the PM-based diets to levels even lower than 8%, further reducing the detrimental effects of FS on egg quality as well as on the health of the hens. However, in the same experiment, yolk pigmentation score of the egg yolks (Roche® color fan) produced by birds fed PM-based diet (1.60±0.24) was lower than those produced by birds fed a corn-based diet (6.00±0.01). The inclusion of 0.2% Marigold petal extract in wheat-barley based diets restores yolk pigmentation from 1.58±0.07 to levels as high as 8.84±0.13 (Karadas *et al.*, 2006). Hence, the inclusion of similar quantities of marigold petal extract to a PM based diet may produce comparable results, restoring the pigmentation to marketable levels.

Thus, it may be possible to develop a nutrition program to produce n-3 FA enriched eggs using a PM-based diet, FS levels lower than 8% and natural PG, in order to reduce off-flavour in the eggs, preserve the health and well-being of the hens and at the same time, obtain acceptable levels of yolk pigmentation. The objectives of this study were to determine the lower amount of FS supplementation required to obtain omega 3-enriched eggs, and to evaluate the efficacy of a natural pigment to restore egg yolk pigmentation to marketable levels, in a longer period of FS supplementation (12 weeks).

Materials and Methods

Animals and treatments: Fifty four week old white leghorn hens of the Shaver White strain (ISA Poultry, Cambridge ON, N1R 5V9, Canada) were used. Birds were housed in commercial type laying cages in a room with automatic light and ventilation control. Three hens were accommodated in each cage (434 cm^2/hen). Hens were subjected to a 16 h light, 8 h dark lighting program throughout the experiment. Feed and water were provided *ad libitum*. All experimental procedures involving animals were conducted according to a protocol reviewed and approved by McGill University Institutional Animal Care and Use Committee.

The diets were based on PM and soybean meal, with three inclusion levels of ground FS (4%, 6% and 8%) and two levels of marigold petal extract (Oro Glo 15®, 0.1% and 0.2%; Kemin Industries Inc., Des Moines, IA), in a 3 by 2 factorial arrangement of treatments (Table 1). Six cage replicates (3 hens per cage) were randomly assigned to each treatment, for a total of 108 hens. All diets contained a fixed level of 4.1% canola oil. The diets were formulated to be isocaloric and isonitrogenous and to meet or exceed NRC (1994) requirements. The PM grain used in the experiment (Canadian Grain Pearl Millet Hybrid, CGPMH-1) was developed by AERC (Agriculture Environmental Renewal Canada Inc., Ottawa, ON Canada) and was milled by a hammer mill through a 1.5 mm sieve.

Flock Performance Parameters: The experiment lasted for 12 weeks. Feed intake and BW were recorded every two weeks, whereas egg production (number of eggs and egg mass produced) was recorded daily. The flock performance parameters determined included hen-day egg production, egg mass produced per bird per day, feed consumption per bird per day and feed conversion ratio. At the end of the experiment, all the hens were euthanized to macroscopically determine liver haemorrhage score. Macroscopic scores of liver haemorrhage were scored based on a 1-5 scale, where a score of 1 indicated no haemorrhaging and a score of 5 denoted excessive haemorrhaging (Schumann *et al.*, 2000).

Eggs were collected for two consecutive days at biweekly intervals and three eggs per each cage replicate were randomly selected for measuring egg weight, yolk weight and shell weight and thickness. Albumen weight was calculated accordingly. Yolk pigmentation was determined using the Roche® color fan on a 1-15 scale.

Fatty acid analysis: Three yolks from each cage replicate were pooled at the end of every two weeks, lyophilized and stored at -20°C. Yolk fat was extracted according to the methods described by Folch *et al.* (1957) and converted to methyl esters using the Meth-Prep II methylation kit (Alltech Associates, Inc. Deerfield, IL). Approximately 150 mg of the lyophilized egg yolk sample was weighed into a 50 mL centrifuge tube, mixed with 8 mL of Folch solution (2:1 chloroform-methanol mix) and homogenized with a Polytron (Brinkmann Instruments, Rexdale, ON, Canada) for two min on ice. Then 200 µL recovery standard (C 17:0; 5 mg/mL) was added to each tube and the extraction mixture was sonicated on ice water for 30 min, vortexing occasionally. The mixture was allowed to stand for 30 min on ice and then was filtered through a funnel with a bed of glass wool and sodium sulphate and the tube and funnel were rinsed twice, using 4 and 3 mL of Folch solution, respectively. Four mL of 0.73% NaCl solution was added to each tube and vortexed and then the tube was centrifuged at ~800 g for 5 min. Then 1.2 mL of the lower layer extracted solution was transferred to the GC auto sampler vials and 0.2 mL of Meth-Prep II was added and allowed to stand for one hour at room temperature for the methylation reaction to take place. An internal standard (70 µL; C 19:0 fatty acid methyl ester, 20 mg/mL) was added to the vial shortly before injection. Reaction mixture was then injected into the Gas Chromatograph (GC).

Gas chromatography: A fused silica capillary column (100 m x 0.25 mm i.d.) with 0.25 µm film thickness on a Varian 3400 CX gas chromatograph (Varian 3400, Varian Canada Inc., Mississauga, ON), equipped with an

Table 1: Composition (g/kg) and calculated analyses of pearl millet based diets with different inclusions of flaxseed (FS 4, 6, and 8%) and two levels of natural pigment (PG1 = 0.1%; PG2 = 0.2%)

	Dietary Treatments					
Ingredients	FS4/PG1	FS4/PG2	FS6/PG1	FS6/PG2	FS8/PG1	FS8/PG2
Pearl Millet	583	583	561	561	539	539
Soybean Meal 48	202	202	198	198	194	194
Flaxseed	40	40	60	60	80	80
Canola oil	41	41	41	41	41	41
Limestone	65	65	65	65	65	65
Dicalcium Phosphate	17	17	17	17	17	17
DL-Methionine	2	2	2	2	2	2
L-Lysine	2	2	2	2	2	2
Premix[1]	44	44	44	44	44	44
Sodium chloride	2	2	2	2	2	2
Pigment	1	2	1	2	1	2
Inert filler (sand)	1	0	7	6	13	12
Calculated analysis						
ME (kcal/kg)	2781	2781	2781	2781	2780	2780
CP	161.1	161.1	161.1	161.1	161.2	161.2
Ca	42.3	42.3	42.3	42.3	42.3	42.3
P	8.4	8.4	8.4	8.4	8.4	8.4
Methionine	4.9	4.9	4.9	4.9	4.9	4.9
Lysine	10.2	10.2	10.2	10.2	10.1	10.1

[1]Contained: calcium 316 g/kg, phosphorus 28 g/kg, sodium 18 g/kg, cobalt 4 mg/kg, copper 100 mg/kg, Fe 2215 mg/kg, iodine 10 mg/kg, manganese 930 mg/kg, zinc 730 mg/kg, selenium 3 mg/kg, retinol 34.4 mg/kg, cholecalciferol 0.625 mg/kg, DL-alpha-tocopherol 400 mg/kg.

auto sampler and a flame ionization detector, was used to separate and quantify the fatty acid methyl esters. The injector and detector temperatures were set at 250°C and 275°C, respectively. Helium was used as the carrier gas at a flow rate of 2.0 mL/min. The initial column temperature was set at 80°C, held for 1 min and then increased by 30°C/min to 180°C. Then it was increased to 195°C at the rate of 1°C/min and finally increased by 20°C to a final temperature of 230°C, held for 19 min. Fatty acid methyl esters were identified by comparison with retention times of authentic standards (Nu-Chek Prep Inc., Elysian, MN 56028). Peak areas and percentages were calculated using the Varian Saturn GC/MS Workstation Software (Version 5.52).

Statistical analysis: Data were analyzed as a two way ANOVA, with FS level and PG as fixed effects. For the flock performance parameters and egg fatty acid analysis data, cage served as experimental unit and data were analyzed with PROC-GLM procedure of SAS (2003). For egg trait results, egg served as the sampling unit and cage was included as a random variable and data were analyzed using the PROC-MIXED procedure of SAS (2003). Significant differences among treatment means were separated using the Scheffe's multiple comparison test. Statistical differences were declared at $P < 0.05$.

Results

Flock performance and egg parameters: The dietary treatments did not affect BW and flock performance parameters, including hen-day egg production, egg mass produced per bird per day, feed consumption and feed conversion ratio (data not shown). In the same way, different levels of FS or PG in the diet did not have significant effects on albumen and shell weight (data not shown). During the first 6 weeks of the experiment, the dietary level of FS did not affect the egg weight. However, in week 8, 10 and 12 of the experiment, diet treatments containing 8% FS, significantly lowered egg weight compared to diets with 4% inclusion of FS (Table 2). Similarly, the FS level did not affect yolk weight during the first 8 weeks of the experiment, but in the last two weeks of the trial, hens fed 8% FS had lower yolk weights (16.16±0.26 and 16.63±0.25 g, weeks 10 and 12, respectively) than birds fed 4% FS (17.69±0.26 and 17.59±0.25 g in the same order), whereas hens fed 6% FS had an intermediate yolk weight and not different from the other two FS levels (17.22±0.02 and 17.09±0.03 g, in the same order) . Interactions effects between FS and PG, and the main effects of PG on egg weight and yolk weight were not significant. Liver haemorrhage scores were not different among treatments at the end of the trial (data not shown). The FS level did not affect the pigmentation score (data not shown), but hens fed 0.2% PG in the PM-based diets had higher pigmentation scores compared with hens supplemented 0.1% PG diets throughout the whole experimental period (9.53±0.08 vs 7.40±0.08 at week 2; 10.18 ±0.13 vs 7.25±0.13 at week 12). There were no interaction effects of FS and PG on any of the flock performance parameters, or egg traits measured.

Table 2: Egg weight (g) produced by laying hens fed a pearl millet based diet with different levels of flaxseed (FS, 4, 6, and 8%) and two levels of natural pigment (PG1= 0.1%; PG2=0.2%)[1]

	Weeks					
	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12
Flaxseed (FS)						
FS4[2]	63.57	64.40	64.39	65.04[a]	64.83[a]	65.44[a]
FS6	62.09	64.04	63.17	63.30[ab]	63.36[ab]	63.49[ab]
FS8	62.05	62.87	62.23	60.81[b]	62.43[b]	62.38[b]
SEM	0.60	0.68	0.74	0.94	0.61	0.70
P- value	0.149	0.053	0.138	0.013	0.031	0.014
Pigment (PG)						
PG1[3]	62.23	64.06	62.69	63.24	63.14	63.29
PG2	62.90	64.14	63.83	62.87	63.93	64.26
SEM	0.49	0.55	0.61	0.77	0.50	0.57
P- value	0.3461	0.9211	0.1943	0.7357	0.2701	0.2404

[1]Interaction effects were not significant [2]Mean of 36 observations, [3]Mean of 54 observations, [a,b]Values in the same column and within the same factor not sharing a common superscript are statistically different ($P < 0.05$).

Fatty acids: The n-3 FA content of the eggs increased with increasing levels of FS, except at week 6 and 12, when the n-3 FA of the eggs produced by hens fed the PM diet containing 4% and 6% FS were not different (Table 3). Conversely, the n-6/n-3 FA ratio was consistently higher in the eggs produced by hens fed 4% FS than in those produced by hens fed 8% FS (Table 4). Increasing FS in the PM-based feed from 6-8% also reduced the n-6/n-3 FA ratio, except in week 4 and 8, when n-6/n-3 FA ratio in the eggs produced by feeding 6% FS and 8% FS were not different. Interaction effects between FS and PG were not significant. Different inclusion levels of PG did not affect the n-3 FA content or n-6/n-3 ratio in the eggs.

Discussion

We have previously reported that PM could replace corn in laying hen diets, reducing the amount of FS needed to obtain n-3 FA enriched eggs to 8%, while maintaining flock productivity and health in a 6 week trial (Amini and Ruiz-Feria, 2007). In the same study we found that when corn is totally replaced by PM, there was a significant reduction in yolk pigmentation, which will reduce the marketability of the eggs. The objectives of this study were to evaluate lower FS supplementation levels that could produce n-3 FA enriched eggs, and the addition of natural pigments to restore yolk pigmentation. To further assess the safety of these dietary treatments for laying hens during long-term FS supplementation, we doubled the evaluation time to 12 weeks.

Overall, different inclusion levels of FS and PG in the PM based diets did not affect eggshell quality parameters, which is in agreement with our previous findings (Amini and Ruiz-Feria, 2007) and with the results reported by Caston *et al.* (1994), in which increasing levels of 0, 10% and 20% FS did not have any significant impact on shell weight. Mazalli *et al.* (2004a) obtained comparable results after feeding 3% flax oil or 9% flaxseed to the birds.

In this study, in week 8, 10 and 12, birds fed 8% FS produced smaller eggs compared to hens fed 4% FS, indicating that FS may manifest a negative effect on egg weight after several weeks of use, even at levels as low as 8%. We also found that, in week 10 and 12, diet treatments containing PM and 8% FS had a lower yolk weight compared to PM-based diets with 4% inclusion of FS, which may explain the reduced egg weight (Scheideler and Froning, 1996; Whitehead *et al.*, 1993). Van Elswyk (1997a) suggested that the reduction in egg weight when feeding FS to hens could be mediated by a low estradiol level in the blood, which may limit lipid availability for yolk formation. Whitehead *et al.* (1993) postulated that the phyto-estrogenic compounds contained in FS, or the changes in circulating estradiol as a consequence of either the latter substances or an effect of n-3 FA per se could be the reason for reduced yolk and consequently egg weight. Different inclusion levels of FS did not have any significant impact on albumen weight, which concurs with our previous findings (Amini and Ruiz-Feria, 2007) and the results reported by Mazalli *et al.* (2004a). Nevertheless, we did not find any significant effect of FS level on hen-day egg production or on daily egg mass produced per bird. Aymond and Van Elswyk (1995) reported decreased production in hens fed 15% FS compared to hens fed a control diet or diets containing 5% FS over a 5 week period in 22-week-old hens. This further indicates that low levels of FS do not have negative effects on egg production.

There were no differences in BW due to dietary treatment. Novak and Scheideler (2001) reported lower BW in young 21-week-old hens fed diets with 10% FS. This effect has been attributed to anti-nutritional factors in FS that may reduce the digestion and absorption of feedstuffs providing energy (Gonzalez-Esquerra and Leeson, 2000; Ortiz *et al.*, 2001; Rodriguez *et al.*, 2001). Mature birds seem to be less susceptible than younger birds to the anti-nutritional factors found in FS

Table 3: Total n-3 fatty acid content in the eggs (g/Egg) produced by laying hens fed a pearl millet based diet with different levels of flaxseed (FS, 4, 6, and 8%) and two levels of natural pigment (PG1= 0.1%; PG2=0.2%)[1]

	Weeks					
	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12
Flaxseed (FS)						
FS4[2]	0.301[c]	0.369[c]	0.382[b]	0.367[c]	0.336[c]	0.394[b]
FS6	0.357[b]	0.411[b]	0.402[b]	0.420[b]	0.400[b]	0.401[b]
FS8	0.431[a]	0.504[a]	0.498[a]	0.505[a]	0.518[a]	0.503[a]
SEM	0.010	0.010	0.013	0.010	0.011	0.010
P- value	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0055
Pigment (PG)						
PG1[3]	0.379	0.423	0.429	0.428	0.438	0.415
PG2	0.381	0.418	0.427	0.411	0.437	0.410
SEM	0.015	0.009	0.011	0.008	0.009	0.007
P-value	0.7768	0.3943	0.5353	0.0649	0.5617	0.3663

[1]Interaction effects were not significant [2]Mean of 36 observations , [3]Mean of 54 observations, [a,b,c]Values in the same column and within the same factor not sharing a common superscript are statistically different (P < 0.05).

Table 4: n-6/n-3 fatty acid ratio in the eggs produced by laying hens fed a pearl millet based diet with different levels of flaxseed (FS, 4, 6, and 8%) and two levels of natural pigment (PG1= 0.1%; PG2=0.2%)[1]

	Weeks					
	Week 2	Week 4	Week 6	Week 8	Week 10	Week 12
Flaxseed (FS)						
FS4[2]	3.69[a]	3.09[a]	3.32[a]	3.20[a]	3.26[a]	3.08[a]
FS6	3.36[b]	2.82[b]	2.78[b]	2.63[b]	2.81[b]	2.57[b]
FS8	3.01[c]	2.64[b]	2.54[c]	2.41[b]	2.45[c]	2.38[c]
SEM	0.06	0.06	0.06	0.09	0.08	0.04
P- value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Pigment (PG)						
PG1[3]	3.01	2.87	2.90	2.83	2.89	2.73
PG2	3.02	2.82	2.86	2.66	2.79	2.69
SEM	0.05	0.05	0.05	0.07	0.06	0.04
P- value	0.4044	0.4928	0.4948	0.1189	0.3262	0.0537

[1]Interaction effects were not significant, [2]Mean of 36 observations , [3]Mean of 54 observations, [a,b,c]Values in the same column and within the same factor not sharing a common superscript are statistically different (P < 0.05).

(Klosterman, 1974; Jiang *et al*., 1991; Scheideler and Froning, 1996). We used older birds (54 weeks) and lower FS supplementation in this study, which explains the lack of FS effects on BW. Increasing levels of FS in this experiment did not have any significant impact on feed efficiency (FCR), which agrees with the reports of Baucells *et al*. (2000); Mazalli *et al*. (2004a) indicating that the combination of PM and low levels of FS in layers diets has no detrimental effect on flock performance. Moreover, we did not observe any significant liver haemorrhage in a 12 week trial period, which suggests that dietary levels of FS at 8% or lower are safe to use for longer periods of time as a feed ingredient in layer diets. A potential disadvantage of PM could be its lower ME content compared to corn. According to Singh *et al*. (2005) different varieties of PM have different ME contents. The analysis of PM used in this study indicated a ME content of 3000 kcal/kg. We used canola oil in the dietary treatments, in order to meet the energy requirements of the hens. The advantage of canola oil is that it has lower n-6/n-3 FA ratio compared to other vegetable oils (NRC, 1994).

Another disadvantage is that, compared with corn, PM contains insufficient quantities of xanthophylls, the pigment that imparts a golden yellow color in yolks. This causes lower pigmentation scores in eggs produced by hens fed PM based diets, compared to those produced by birds fed corn. In this experiment, the inclusion of either 0.1% or 0.2% PG to PM-based diets restored yolk pigmentation. Addition of 0.1% PG restored the yolk color score to an average of 7.44±0.11 (Roche® pigmentation score) during the 12 week experimental period, while inclusion of 0.2% PG increased the average score to 9.90±0.12. Different egg markets demand different egg pigmentation scores, but both inclusion levels of PG produced eggs with acceptable pigmentation to meet the requirements of the North American market. According to Leeson and Summers (2005), a pigmentation score of 7-8 is acceptable for grade A eggs. We reported previously (Amini and Ruiz-Feria, 2007) that higher levels of FS (12%) in combination with PM slightly improved yolk pigmentation, although it remained lower than the pigmentation score obtained by feeding a corn based diet. The results of this study

indicate that there is no interaction effect of FS and PG on yolk pigmentation.

It is well established that dietary polyunsaturated fatty acids can cause major changes on egg FA profile (Noble *et al*. 1990; Mazalli *et al*. 2004b). In this study, the n-3 FA content of eggs was increased with increasing levels of FS, except at week 6 and 12, when the yolk n-3 FA content of eggs produced by PM-based diets containing 4% and 6% FS were not significantly different. The n-3 FA acid content was maintained at high levels during the whole length of the experiment. This agrees with findings of other workers who used high levels of n-3 FA in the diets and observed incremental trends in the n-3 FA content of the eggs in the first 2-3 weeks of treatment, after which the n-3 FA content reached a plateau (Herber and Van Elswyk, 1996; Van Elswyk, 1997a). As expected, different inclusion levels of the PG did not affect the deposition of n-3 FA in the eggs. Supplementation of 4% FS to a PM based diet was not sufficient to consistently supply enough n-3 FA to obtain the required 350 mg of n-3 FA per egg, necessary for the production of "omega-3 enriched egg" (Scheideler and Lewis, 1997). However, hens fed 6% FS consistently deposited enough n-3 FA in their egg yolks to be considered as "omega-3 enriched eggs". Higher levels of FS in diets may produce off-flavours in the eggs (15% FS; Jiang *et al*. 1992) and are associated with high incidence of liver haemorrhages in laying hens in long term use (Bean and Leeson, 2003). Therefore a dietary treatment containing PM as the sole grain source and FS with inclusion levels as low as 6%, can eliminate the incidence of liver haemorrhages and production of off-flavours in the eggs and meanwhile provide hens with enough n-3 FA for production of "omega-3 enriched eggs".

The hens' efficiency in depositing n-3 FA in the yolk is also dependent on age. Scheideler *et al*. (1998) reported that hens younger than 35 week deposit 25% to 50% less n-3 FA in their eggs than older birds. Caston and Leeson (1990) reported that 32 week old hens fed 0%, 10%, 20% and 30% of dietary FS deposited 21, 247, 478 and 618 mg n-3 FA in their eggs, respectively. In this experiment we used 54 week old birds, which may explain, at least in part, the high amount of n-3 FA in the yolks, using PM based diets with rather low levels of FS. The contribution of n-3 FA of the canola oil added to the diet is too small to explain this difference. For example, birds fed 8% FS deposited around 500 mg of n-3 FA per egg (Table 4), twice as much as the birds fed 10% FS in the Caston and Leeson (1990) report.

In this study, we also observed a dose-related improvement in the n-6/n-3 FA ratio in response to increasing levels of dietary FS. The n-6/n-3 FA ratio was consistently lowered in the eggs produced by hens fed 8% FS compared to those produced by hens fed 4% FS. Increasing levels of FS in the PM-based feed from 6% to 8% also improved the n-6/n-3 FA ratio, except in week 4 and 8, when 8% FS in the feed did not reduce the n-6/n-3 FA ratio in the eggs compared to 6% FS diets. As expected, dietary level of PG in the diets did not have a significant effect on n-6/n-3 FA ratio in the eggs.

Clinical studies indicate that the ingested ratio of n-6/n-3 FA is important for maintaining cardiovascular health (Okuyama, 2001). An increased consumption of n-6 FA in Western diets favours the eicosanoid metabolic products from arachidonic acid, specifically prostaglandins, thromboxanes, leukotrines, hydroxy fatty acids, and lipoxins, leading to the formation of thrombus and atheromas, allergic and inflammatory disorders, and cell proliferation (Simopoulos, 2006). The typical western diet has an n-6/n-3 FA ratio of 15-16.7, whereas the ideal dietary ratio of n-6/n-3 has been reported to be lower than 5 (Simopoulos, 2000, 2006). Therefore, the eggs produced as a result of feeding PM and low levels of FS to the hens, with a n-6/n-3 FA ratio of less than 3 (FS6), can constitute an optimum food for human consumption, compared with normal table eggs, with a n-6/n-3 ratio of 10 (Amini and Ruiz-Feria, 2007).

In general, these results confirm that for the purpose of n-3 FA enriched egg production, the use of PM as the sole grain source in a diet containing 4.1% of canola oil reduces the requirements for FS to levels as low as 6% in the diet of laying hens. In contrast, in a corn based diet, it is necessary to use inclusion levels of 10-15% of FS to obtain an n-3 FA enriched egg (Scheideler and Froning, 1996). As previously discussed, the use of high levels of FS inclusion in layers feed is associated with liver haemorrhage and off-flavours, reducing market acceptability of the eggs. Therefore, reduced inclusion of FS in a PM based diet can eliminate these problems without compromising productivity. The reduced yolk pigmentation as a result of feeding PM can be totally restored by low levels (0.1%) of natural pigment supplementation.

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