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Dietary enrichment of *n*-3 PUFA for laying hens: effect of different sources on production, composition and quality of eggs

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Seventy-two Warren strain laying hens were fed 4 isonitrogenous diets *ad libitum* for 61 days in order to investigate the effects of different sources of *n*-3 polyunsaturated fatty acids (PUFA) on hen performance, egg production, fatty acid (FA) composition and egg quality. Extruded linseed (EL), ground linseed (GL) and a commercial fish oil source (NF) were added to the control diet (C) at 10.0, 10.0, and 3.4%, respectively. The eggs were collected daily and subjected to measurement and analysis ten times throughout the experimental period. Compared to the C diet, the NF diet significantly improved egg production efficiency (P<0.01) and overall egg weight/hen ratio (P<0.05), whereas the GL and NF diets led to a reduction in hen weight gain (P<0.01). Equilibrium in the yolk FA profile was reached after 14 days of dietary n-3 PUFA source inclusion. All experimental diets significantly affected yolk FA composition and reduced the *n*-6/*n*-3 ratio (11.4, 2.0, 2.3, and 2.0 at equilibrium for C, EL, GL, and NF, respectively; P<0.05). Yolk redness (a* values: -1.13 *vs* -2.25 and -2.96) and yellowness (b* values: 50.7 *vs* 48.5 and 48.2) were significantly reduced by both linseed-supplemented diets (C *vs* EL and GL, respectively; P<0.05). Moreover, the form of linseed in the diet (extruded *vs* ground) significantly affected yolk n-3 PUFA content (7.1 *vs* 6.4 % total FAME for EL and GL, respectively; P<0.05), and the extruded form significantly enhanced the rate of inclusion, thereby illustrating the importance of feed source processing in egg quality traits.

KEY WORDS: laying hen / dietary *n*-3 PUFA / egg production / egg composition/ egg colour

Considering the positive effects of highly polyunsaturated fatty acids (HUFA) of the omega-3 series (*n*-3), namely EPA (20:5 *n*-3) and DHA (22:6 *n*-3), on human

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health [Simopoulos 2000, Poławska *et al.* 2013] and the recommendation that the desirable ratio of *n*-6/*n*-3 polyunsaturated fatty acids (PUFA) should be less than 4:1 in human diet [Kralik *et al.* 2008], *n*-3 PUFA sources are being introduced in the feeding of poultry to improve the fatty acid (FA) composition of their meat and eggs. A proven ability to modify egg yolk FA profile would provide a meaningful marketing tool for egg producers. Among the numerous dietary supplements for laying hens, fish oil [Ebeid 2011], marine algae [Lemahieu *et al.* 2013], and linseed [Nain *et al.* 2012] are reported to be effective in enhancing yolk *n*-3 FA content. The different sources of *n*-3 FA, however, do not have the same effect on FA composition [Kirubakaran *et al.* 2011, Van Elswyk 1997], laying performance, egg quality and sensory traits [Ebeid 2011, Yi *et al.* 2014], and generally have both advantages and disadvantages. Fish oil, for example, readily promotes the deposition of EPA and DHA in egg yolk [Cachaldora *et al*. 2006], but has also been seen to decrease yolk weight [Novak and Scheideler 2001, Whitehead *et al*. 1993]. Other sources of n-3 FA have shown different levels of lipid oxidation and consequently result in different egg shelf life [Botsoglou *et al.* 2012, Meynier *et al.* 2014]. Linseed, which is rich in α-linolenic acid (ALA) that is partially converted in EPA and DHA and transferred to the egg [Surai and Sparks 2001], has been found by some authors [Bean and Leeson 2003, Leeson *et al.* 2000, Novak and Scheideler 2001] to decrease body weight, egg production, and yolk weight when included in laying hen diets, even if other authors have not reported these negative effects [Nain *et al.* 2012]. Those differences could depend on the technological treatment given to feed components, which in some cases alleviate the negative effects of anti-nutritional factors such as trypsin inhibitors, mucilage, and cyanogenic glycosides present in linseed [Nain *et al*. 2012]. Although some authors [Thacker *et al.* 2005] have observed the linseed extrusion process to degrade mucilage and improve digestibility and feed conversion in broilers, to our knowledge little is known on the effect of linseed extrusion on production, FA composition and the quality of eggs in laying hens.

The purpose of this study was to compare the effects of feeding different forms of *n*-3 FA sources (extruded linseed, ground linseed and microencapsulated fish oil) on hen performance, egg production, FA composition, cholesterol content, lipid oxidation, and colour and pH of the yolk. Moreover, given that egg composition shows some changes throughout the oviposition cycle [Nain *et al.* 2012], the effect of time on yolk colour and *n*-3 FA profile was investigated in order to understand when the transfer of nutrients from the feed to the egg is complete and leads to constant FA and colour trait values and the achievement of a status of equilibrium.

Material and methods

Experimental design and animal management

Four isonitrogenous diets containing different sources of *n*-3 PUFA were tested. The control diet was a commercial diet (C), whereas the three experimental diets

consisted in adding the following *n*-3 PUFA-rich ingredients to the C diet: extruded linseed (EL), ground linseed (GL), and a commercial product derived from refined and microencapsulated fish oil (NF: Nordos Fat®, produced by Trouw Nutrition Italia S.p.A., Bussolengo, Verona, Italy) at 10.0, 10.0 and 3.4% of the C diet, respectively. Each diet was enriched with 300 mg α-tocopheryl acetate/kg.

Seventy-two Warren strain laying hens of 24 weeks of age were randomly housed in pairs in enriched cages with 750 cm^2 floor space per hen in an experimental farm with the controlled environment conditions. Hens were allocated into four experimental groups (nine replicates per treatment) corresponding to the four experimental diets fed *ad libitum* for 61 days. Water was provided *ad libitum* with a nipple waterer. The lighting regime was 16 h of light and 8 h of darkness, and the temperature ranged between 15 and 22°C. Hen body weight was measured at the 1st and 61st day of the experiment. Egg production (number and weight) and feed intake (unconsumed feed was weighed back daily) were measured daily in each cage and calculated on a hen-day basis. All animals were handled according to the principles stated in the EC Directive 86/609/EEC regarding the protection of animals used for experimental and other scientific purposes.

Sample collection and analysis

Diets were analysed (Tab. 1) to determine dry matter (DM), crude protein (CP), ether extract (EE), and ash according to AOAC [AOAC 2000], whilst NDF, ADF and ADL were analysed according to Van Soest et al. [1991] using a Fibre Analyser (ANKOM/ 2000; ANKOM Technology, New York, NY, USA). The FA profile of diets was determined using gas chromatography and it is shown in Table 1. In brief, fatty acids were extracted from ground and mixed samples using chloroform:methanol (2:1. vol/vol) [Folch *et al*. 1957]. For each sample 20 mg of fat were exposed to acid derivatization with 2 mL of methanolic-H_2SO_4, 10% vol/vol (Sigma-Aldrich, St. Louis, MO) at 65°C for 1 h and mixed. After dilution with deionized water, fatty acid methyl esters were extracted using exhane and then separated and quantified using a gas chromatograph (Shimatzu GC17A, with a FID detector and an Omegawax 250 column 30 m x 0.25 mm x 0.25 μm).

As mentioned above, all eggs laid during the trial were counted and weighed, and total egg weight/hen was also calculated. Egg production efficiency was calculated by dividing the number of laid eggs/hen by days of egg collection ×100. The eggs laid at days 0, 2, 4, 8, 14, 21, 28, 35, 42 and 56 were collected (No.=720). The day after each collection, the weight of the whole egg and yolk were measured, and also the pH of yolk and albumen. The pH was measured in duplicate with a portable pH-meter (Piccolo, Hanna Instruments, Villafranca Padovana, Italy). At each collecting date, the above-mentioned egg measurements of the two hens per cage were averaged, and 90 eggs per dietary treatment were considered. Cholesterol content of the yolk was determined using high performance liquid chromatography (HPLC) and the method described by Casiraghi *et al.* (1994), whereas FA composition was determined using the

Table 1. Chemical composition (g/kg as fed), nutritive value and fatty acid (FA; % total FAME) profile of the experimental diets

Item	Control (C)	Extruded linseed (EL)	Ground linseed (GL)	Microencapsulated fish oil (NF)
Inclusion level (%)	-	10	10	3.4
Analysed composition				
dry matter	910	914	912	913
crude Protein	175	176	178	178
ether Extract	56	66	59	60
crude Ash	128	120	101	139
N-Free Extracts	512	504	522	479
NDF	140	168	145	125
ADF	35	49	47	36
ADL	3.4	7.9	8.9	4.9
calculated ME* (MJ/kg)	12.9	13.0	13.0	12.2
FA profile				
SFA	33.4	19.1	17.0	38.6
MUFA	34.4	22.5	20.2	21.5
PUFA	32.2	58.4	62.9	39.9
C18:2 (*n*-6)	30.4	27.0	32.5	30.0
C18:3 (*n*-3)	1.87	31.4	30.4	5.64
C20:4 (*n*-6)	0	0	0	0.18
C20:5 (*n*-3)	0	0	0	1.86
C22:6 (*n*-3)	0	0	0	2.24
n-6	30.4	27	32.5	30.1
n-3	1.87	31.4	30.4	9.74
n-6/*n*-3	16.2	0.86	1.07	3.09
PUFA/SFA	0.96	3.06	3.70	1.03

*Sibbald [1980].

method described for diets [Folch *et al.* 1957]. Thiobarbituric acid reactive substance (TBARS) analysis was performed by HPLC [Bergamo *et al*. 1998] and the results were expressed as ng malondialdehyde (MDA) equivalents/g yolk. Cholesterol and TBARS contents were determined on 36 eggs (9 eggs per dietary treatment) collected at day 56.

Instrumental yolk colour expressed as L* (lightness), a* (redness), and b* (yellowness) according to the CIELab system [CIE, 1976] was measured with a Minolta CR300 chromameter (Minolta, Osaka, Japan). The illuminant was D65, and an incidence angle of 0 was used. The values corresponded to the average of two measurements per sample.

Statistical analysis

Data on hen performance and production and egg composition were subjected to two-way analysis of variance (with interaction) using the ANOVA procedure [SAS

2008], adopting a linear model that considered the effect of the four experimental diets. Data of yolk FA composition and colour were also analysed by adopting a linear model that considered the diet effect (D), the time of experimental diet supplementation (T), and their interaction DxT, where T has three levels: initial (d0, first day of diet supplementation), pre-equilibrium (d2 to d14), and equilibrium (d21 to d56). In this procedure, the repeated measurement option was used to test the significance of T. The adjusted Bonferroni correction for multiple comparison was used.

Results and discussion

Performance data

Overall hen body weight and feed intake showed no significant effect of dietary PUFA sources (Tab. 2). Our observations confirmed those of other authors [Baucells *et al.* 2000, Ebeid 2011],who compared several dietary fat sources, such as fish oil, linseed oil, rapeseed oil, sunflower oil and tallow, at different inclusion levels.

In contrast to the observations reported by Scheideler and Froning [1996] and by Novak and Scheideler [2001], linseed-supplemented diets did not reduce hen body weight. However, when considering weight gain during the 61 days of experimental feed supplementation, we observed that the GL and NF groups gained less weight than the C group ($P<0.01$), whereas the EL group showed an intermediate value. Dietary *n*-3 PUFA sources did not modify feed efficiency in egg production (Tab. 2), as had already been demonstrated by Baucells *et al.* [2000], Grobas *et al.* [2001] and Nain *et al*. [2012] when several different fat sources were used in the diet. The NF diet increased egg production significantly (both total egg weight and egg number) compared to the C diet, whereas both linseed treatments led to intermediate yields. The average weight of the eggs was not significantly affected by dietary treatment. These findings seem to suggest that enrichment with *n*-3 PUFA leads to an overall improvement in egg production, although it tends to reduce the weight gain.

Even if many authors have not reported any effect of *n*-3 PUFA supplementation on egg production or egg weight [Baucells *et al*. 2000, Ebeid 2011, Novak and Scheideler 2001], others [Aziza *et al.* 2013, Scheideler and Froning 1996] have observed increased egg production after supplementation with linseed, fish oil or camelina meal. Furthermore, Scheideler and Froning [1996] showed that linseed inclusion led to a reduction in egg weight. No significant effect of the physical form of linseed (extruded vs ground) on hen performance, egg production or weight was found (Tab. 2).

Egg quality and composition

Yolk weight was affected by the treatment ($P<0.05$), and its highest value was found in hens fed the C diet, even though Bonferroni test showed no significant differences in pairwise comparisons (Tab. 2). Previously, other authors had reported a decrease in yolk proportion when diets were supplemented with fish oil or linseed

Table 2. The effect of feeding *n*-3 PUFA sources on laying hen performance, egg production, yolk cholesterol content, and TBARS index (wet basis)

Item	Diet				P-value	SEM[1]
	control (C)	extruded linseed (EL)	ground linseed (GL)	microen-capsulated fish oil (NF)		
No. of hens	18	18	18	18		
Initial body weight	1866	1867	1859	1866	0.663	165
Weight gain 0-61 day (g/d)	2.6^{B}	1.8^{AB}	1.1^{A}	1.3^{A}	0.007	1.3
Feed intake 0-61 days[2] (g/hen/d)	119	123	117	119	0.652	5.7
Feed efficiency 0-61 day (kg feed/kg egg)	2.14	2.09	2.01	1.97	0.784	0.15
Overall eggs weight/hen[3] (61 days) (g)	3370^{a}	3525^{ab}	3506^{ab}	3623^{b}	0.035	257
Eggs laid/hen (61days) (No.)	54.6^{A}	57.9^{AB}	57.7^{AB}	59.9^{B}	0.009	3.6
Egg production efficiency[3] (%)	89^{A}	95^{AB}	95^{AB}	98^{B}	0.006	6
Egg weight[4] (g)	61.8	60.9	60.8	60.5	0.681	4.7
Yolk weight[4] (g)	14.7	14.1	14.3	14.0	0.042	1.3
Yolk pH[4]	6.20	6.17	6.20	6.22	0.367	0.12
Albumen pH[4]	9.00	8.99	8.98	8.99	0.137	0.12
Yolk cholesterol[5] (mg/g)	10.96^{Aa}	11.83^{B}	11.79^{B}	11.54^{ABb}	0.002	0.74
Yolk cholesterol[5] (mg/yolk)	176.1^{β}	167.7^{α}	169.3^{α}	160.3^{α}	0.089	20.2
TBARS[6] (ng MDA/g)	37.4^{A}	70.0^{C}	69.5^{C}	57.3^{B}	<0.001	0.73

[1]SEM is the standard error of the least squares means.
[2]Mean value calculated considering the cage consumption.
[3]Egg production considering the average output of two hens per cage.
[4]On 360 eggs (90 eggs per dietary treatment).
[5]On 95 eggs (20, 22, 26 and 27 eggs per C, EL, GL and NF, respectively).
[6]On 36 eggs (9 eggs per dietary treatment, of the last collection day).
[aA...]Means within a rows bearing different superscripts differ significantly at: small letters – $P<0.05$; capitals – $P<0.01$.

[Novak and Scheideler 2001, Scheideler and Froning 1996, Whitehead *et al.* 1993]. One possible reason is that the diet's long chain FA composition can influence the synthesis of estradiol, leading to a reduction in the formation of yolk FA precursors and their transport into the ovarian follicles [Novak and Scheideler 2001, Whitehead *et al.* 1993]. Other authors, however, after feeding hens different *n*-3 PUFA sources, such as fish oil, linseed, and microalgae, did not report any effects on yolk percentage [Ebeid 2011, Kim *et al.* 2014, Nain *et al.* 2012]. Generally, using supplemented-PUFA sources does not seem to negatively affect the weight of eggs or that of their components, and the reduction of egg components sometimes observed is amply compensated for by increased egg production, as demonstrated in the present study.

The pH of yolk and albumen did not show differences associated with dietary treatment (Tab. 2), neither did Ahn *et al.* [1999] observe any significant pH variation in egg yolk or albumen when feeding hens a conjugated linoleic acid (CLA) supplement.

Yolk cholesterol concentration, expressed as mg/g of yolk, increased in both linseed diets (P<0.01) and also in the NF diet (P<0.05) when compared to the C diet (Tab. 2). When considering cholesterol content per yolk, however, it was lower (P<0.10) in the hens fed the *n*-3 PUFA-supplemented diets due to their lower yolk weight, and it was below the standard total cholesterol egg content (210 mg/egg) reported by other authors [Ebeid *et al.* 2008].

TBARS, a measure of lipid peroxidation expressed as ng MDA/g yolk, was higher in all the *n*-3 PUFA-supplemented diets when compared to the C diet, and this increase was particularly relevant (P<0.001) in both linseed diets (Tab. 2). The level of yolk unsaturation, in fact, was enhanced by supplementation with *n*-3 PUFA sources (Tab. 1), leading to a greater susceptibility to lipid peroxidation [Botsoglou *et al.* 2013]. Although a direct comparison of TBARS values in different studies should be considered with caution, in this study TBARS was observed to be low when compared to the findings of some authors [Cortinas *et al*. 2003] concerning fresh eggs (100-180 ng MDA/g yolk), and this was probably due to the addition of α-tocopheryl acetate in all the diets.

FA composition of yolk

Dietary supplementation of *n*-3 PUFA led to a gradual increase in ALA in the yolk from day 2 to day 14 of feeding (pre-equilibrium phase), after which a status of equilibrium was achieved and ALA content was fairly constant (Fig. 1). This result is in line with the findings of Nain *et al*. [2012] and Van Elswyk [1997], who reported that the deposition of lipids in the yolk in response to PUFA supplementation in the diet reaches a plateau after 6 and 9 days of diet enrichment, respectively. For this reason, the time effect (T) on yolk composition was represented by 3 time phases: initial (d0), pre-equilibrium (d2-d14), and equilibrium (d21-d56). As expected, the FA composition varied depending on T as reported in Tables 3 and 4, and the diet × time (D×T) interaction was significant for all FAs. Hens fed *n*-3 PUFA-supplemented diets

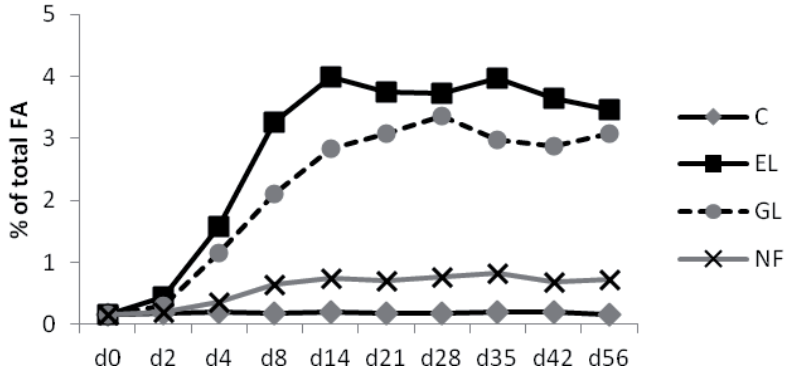

Fig. 1. The α-Linolenic acid trend in egg yolks throughout the dietary supplementation period (d0-d56, days of egg collection).

showed a strong increase of *n*-3 PUFA and a decrease of *n*-6 PUFA from the initial to the equilibrium phase, with a consequent reduction of the *n*-6/*n*-3 ratio (Tab. 3 and 4).

As expected, while no significant differences were observed in the yolk FA composition among diets in the initial phase (d0), in both pre-equilibrium and equilibrium phases the *n*-3 PUFA-supplemented diets significantly modified the yolk FA profile (Tab. 3 and 4). Compared to the C diet, already from the pre-equilibrium phase all *n*-3 PUFA-supplemented diets decreased total MUFA and *n*-6/*n*-3 ratio, whereas they increased the concentration of total and *n*-3 PUFA. This was due to the lower proportion of MUFA and to a higher concentration of total and *n*-3 PUFA in the

Table 3. Effect of feeding n-3 PUFA sources on yolk fatty acid (FA) composition (% total FAME) at three times of diet supplementation

Item	Time (T)[1]	Diet				P-value			SEM[2]
		control (C)	extruded linseed (EL)	ground linseed (GL)	microen-capsulated fish oil (NF)	D	T	DxT	
Hens (No.)		18	18	18	18				
Eggs (No.)[3]		90	90	90	90				
SFA	Initial	43.8	43.6	44.0^{y}	44.0^{x}	0.651			0.78
	Pre-equilibrium	44.3^{b}	42.7^{a}	43.9^{by}	44.3^{bx}	<0.001			0.62
	Equilibrium	44.3^{b}	42.0^{a}	43.1^{ax}	45.4^{cy}	<0.001			0.75
	P-value Time	0.359	0.389	0.235	0.043	<0.001	0.241	0.002	1.58
MUFA	Initial	37.0^{x}	36.8	36.5	36.5	0.752			1.08
	Pre-equilibrium	38.0^{b}	36.4^{a}	36.4^{a}	36.5^{a}	<0.001			0.62
	Equilibrium	38.5^{by}	36.1^{a}	35.9^{a}	36.1^{a}	<0.001			0.70
	P-value Time	0.008	0.468	0.618	0.238	<0.001	0.213	0.035	1.50
PUFA	Initial	19.3^{y}	19.6^{x}	19.5^{x}	19.6^{y}	0.925			1.22
	Pre-equilibrium	17.7^{ax}	20.9^{cy}	19.7^{bx}	19.3^{by}	<0.001			0.62
	Equilibrium	17.2^{ax}	21.4^{cy}	21.0^{cy}	18.5^{bx}	<0.001			0.78
	P-value Time	<0.001	<0.001	0.005	0.016	<0.001	0.703	<0.001	1.41
n-6	Initial	17.7^{y}	17.9^{z}	17.8^{z}	17.8^{z}	0.985			1.13
	Pre-equilibrium	16.3^{bx}	15.9^{by}	16.0^{by}	15.2^{ay}	<0.001			0.48
	Equilibrium	15.8^{cx}	14.3^{bx}	14.6^{bx}	12.2^{ax}	<0.001			0.49
	P-value Time	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	1.11
n-3	Initial	1.57	1.74^{x}	1.72^{x}	1.74^{x}	0.321			0.23
	Pre-equilibrium	1.45^{a}	4.98^{cy}	3.77^{by}	4.06^{by}	<0.001			0.41
	Equilibrium	1.41^{a}	7.07^{cz}	6.36^{bz}	6.29^{bz}	<0.001			0.43
	P-value Time	0.896	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	1.30
n-6/*n*-3	Initial	11.3	10.6^{z}	10.4^{z}	10.4^{z}	0.449			1.45
	Pre-equilibrium	11.4^{c}	4.5^{ay}	5.6^{by}	5.1^{aby}	<0.001			0.65
	Equilibrium	11.4^{c}	2.0^{ax}	2.3^{bx}	2.0^{ax}	<0.001			0.30
	P-value Time	0.372	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	1.97
PUFA/SFA	Initial	0.44^{y}	0.45^{x}	0.44^{x}	0.44^{y}	0.913			0.011
	Pre-equilibrium	0.40^{ax}	0.49^{cxy}	0.45^{bx}	$0.44b^{y}$	<0.001			0.008
	Equilibrium	0.39^{ax}	0.50^{by}	0.49^{by}	0.41^{ax}	<0.001			0.006
	P-value Time	<0.001	0.015	0.002	0.002	<0.001	0.832	<0.001	0.009

[1]Initial (d0, first day of diet supplementation), Pre-equilibrium (d2 - d14), Equilibrium (d21 - d56).
[2]SEM is the standard error of the least squares means.
[3]No.= 36 eggs for Initial, No.=144 eggs for Pre-equilibrium and No.=180 eggs for Equilibrium measurements.
[a, b, c, d]Means within a rows bearing different superscripts differ significantly at P<0.05.
[x, y, z]Means within a columns bearing different superscripts differ significantly at P<0.05.

Table 4. The effect of feeding n-3 PUFA sources on yolk polyunsaturated fatty acid (PUFA; % total FAME) profile at three times of diet supplementation

Item	Time (T)[1]	Diet (D)				P-value			SEM[2]
		control (C)	extruded linseed (EL)	ground linseed (GL)	microen-capsulated fish oil (NF)	D	T	DxT	
Hens (No.)		18	18	18	18				
Eggs (No.)[3]		90	90	90	90				
C18:2 *n*-6	Initial	12.7^{y}	12.8	12.7	12.6^{y}	0.963			0.87
	Pre-equilibrium	12.0^{ax}	12.4^{bc}	12.5^{c}	12.1^{by}	0.021			0.41
	Equilibrium	11.9^{bx}	12.3^{c}	12.5^{c}	10.7^{ax}	<0.001			0.42
	P-value Time	0.006	0.101	0.640	<0.001	0.002	<0.001	<0.001	0.73
C18:3 *n*-3	Initial	0.15	0.16^{x}	0.16^{x}	0.16^{x}	0.911			0.02
	Pre-equilibrium	0.18^{a}	2.31^{dy}	1.60^{cy}	0.49^{b}	<0.001			0.18
	Equilibrium	0.18^{a}	3.71^{dz}	3.07^{cz}	0.75^{by}	<0.001			0.25
	P-value Time	0.935	<0.001	<0.001	0.028	<0.001	<0.001	<0.001	0.64
C20:4 *n*-6	Initial	5.01^{z}	5.10^{z}	5.07^{z}	5.23^{z}	0.809			0.49
	Pre-equilibrium	4.26^{cy}	3.44^{by}	3.48^{by}	3.14^{ay}	<0.001			0.25
	Equilibrium	3.92^{cx}	2.00^{bx}	2.14^{bx}	1.52^{ax}	<0.001			0.16
	P-value Time	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.64
C20:5 *n*-3	Initial	-	-	-	-	-	-	-	-
	Pre-equilibrium	$<0.000^{a}$	0.093^{cx}	0.030^{bx}	0.150^{dx}	<0.001			0.02
	Equilibrium	$<0.000^{a}$	0.193^{cy}	0.153^{by}	0.341^{dy}	<0.001			0.03
	P-value Time	-	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.07
C22:6 *n*-3	Initial	1.42	1.58^{x}	1.57^{x}	1.58^{x}	0.343			0.23
	Pre-equilibrium	1.26^{a}	2.58^{cy}	2.14^{bx}	3.42^{dy}	<0.001			0.36
	Equilibrium	1.23^{a}	3.17^{bz}	3.13^{by}	5.20^{cz}	<0.001			0.31
	P-value Time	0.885	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.82

[1]Initial (d0, first day of diet supplementation), Pre-equilibrium (d2 - d14), Equilibrium (d21 - d56).
[2]SEM is the standard error of the least squares means.
[3]No.= 36 eggs for Initial, No.=144 eggs for Pre-equilibrium and No.=180 eggs for Equilibrium measurements.
[a, b, c, d]Means within a rows bearing different superscripts differ significantly at P<0.05.
[x, y, z]Means within a columns bearing different superscripts differ significantly at P<0.05.

n-3 PUFA-supplemented diets, with the latter being reported to exert an inhibitory effect on the Δ9-desaturase necessary for the synthesis of MUFA [Garg *et al.* 1988]. In the pre-equilibrium phase *n*-6 FA contents were significantly lower only in NF yolks, and at equilibrium even EL and GL diets lowered the yolk *n*-6 PUFA. In the pre-equilibrium phase, yolk SFA proportions were lowered only by EL, whereas at equilibrium the SFA in yolks were decreased also by GL, while they were increased by NF; the SFA proportions found in yolks were consistent with those of the diets (Tab. 1). The ratio between PUFA and SFA in the yolk is consistent with that in the diets, showing the highest values in EL and GL, particularly at equilibrium.

EPA (C20:5 *n*-3) and DHA (C22:6 *n*-3) showed the greatest concentration in yolks of the NF group, followed by linseed-based diets and then by the C diet (Tab. 4). In contrast, ALA (C18:3 *n*-3) showed the highest concentration in EL yolk, followed by GL, NF, and C. The C diet maintained the highest concentration of arachidonic acid (C20:4 *n*-6), followed by the GL, EL and NF diets (Tab. 4). The linoleic acid (C18:2 *n*-6) proportion in pre-equilibrium was lowest in the C diet, whereas after the

achievement of equilibrium it showed its minimum concentration in the NF group. As reported by other authors [Ebeid 2011, Jia *et al.* 2008], hens consuming both linseed and fish oil increased total *n*-3 FA contents in egg yolk and decreased the *n*-6/*n*-3 ratio to below 4:1, the recommended value for human diet [Kralik *et al.* 2008].

Eggs from linseed-fed hens were not enriched only with ALA, but also with EPA and DHA, because ALA is their precursor through the desaturation-chain elongation pathway in the liver [Nain *et al.* 2012]. This conversion requires the same desaturase and elongase enzymes necessary for the synthesis of arachidonic acid from linoleic acid, and due to this competition the concentration of arachidonic acid in yolks produced by hens fed the *n*-3 PUFA-supplemented diets is lower than those from hens fed the C diet [Jia *et al.* 2008, Nain *et al.* 2012]. After the achievement of equilibrium, compared to GL diet, the EL diet was responsible for higher concentrations of *n*-3 PUFA, ALA, and EPA in egg yolks, with a subsequent reduction of their *n*-6/*n*-3 ratio. The ability of the extruded form of linseed (EL diet) to anticipate and improve the deposition of all the *n*-3 FAs in yolks (Tab. 3 and 4) needs to be stressed here. This likely means that the extruded form of linseed led to a higher and faster absorption of PUFA when compared to the ground form, especially with regard to *n*-3 PUFA. This could be explained by the fact that the extruded form was deprived of anti-nutritional factors and became more readily digestible, as reported by Thacker *et al.* [2005].

Colour of egg yolk

Yolk colour is a relevant quality trait that affects egg consumer acceptance [Ebeid 2011, Krawczyk 2009, Laca *et al*. 2009]. In the present study yolk colour was modified by the dietary *n*-3 PUFA sources and by the diet × phase of feeding interaction. On the whole, dietary n-3 PUFA enrichment did not enhance yolk colour when compared to the C diet. Although it is well established that the absorption and further deposition of oxicarotenoids responsible for egg yolk pigmentation is expected to increase with the concurrent increase in dietary lipid concentration [Ebeid 2011, Grobas *et al*. 2001], Cachaldora *et al*. [2006] found that the composition of the dietary FA source affects yolk lipid content, and consequently also yolk colour.

The variation of yolk colour throughout the experimental diet supplementation (T) was not significant in terms of lightness (L*) and yellowness (b*) values, whereas a non-linear trend was observed for redness (a*) that did not seem to indicate a biological relationship with T (Tab. 5).

As far as the effects of the experimental diets are concerned, the L* value was significantly affected in the pre-equilibrium phase and showed a tendency to gain in significance after equilibrium ($P<0.1$); in the pre-equilibrium phase L* was found to be lowest in the EL and highest in the GL group. Egg yolks from the linseed-supplemented diets exhibited on average lower a* and b* values when compared to those of C and NF diets both in the pre-equilibrium and equilibrium phases. This could in part be due to linseed's lower oxicarotenoid pigment content in comparison to that of other natural sources of *n*-3 PUFA [Karunajeewa *et al.* 1984] and in part to

Table 5. Effect of feeding n-3 PUFA sources on L*a*b* colour of egg yolks at three times of diet supplementation

Item	Time (T)[1]	Diet (D)				P-value			SEM[2]
		control (C)	extruded linseed (EL)	ground linseed (GL)	microen-capsulated fish oil (NF)	D	T	DxT	
Hens (No.)		18	18	18	18				
Eggs (No.)[3]		90[2]	90	90	90				
Lightness (L*)	Initial	59.8	60.5	60	59.9	0.792			1.59
	Pre-equilibrium	59.6^{bx}	58.2^{a}	60.8^{c}	$60.2b^{c}$	<0.001			1.02
	Equilibrium	61.0^{by}	60.4^{ab}	61.1^{b}	60.1^{a}	0.084			0.93
	P-value Time	0.135	0.123	0.205	0.160	0.250	<0.001	0.008	1.94
Redness (a*)	Initial	-2.16^{x}	-2.38	-1.89^{z}	-2.18^{x}	0.560			0.71
	Pre-equilibrium	-1.96^{ax}	-3.23^{b}	-3.65^{bx}	-2.48^{ax}	<0.001			0.66
	Equilibrium	-1.13^{ay}	-2.25^{b}	-2.96^{by}	-1.13^{ay}	<0.001			0.43
	P-value Time	0.003	0.125	0.002	0.002	<0.001	<0.001	0.009	1.09
Yellowness (b*)	Initial	47.6^{x}	49.2	48.1	48.8	0.686			2.98
	Pre-equilibrium	49.8^{by}	48.0^{a}	47.4^{a}	49.1^{b}	0.008			1.38
	Equilibrium	50.7^{cy}	48.5^{ab}	48.2^{a}	49.5^{bc}	0.009			1.27
	P-value Time	<0.001	0.340	0.484	0.181	0.239	0.049	0.125	2.43

[1]Initial (d0, first day of diet supplementation), Pre-equilibrium (d2 - d14), Equilibrium (d21 - d56).
[2]SEM is the standard error of the least squares means.
[3]No.= 36 eggs for Initial, No.=144 eggs for Pre-equilibrium and No.=180 eggs for Equilibrium measurements.
[a, b, c, d]Means within a rows bearing different superscripts differ significantly at P<0.05.
[x, y, z]Means within a columns bearing different superscripts differ significantly at P<0.05.

an enhanced PUFA peroxidation process that decreases the availability of pigments for deposition in yolk [Calchadora *et al.* 2006, Zaghini *et al.* 2005]. Other authors [Cachaldora *et al.* 2006; Sukombat *et al.* 2006] have reported a decrease in egg yolk colour when adding *n*-3 PUFA sources.

This study demonstrated that dietary enrichment with different *n*-3 PUFA sources partly modified hen weight gain, egg production, yolk weight, egg FA composition, and yolk colour parameters. Compared with the C diet, the fish oil source and the linseed sources significantly improved egg production, but hens showed a reduction in weight gain. Although egg weight was not influenced by dietary treatment, its FA composition was strongly affected, leading to an advantageous reduction of the *n*-6/*n*-3 ratio, while yolk colour was reduced by linseed supplements. When compared to ground linseed, extruded linseed significantly enhanced the rate of inclusion and the relative amount of *n*-3 PUFA in the egg yolk, thus showing the importance of selecting a proper feed form in the formulation of rations.

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