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Original Article

Different N6:N3 Ratios on Performance, Egg Quality, and Health of 81-Week-Old Laying Hens

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ABSTRACT

This study aimed to evaluate different n6:n3ratios on performance, serum biochemical variables, and egg quality in 81-week-old laying hens. A total of 224 laying hens, 81-week-old Hysex White, were utilized and distributed in a completely randomized design consisting of seven treatments and eight replications of four birds per cage, totaling 56 cages. The experimental treatments consisted of seven different n6:n3fatty acid ratios: 1.0:1.0, 2.0:1.0, 4.0:1.0, 8.0:1.0, 16.0:1.0, 32.0:1.0, and 64.0:1.0. For diet formulation, sunflower oil, rich in omega-6, and linseed oil, rich in omega-3, were used. Productive performance, egg quality and serum biochemical variables of laying hens were evaluated at the end of the cycle at 26, 27, and 28 days. Analysis of variance (ANOVA) was conducted and orthogonal contrasts were used to obtain the sum of squares of the treatment of the analysis of variance in polynomial regression effects. Egg quality variables did not vary significantly between the diets. Therefore, diet supplementation with polyunsaturated fatty acids at different ratios was possible without altering egg quality. The only exception was Haugh unit, which displayed a quadratic effect indicating that the best value was the n6:n3ratio of 32, according to data significance. It was concluded that an increase in n6:n3ratios decreases laying hens' feed intake. The n6:n3 ratio of 34.64 provides greater eggs' Haugh units, decreasing from that value on. The ratios of n6:n3 did not influence the hens' serum biochemical variables.

INTRODUCTION

In the past decades, the population's eating habits have been changing due to the increasing search for foods that provide a healthier, longer life. Aiming to reach these consumers that are more and more concerned with healthy food intake, the poultry industry has been searching for technological and nutritional innovations that meet the demands of this new market niche (Bernardino, 2009; Calder, 2018; Bernaerts *et al.*, 2020).

The search for alternatives to improve the final product (i.e. the egg) through the feeding of laying hens is extremely important. It is known that the addition of fat sources to diet compositions is necessary, since they are capable of providing several productive and qualitative benefits to the poultry production system, and have thus been receiving special attention from producers and researchers (Harris *et al.*, 2007; Oliveira *et al.*, 2010a; Dalle Zotte *et al.*, 2015).

A lot of researchers aim to enrich and change fats in animal products by managing feed (Rodrigues *et al.*, 2005; Costa *et al.*, 2008; Santos *et al.*, 2009; Omidi *et al.*, 2015). One of these alternatives, seen as an opportunity for the poultry industry to add value to their product, is feed supplementation with polyunsaturated oils, which are considered essential for birds and are exclusively obtained through lipids from feed (Garcia *et al.*, 2013; Kralik *et al.*, 2020).

Aquatic (fish and algae) and vegetal sources (linseed, canola, and sunflower) of polyunsaturated fatty acids have been studied as ingredients for monogastric animals' diet that increase the presence of these fatty acids in eggs (Harris *et al.*, 2007; Pita *et al.*, 2011; Dalle Zotte *et al.*, 2015; Araujo *et al.*, 2019; Kralik *et al.*, 2020).

A number of studies have been carried out to demonstrate the possibility of enriching eggs through the addition of n-3 polyunsaturated fatty acids (PUFA) in poultry diets (Santos *et al.*, 2009; Cedro *et al.*, 2010; Oliveira *et al.*, 2010b; Ahmad *et al.*, 2013; Hamady *et al.*, 2013; Antruejo *et al.*, 2014; Omidi *et al.*, 2015; Rakita *el al.*, 2016; Panaite *et al.*, 2016). These studies indicated that n-3 PUFA can improve egg quality. However, it is necessary to be careful not to unbalance the diet's n6:n3 ratio, since a large omega-3 supplementation in order to enrich eggs may influence or even harm the birds' health and performance.

The n6:n3fatty acid ratio in the diet is an important factor to determine the appropriate intake of fatty acids, and also to prevent the occurrence of diseases that can interfere in the performance, egg quality, and plasmatic lipid behavior of laying hens (Basmacoglu *et al.*, 2003; Hamady *et al.* 2013; Neijat *et al.*, 2016).

n-6 and n-3 PUFA have several effects on immune and inflammatory response. PUFA balance in the diet and, consequently, their incorporation to the immune cell membrane is important to determine the severity of inflammatory processes (Perini *et al.*, 2010; Yang *et al.*, 2018; Oppedisano *et al.*, 2020).

Thus, this study aimed to evaluate the effect of different n6:n3ratios in incrementing the performance, serum biochemical variables, and egg quality from 81-week-old laying hens.

MATERIAL AND METHODS

The experiment was carried out at the São Paulo State University (Unesp), College of Agricultural and Technological Sciences, Campus of Dracena, Brazil, in the shed for laying hens of the poultry sector. It followed the principles and rules of the Committee for Ethics in Animal Use - CEUA of Unesp, Campus of Dracena (registration number 35/2016).

A total of 224 laying hens, 81-week-old Hysex White, were utilized for a 28-day experimental period. The birds were kept in a production aviary with galvanized wire cages (1.00 × 0.50 m) and provided with frontal feeders and *nipple* drinkers. The adopted experimental design was completely random, consisting of seven treatments with eight replications of four birds per battery cage. The experimental treatments consisted of seven different diet n6:n3 fatty acid ratios: 1.0:1.0, 2.0:1.0, 4.0:1.0, 8.0:1.0, 16.0:1.0, 32.0:1.0, and 64.0:1.0. Due to the lack of information in the literature about the ideal n-6:n3 ratio in diets, we sought to study the largest possible range, since the lack of an initial parameter (value) on this information has not allowed us to start from a specific interval. Thus, the highest possible n-3 inclusion was the 1: 1 ratio, and the highest possible n-6 inclusion was obtained with the 64: 1 ratio. Exponential values were chosen for the study due to the biological aspect of bird physiology relations: in theory, when the relationship is close to 1: 1, any changes have major impacts; while when the ratio is higher (64: 1), a greater change in the ratio is needed to have an effect on the chicken's organism.

For diet formulation, sunflower oil (Cargill Incorporated) containing 50.78% of n-6 and linseed oil (Indústria de Óleos Vegetais Longa Vida Ltda - RS) containing 50.10% of n-3 were used, after determining the profile of fatty acids using a gas chromatographer (Focus CG-Finnigan). The experimental diet formulation (Table 1), with corn and soybean meal, was adapted from Rostagno *et al.* (2017), differing only in relation to the levels of n-6 and n-3. Feeding was *ad libitum,* and feed was distributed in the morning and in the afternoon.

Productive performance of hens

Feed intake (g/bird/day), average egg weight (g), egg production (% eggs/bird/day), egg mass (g/bird/day), feed conversion per egg mass (kg/kg), and feed conversion per egg dozen (kg/dozen) were measured at the end of the 28-day period and evaluated.

Egg quality

On the last days of the cycle (days 26, 27, and 28), three eggs from each plot were collected for egg quality measurements. The height of the albumen was measured halfway between the yolk and the edge of the inner thick albumen by using a vernier caliper (Digimess®). Haugh unit (HU) was measured using a DET 6000, Digital Egg Tester (Tokyo, Japan). Haugh units were calculated from the recorded egg weights and albumen heights using the formula proposed by Card & Nesheim (1978): $HU = 100 \times \log (H - 1.7 \times W^{0.37} + 7.57)$, where HU = Haugh unit, H = height of the albumen (mm), and W = egg weight (g). Albumen index and yolk index were calculated using the following formulas:

Table 1 – Ingredients and nutrient composition of experimental diets.

Composition (g/kg)	Diets (ration n6:n3)						
	1.0:1.0	2.0:1.0	4.0:1.0	8.0:1.0	16.0:1.0	32.0:1.0	64.0:1.0
Corn	563.80	565.60	566.90	567.70	568.02	568.50	568.40
Soybean meal	250.60	250.20	250.00	249.90	249.80	249.70	249.70
Linseed oil	37.06	23.60	13.65	7.38	3.81	1.91	0.92
Sunflower oil	0.44	13.90	23.85	30.12	33.68	35.59	36.62
Choline chloride 60	0.52	0.52	0.52	0.52	0.52	0.52	0.52
Vitamin premix[1]	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Mineral premix[2]	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Kaolin	9.87	8.41	7.34	6.67	6.28	6.07	6.04
L-Lysine	0.83	0.83	0.84	0.84	0.84	0.84	0.84
DL- methionine	3.38	3.38	3.38	3.38	3.38	3.38	3.38
L-threonine	0.73	0.73	0.73	0.73	0.73	0.73	0.73
Limestone	108.11	108.11	108.12	108.12	108.12	108.12	108.12
Dicalcium phosphate	17.25	17.24	17.23	17.23	17.23	17.23	17.23
Salt	3.50	3.50	3.50	3.50	3.50	3.50	3.50
Sodium bicarbonate	2.42	2.42	2.42	2.42	2.42	2.42	2.42
Total	100.0	100.0	100.0	100.0	100.0	100.0	100.0
	Nutritional values						
Metabolizable energy (MJ/kg)	12,14	12,14	12,14	12,14	12,14	12,14	12,14
Crude Protein (g/kg)	158.8	158.8	158.8	158.8	158.8	158.8	158.8
Met dig. (g/kg)	5.58	5.58	5.58	5.58	5.58	5.58	5.58
Met + Cis dig. (g/kg)	7.77	7.77	7.77	7.77	7.77	7.77	7.77
Lysine dig. (g/kg)	7.93	7.93	7.93	7.93	7.93	7.93	7.93
Threonine dig. (g/kg)	6.11	6.11	6.11	6.11	6.11	6.11	6.11
Valine dig. (g/kg)	6.53	6.53	6.53	6.53	6.53	6.53	6.53
Calcium (g/kg)	4.56	4.56	4.56	4.56	4.56	4.56	4.56
Phosphorus dig (g/kg)	3.44	3.44	3.44	3.44	3.44	3.44	3.44
Sodium (g/kg)	2.15	2.15	2.15	2.15	2.15	2.15	2.15
Choline (mg/kg)	270.0	270.0	270.0	270.0	270.0	270.0	270.0
n-6 (g/kg)	18.58	23.70	27.48	29.87	31.23	31.95	32.34
n-3 (g/kg)	18.58	11.85	6.87	3.73	1.95	1.00	0.50
n6:n3	1.000	2.000	4.000	8.000	16.00	32.00	64.00

[1] Vitamin supplement for laying hens (levels of guarantee per kg of feed): retinyl acetate: 2.4 mg, cholecalciferol: 0,057 mg, α-tocopherol acetate: 15 mg, pantothenic acid: 6.44 mg, biotin 0.015 mg, folic acid: 0.5 mg, niacin: 20 g, pyridoxine: 1.7 mg, riboflavin: 3 mg, thiamine: 0.2 mg, cyanocobalamin: 0.01 mg, menadione: 1 mg, and selenium: 0.25 mg. [2] Mineral supplement for laying hens (levels of guarantee per kg of feed): iron 30 mg, copper: 9 mg, manganese 60 mg, zinc 60 mg, and iodine: 1 mg.

Albumen index = Albumen height (mm)/[Albumen length (mm) + Albumen width (mm)] × 100

Yolk index = Yolk height (mm)/Yolk diameter (mm) × 100

Eggshells were washed, oven-dried at 105°C for 6h, and then individually weighed using an electronic scale with accuracy of 0.01 g. Shell thickness was determined by the average value of measurements at three points (air cell, equator, and sharp end) using a digital micrometer (0-25 mm, Digimess®).Specific egg gravity (measured immediately after daily egg collection) was calculated using sodium chloride and water solutions with molar concentrations ranging from 1.065 to 1.125 in increments of 0.005 at volumes of 1,000 mL, following the methodology proposed by Castelló *et al.* (1989). Eggs were individually tested starting from the solution with lowest molarity to that with the highest molarity, with the solution in which the egg began floating being recorded for the specific gravity measurement.

Serum biochemical variables

On the last day of the experimental cycle, five mL of blood were collected from the brachial vein of the birds' wings for posterior analysis of cholesterol and triglycerides. Two birds from each replication were randomly chosen (totaling 16 birds/treatment). The material was stored in previously identified tubes and centrifuged at 3.000G for 10 minutes. The serum was collected and stored in a freezer at -20°C for posterior analysis. The levels of cholesterol and triglycerides were determined by colorimetric enzymatic method utilizing a commercial kit (Gold Analisa Diagnóstico LTDA- Batches: 7015 and 7016), with reading at 500 nm in a spectrophotometer, according to Lumeij's method (1997).

The calculations were performed using the Calibration Factor (FC):

SC = Standard Concentration TC = Test Concentration SA = Standard Absorbance TA = Test Absorbance

FC = SC÷SA

Statistical analysis

The data were analyzed by SAS software (2012) at 5% of probability. An analysis of variance was performed and the effect of the treatments was determined through a regression analysis using orthogonal polynomials adjusted to the exponential levels of n6:n3 ratios.

RESULTS

Productive performance of hens

There was no difference between the treatments for average egg weight, egg production, egg mass, and feed conversion per mass and per egg dozen. The average results referring to the performance of laying hens submitted to diets with different n6:n3ratios can be observed in Table 2.

Regarding feed intake, it was possible to note that it decreased as the n6:n3 ratio of the diet increased, with a linear effect (p=0.042) among treatments. The exception was the 64: 1 ratio, in which there was an increase in consumption as compared to other treatments.

Table 2 – Performance of laying hens at 81-week-old as a function of different n6:n3 PUFA ratios in the diet.

Variables[1]	n6:n3							SEM[2]	Probability [3]	
	1:1	2:1	4:1	8:1	16:1	32:1	64:1		L	Q
FI	123.0	121.6	120.3	123.2	122.0	120.9	128.1	0.872	0.042	0.165
EW	64.07	63.64	64.03	64.07	64.15	65.46	64.77	0.251	0.128	0.249
EP	89.51	83.64	85.49	83.04	88.50	84.26	87.60	0.889	0.654	0.523
EM	57.32	53.29	54.69	53.25	56.77	55.07	56.71	0.591	0.330	0.820
FC (kg/kg)	2.14	2.25	2.20	2.31	2.14	2.20	2.25	0.023	0.651	0.615
FC (kg/dozen)	1.651	1.740	1.701	1.788	1.655	1.731	1.748	0.019	0.464	0.910

[1]FI: Feed intake (g/hen/day); EW: Average egg weigh (g); EP: Egg production (%); EM: Egg mass (g/hen/day); FC (kg/kg): Feed conversion per egg mass (kg feed/kg eggs); FC (kg/dozen): Feed conversion per eggs dozen (kg feed/eggs dz).

[2]SEM: Standard error of means.

[3]L, linear effect; Q, quadratic effect.

Egg quality

The average results egg quality are shown in Table 3. The values found for albumen height, yolk index, albumen index, shell thickness, shell weight, and specific gravity did not present any significant difference between the treatments.

For Haugh unit, it was possible to observe a quadratic effect (p=0.022), with continuous increase of this variable up to a n6:n3ratio of 34.64. From this point on, the Haugh unit decreased again.

Serum biochemical variables

The average results regarding plasmatic cholesterol and triglycerides of laying hens subject to diets with different n6:n3 ratios are shown in Table 4.

There was no significant difference between the treatments for plasmatic cholesterol and triglycerides. These results indicate that different n6:n3 ratios in laying hens' diets did not influence these serum biochemical variables.

Table 3 – Egg quality of laying hens at 81-week-old as a function of different n6:n3 PUFA ratios in the diet.

Variables[1]	n6:n3							SEM[2]	Probability[3]	
	1:1	2:1	4:1	8:1	16:1	32:1	64:1		L	Q
AH	6.46	6.18	6.90	6.83	6.51	6.48	6.67	0.074	0.684	0.939
HU	78.64	78.46	80.81	80.71	81.88	82.93	80.05	0.565	0.414	0.022
YI	0.413	0.409	0.416	0.405	0.402	0.406	0.415	0.002	0.720	0.079
AI	0.073	0.068	0.078	0.077	0.073	0.072	0.074	0.001	0.943	0.883
ST	0.404	0.408	0.412	0.404	0.415	0.400	0.399	0.002	0.117	0.811
SW	6.11	6.24	6.33	6.25	6.43	6.45	6.42	0.043	0.073	0.125
SG	1.103	1.104	1.101	1.104	1.104	1.103	1.103	0.001	0.986	0.660

[1]AH: Albumen Height (mm); HU: Haugh unit; YI: Yolk index; AI: Albumen index; ST: shell thickness (mm); SW: Shell weight (g); SG: Specific gravity.

[2]SEM: Standard error of means.

[3]L, linear effect; Q, quadratic effect.

Table 4 – Serum biochemical variables of laying hens at 81-week-old as a function of different n6:n3 PUFA ratios in the diet.

Variables[1]	n6:n3							SEM[2]	Probability[3]	
	1:1	2:1	4:1	8:1	16:1	32:1	64:1		L	Q
CHO	117.0	112.3	104.6	114.9	110.8	113.8	109.4	3.05	0.814	0.868
TG	77.35	76.98	73.90	77.62	75.23	73.04	71.89	1.427	0.238	0.831

[1]CHO: Cholesterol (mg/dL); TG: Triglyceride (mg/dL).

[2]SEM: Standard error of means.

[3]L, linear effect; Q, quadratic effect.

DISCUSSION

Productive performance of hens

The average egg weight, egg production, egg mass, feed conversion per mass, and feed conversion per egg dozen were not affected by different n6:n3ratios in laying hens' diets. Studies with different n6:n3ratios did not find differences for these performance variables, either (Filardi *et al.*, 2005; Costa *et al.*, 2008).

With respect to feed intake, it was observed that as the n6:n3ratio increased, there was a reduction in this intake until the ratio 32:1, with an increase in the 64: 1 ratio.

Birds are capable of accurately selecting the nutritional balance that meets their physiological demands because they regulate feed intake by balancing the amount of essential nutrients to satisfy their needs and production (Forbes, 1995; Pousga *et al.*, 2005).

For Emmans (1991), energy, nutrients, protein, and minerals are essential in the diet and laying hens regulate the feed intake based on their nutritional requirements. Flavors may initially influence intake and preference, but they soon stop doing so as birds learn that there is no nutritional implication in different flavors (Balog & Millard, 1989).

In this study, laying hens consumed a smaller amount of feed as the n6:n3ratio increased, ie, birds had to consume less food for to meet their needs because there were more n-6 than n-3 (Parra *et al.*, 2008).

Hamady *et al.* (2013) analyzed diets with different n6:n3ratios and verified that feed intake reduces significantly as n6:n3ratios decrease. On the other hand, Puthpongsiriporn & Scheideler (2005), by supplementing laying hens' diets with linseed and corn oils, obtained n6:n3 ratios of 17:1, 8:1, 4:1, and 2:1, and observed that there was no interference in the birds' feed intake and weight gain.

In diets enriched with linseed oil (N-3), laying hens consumed less feed when compared to diets formulated only with soybean and cottonseed oil in the same amounts (Santos *et al.*, 2009).

Egg quality

The analysis of egg quality showed that there were no significant differences for the analyzed variables, except for Haugh unit. Ratios of n-6 and n-3 of 40, 14, and 7 were studied by Hamady *et al.* (2013), who obtained greater egg weight, yolk weight, and shell weight for the greatest studied ratio. The albumen characteristics were not influenced by different ratios. Studies pointed out that n-3 rich lipid sources (linseed oil, fish oil) added to the diet did not alter laying hens' egg quality (Mendonça *et al.*, 2000; Souza *et al.*, 2008; Rakita *et al.*, 2016; Agboola *et al.*, 2016).

Studies showed that some factors that could affect Haugh unit were hen age (Fletcher *et al.*, 1983; Trindade *et al.*, 2007; Ramos *et al.*, 2010; Oliveira *et al.*, 2010b), storage time and temperature (Menezes *et al.*, 2012), and suggested that nutrition contributed little to albumen quality (Roberts Jr., 2004; Samli *et al.*, 2005). Other studies also reported that diet composition did not influence Haugh unit (Kirunda; Mckee, 2000; Grobas *et al.*, 2001; Basmacoglu *et al.*, 2003; Lokaewmanee *et al.*, 2010).

The highest value for HU was obtained in the ratio of 32: 1 and decreased in the ratio of 64: 1, demonstrating that large amounts of n-6 can be bad for this variable. That is explained by fatty acids competing for the same enzymes in the animal's body, which increases plasma concentration of arachidonic acid when there is excess n6 in the diet (Cedro *et al.*, 2011., Santos *et al.*, 2007a).

In this study, it was possible to observe that different n6:n3additions to the diet increased Haugh unit up to 32:1, showing that birds accepted greater n6:n3ratios better in greater proportions than the ones indicated for humans. It is worth to point out that diet addition recommendations for n6:n3fatty acids for both humans and animals are yet to be well defined.

Serum biochemical variables

There was no treatment effect on the amounts of total serum cholesterol and triglycerides. These findings are supported by the literature, which evidences that different n6:n3ratios do not influence the values of triglycerides, total cholesterol HDL, LDL of laying hens (Murata *et al.*, 2003; Ahmad *et al.*, 2014). Likewise, increasing increments in the amount of alpha linolenic acid did not affect cholesterol and triglycerides (Neijat *et al.*, 2016). These results clearly show that different n6:n3 ratios in the diet of laying hens do not influence serum biochemical variables, indicating that a nutritionist may formulate diets concentrated with n-3 or n-6 with no harm.

In conventional diets with corn, soybean meal, and soybean oil, the amount of n-6 is very high (Brown & Hart, 2010). On the other hand, when the aim is to enrich eggs with n-3, diets are overloaded with ingredients that are rich in n-3 such as linseed, increasing the amount of n-3 and providing a diet with different lipid quality from conventional ones. However, our study showed that the serum biochemical variables were not changed in either scenario.

Future research should investigate how increased PUFA concentrations and fatty acid compositions impact birds' health and performance; without negatively impacting final products' taste and quality, while still meeting animals' needs.

CONCLUSION

The increment in n6:n3ratios decreased laying hens' feed intake.The n6:n3 ratio of 34.64 showed the highest value for Haugh Unit, decreasing from this point on, which indicates that this is the best n6:n3 ratio for HU among the studied ones. Other performance and egg quality variables remained the same. Ratios of n6:n3did not influence hens' serum biochemical variables.

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