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Research Article
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Strategy for egg-yolk pigmentation in end-of-cycle laying hens using a combination of natural pigments[1]

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ABSTRACT - The aim of this study was to evaluate performance and egg quality from the addition of two levels of pigment obtained from a combination of annatto seeds (*Bixa orellana*) and hay from the leaves of leucaena (*Leucaena leucocephala*) or moringa (*Moringa oleifera*), or from a mixture of the two. For the study, 280 Lohmann LSL Lite laying hens, 115 weeks old, were used over 84 days, divided into four periods of 21 days. The design was completely randomized in a 3 x 2 + 1 factorial scheme (three types and two levels of pigment, plus a control diet), with seven treatments and five replications of eight birds per replication. Three pigments were used: Pigment 01 – 50% annatto +50% hay from leucaena leaves, Pigment 02 – 50% annatto +50% hay from moringa leaves, and Pigment 03 – 50% annatto + 25% hay from leucaena leaves + 25% hay from moringa leaves. The treatments were T1 – control diet, T2 – diet containing 0.5% Pigment 01, T3 – diet containing 0.5% Pigment 02, T4 – diet containing 0.5% Pigment 03, T5 – diet containing 1.0% Pigment 01, T6 – diet containing 1.0% Pigment 02, and T7 – diet containing 1.0% Pigment 03. The use of pigments comprising a mixture (50%/50%) of annatto seeds with hay, regardless of whether from leucaena, moringa or from both, has no effect on the performance of laying hens or on egg quality. However, the color of the yolk intensifies and the TBARS value is reduced as the pigments are added, with the best result obtained at a level of 1%.

Keywords: *Bixa orellana*. Layers. *Leucaena leucocephala*. *Moringa oleifera*. Pigments.

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INTRODUCTION

Consumer preference for eggs with highly colored yolks, which, according to the region, can range from golden yellow to a deep orange-red, has increasingly required that commercial diets for laying hens be supplemented with pigments to ensure that the egg yolk has the desired color intensity and does not vary over the course of the production cycle (Melo *et al*., 2021).

There is, however, one market for which yolk color is very important; but since this market requires that diets for laying hens be composed of all-natural ingredients, synthetic pigments cannot be used. To meet this ever-growing demand, many studies have been carried out over the years using natural pigments, particularly products from different plant origins (Abou-Elezz *et al*., 2011). According to Moura *et al*. (2011), the most widely used natural pigments in the diets of laying hens derive from annatto (*Bixa orellana*), saffron (*Curcuma longa*), marigold petals (*Tagete serecta*) and paprika (*Capsicum annum*). However, in recent years there has been a growing interest in the pigmentation capacity of hay from such plants as Leucaena (*Leucaena leucocephala*) and Moringa (*Moringa oleifera*), which contain important carotenoid and flavonoid compounds (Abou-Elezz *et al*., 2011).

According to Wood and Carter (1983), the concentration of carotenes in the hay from leucaena leaves varies between 377 and 423 mg/kg, and that of xanthophylls, from 836 to 858 mg/kg. Moringa hay contains 15.25 mg of β-carotene and 100 mg of quercetin per 100 g of hay (Tesfaye *et al*., 2014).

There are several studies in the literature that evaluate the effects of including annatto seeds in the diets of laying hens (Garcia *et al*., 2009, 2015; Garcia; Molino; Berto, 2009; Harder; Canniatti-Brazaca; Arthur, 2007; Nunes Junior *et al*., 2020; Spada *et al*., 2012) with hay from leucaena leaves (Abou-Elezz *et al*., 2011; Atawodi *et al*., 2008; Lopes *et al*., 2014; Okonkwo; Adikpe, 1988; Sekhar *et al*., 1998) or from moringa leaves (Abou-Elezz *et al*., 2011; Ahmad *et al*., 2018; Kakengi *et al*., 2007, Olugbemi; Mutayoba; Lekule, 2010; Sharmin *et al*., 2021; Tesfaye *et al*., 2014; Valdivié; Mesa; Rodríguez, 2016). All of them noted an increase in yolk pigmentation when the ingredients were added, the most appropriate level being the limit at which production remains uncompromised, especially due to the negative effect of the increase in fiber and, in the case of hay, the presence of antinutritional factors.

In studies on the individual use of these ingredients, it was found that 0.5% annatto seeds (Garcia *et al*., 2015; Nunes Junior *et al*., 2020), 2% hay from leucaena leaves (Lopes *et al*., 2014) and 0.5% hay from moringa leaves (Sharmin *et al*., 2021) were enough to improve the color of the yolk in diets where corn is the main source of energy. However, there are no studies in the literature on combining annatto seeds with hay from leucaena or moringa leaves. In view of the above, the aim of this study was to evaluate the productive performance and egg quality of laying hens fed diets based on corn and soybean meal with the addition of two levels of pigments obtained from a mixture of annatto seeds (*Bixa orellana*) and hay made from either leucaena leaves (*Leucaena leucocephala*) or moringa leaves (*Moringa oleifera*), or from a mixture of both.

MATERIAL AND METHODS

Location and facilities

The experimental protocol was approved by the Ethics Committee on the Use of Animals (CEUA – UFC) in Fortaleza, Brazil, under number 0706202101, and is in accordance with the Ethical Principles of Animal Experimentation adopted by the National Council for the Control of Animal Experimentation (CONCEA).

The research was carried out in the Department for Animal Science at the Center for Agricultural Sciences of the Federal University of Ceará – UFC, Pici Campus, Fortaleza, Ceará, from May 27, 2021 to August 18, 2021.

The birds were housed in a conventional brick shed measuring 3 m x 10 m, used for commercial laying hens, that was covered in clay tiles, with a cement floor and a ceiling height of 2.8 m. The shed had a central corridor, with two rows of cages on each side, giving a total of four rows for a total of 320 galvanized wire cages, each measuring 0.25 x 0.45 x 0.40 m (width, length and height) with a capacity for two birds per cage at a density of 562.5 cm^2/bird. The cages were equipped with a 12.5 cm/bird trough feeder and an automatic nipple drinker, with one nipple per cage. The photoperiod was 16 hours, including 12 hours of natural light and 4 hours of artificial light.

The shed had a central fan system, with the temperature and average relative humidity recorded by Datalogger (HOBO®, Bourne, United States). The average temperature and relative humidity in the shed during the experiment was 28.6 ± 1.18 °C and 63.7 ± 7.33%, respectively.

Bird experiment

The study included 280 Lohmann LSL Lite hens, 15 weeks old, with an average weight of 1.650 kg and egg production of 74.86%. The experimental period of 84 days was divided into four periods of 21 days. The birds were selected based on their weight and egg production, and were evenly distributed over the experimental units, four cages with two birds each, so that each replication had birds of similar weight and egg production, as recommended by Sakomura and Rostagno (2016).

Experimental design and diets

The experimental design was completely randomized in a 3 x 2 + 1 factorial scheme, with seven treatments and five replications of eight birds, to evaluate three types of pigment (Table 1) obtained by mixing hay with annatto, two levels of each pigment (0.5% and 1%) and a control diet. The treatments comprised the following diets:

. T1- control diet;

. T2- diet containing 0.5% of pigment 1;

. T3- diet containing 0.5% of pigment 2;

. T4- diet containing 0.5% of pigment 3;

. T5- diet containing 1.0% of pigment 1;

. T6- diet containing 1.0% of pigment 2;

. T7- diet containing 1.0% of pigment 3.

Three pigment additives were prepared based on annatto seeds (*Bixa orellana*) mixed with hay from moringa leaves (*Moringa oleifera*), leucaena leaves (*Leucaena leucocephala*), or a mixture of both.

The materials were first sent to the Animal Nutrition Laboratory (LANA) of the Department of Animal Science at UFC, where the annatto seeds and the different hays were separately ground in a knife-type mill, using a 2 mm sieve. The materials were then weighed and mixed to obtain each pigment in the proportions shown in Table 1.

The experimental bran diets were formulated to be isonutritive, based on the nutritional requirements recommended by the Lineage Manual (Lohmann do Brasil, 2017). The calculations were based on the nutritional values of the ingredients, as suggested by Rostagno *et al*. (2017). The pigments were added to the diets to replace the inert material in the control diet (Table 2).

Assessment of bird performance

When evaluating bird performance, the following variables were analyzed: feed intake (g/bird/day), calculated as the difference in the amount of feed provided at the beginning and end of each phase, discounting any leftovers – in the event of the death of any birds, corrections were made in the respective experimental units; Egg production (%/bird/day), determined from the daily egg production per cage, calculating the production per replication at the end of each period – in the event of the death of any birds, corrections were made in the respective experimental units; egg weight (g/bird/day), obtained by multiplying the number of eggs produced by the average weight of the eggs for each replication during the period; feed conversion by egg weight (g of feed/g of egg) calculated from the ratio of feed intake to egg weight for each replication per period.

Assessment of internal and external egg quality

Egg quality was assessed using specific gravity (g/cm^3), albumen quality (Haugh unit), percentage of yolk, albumen and shell (%), shell thickness (mm) and yolk color.

To determine the above parameters, one day a week during each experimental period all the eggs in each plot were collected, identified and taken to the Egg Quality Assessment Laboratory, located in the Poultry Sector of UFC, where they were stored in air-conditioned rooms at 21 ºC.

After one day in storage, the eggs were weighed individually on a semi-analytical balance with a precision of 0.01 g (Marte Científica™ Model AD1000, max. 1010 g) to determine their average weight. Three eggs were then selected per plot based on the average weight and then submitted to further testing as per Table 3.

Table 1 - Pigments and their Composition

Pigment	Composition
Pigment 01	Annatto (50%) + Leucena (50%)
Pigment 02	Annatto (50%) + Moringa (50%)
Pigment 03	Annatto (50%) + Moringa (25%) + Leucena (25%)

Table 2 - Proximate and nutritional composition of the experimental diets for laying hens

Ingredient (%)	
Corn (CP 7,92%)	64.81
Soya meal (PB 45%)	22.31
Calcitic limestone	9.93
Dicalcium phosphate	1.38
Common salt	0.32
Dl-methionine	0.10
Vitamin supplement[1]	0.10
Mineral supplement[2]	0.05
Inert[3]	1.00
TOTAL	100.00

Continuation Table 2

Calculated Composition	
Metabolizable energy, kcal/kg	2725
Crude protein, %	15.30
NDF[4], %	8.24
ADF[5], %	3.53
Digestible lysine, %	0.32
Digestible methionine + cystine, %	0.54
Digestible methionine, %	0.37
Digestible threonine, %	0.50
Digestible tryptophan, %	0.16
Calcium, %	4.10
Phosphorus, %	0.35
Sodium, %	0.15
Chlorine, %	0.25
Potassium, %	1.02
Magnesium, %	0.11

[1]Composition per kg of product: Vit. A – 9,000,000.00 IU; Vit. D3 – 2,500,000.00 IU; Vit. E – 20,000.00 mg; Vit. K3 – 2,500.00 mg; Vit. B1 – 2,000.00 mg; Vit. B2 – 6,000.00 mg; Vit. B12 – 15.00 mg; Niacin – 35,000.00 mg; Pantothenic acid – 12,000.00 mg; Vit. B6 – 8,000.00 mg; Folic acid – 1,500.00 mg; Biotin – 100.00 mg; [2]Composition per kg of product: Selenium – 250.00 mg; Iron – 100,000.00 mg; Copper – 20.00 g; Manganese – 130,000.00 mg; Zinc – 130,000.10 mg; Iodine – 2,000.00 mg; [3]Inert: Washed sand; [4]Neutral detergent fiber; [5]Acid detergent fiber

First, the specific gravity of the eggs was determined following procedures described by Freitas *et al*. (2004). To obtain the weight of the eggs in both air and water, the egg-weighing system was set up using a semi-analytical balance with a precision of 0.01 g (Marte Científica™ Model AD1000, max. 1010 g). Albumen quality was then assessed by determining the Haugh unit (Haugh, 1937), where the eggs were broken on a flat glass surface and the height (mm) of the dense albumen was measured using an analog depth micrometer (Baxlo Haugh®, Baxlo Precision, Barcelona, Spain). The egg weight and albumen height were applied in the equation: $HU=100 \times \log(H-1.7 \times W^{0.37}+7.6)$, where: UH = Haugh unit; H = albumen height in mm; W = egg weight in g.

The yolk was then immediately separated from the albumen and weighed on a semi-analytical balance with a precision of 0.01 g (Marte Científica™ model AD1000, max. 1010 g), and the percentage obtained by dividing the weight of the yolk by the weight of the egg and multiplying by 100. The color of the yolk was evaluated by comparison using the Digital YolkFan™ application. The broken eggshells were washed and left to dry for 72 hours and then weighed on a semi-analytical balance with a precision of 0.01 g (Marte Científica™ Model AD1000, max. 1010 g); the percentage of shell was determined by dividing the weight of the shell by the weight of the egg and multiplying by 100. The percentage of albumen was obtained by difference, where: albumen = 100 - (percentage of yolk + percentage of shell).

To determine the thickness of the shell, measurements were taken in three regions: the major and minor poles and the equatorial region, using a 0.01 mm digital micrometer (Mitutoyo Company, Kawasaki, Japan) and calculating the average of the results.

Malonaldehyde is found in egg yolks and is an indicator of lipid oxidation, which can alter the color and quality of the yolk. The variable was assessed by determining the level of thiobarbituric acid reactive substances (TBARS) using the aqueous acid extraction method, with adaptations (Cherian *et al*., 2002). Approximately 2 g of the sample were homogenized for one minute with 6.75 ml of perchloric acid (3.86%) and 18.7 µl of BHT (4.5%) in a 15 ml Falcon tube. The homogenate was filtered, and 1 ml was transferred to an Eppendorf tube, together with 1 ml of 2-thiobarbituric acid (20 mM), and heated in an Eppendorf ThermoMixer® for 30 minutes (95 ºC). The solution was then cooled in an ice bath. The reading was taken using a spectrophotometer (700 PLUS - FEMTO®) at 535 nm. The standard curve was obtained using malonaldehyde, and the results were expressed in g of malonaldehyde/kg of yolk.

Statistical analysis of the data

A statistical analysis of the data was carried out using the SAS v. 8.2 software (SAS Institute, 2000). The data were submitted to analysis of variance to evaluate the effect of the treatments and test the factorial. To compare the results of the different treatments with those of the control group, the data from all the treatments were analyzed in a completely randomized design. Dunnett's test was then applied at 5% probability.

The second step was to use the F-test at 5% probability to assess a possible interaction between the type and different levels of pigment. To this end, the data obtained from the control treatment were excluded from the analysis and a 3 x 2 factorial scheme was used (three types and two levels of pigment).

RESULTS AND DISCUSSION

According to the results for the performance parameters of the hens (Table 3), when the effect of the type and level of pigment was evaluated, there was no significant interaction between the factors;

furthermore, the individual factors had no effect on the performance variables. This shows that annatto mixed with hay from leucaena leaves, moringa leaves, or both can be added to the diets of laying hens up to a level of 1% without compromising performance.

The lack of any effect from the pigments on the performance variables may be related to the small amount added to avoid the possibility of negative effects, since the maximum level of annatto seeds and hay in the diet is 0.5% when 1% of each pigment is added. The results are in line with those reported in the literature for the individual use of pigment components in the diets of laying hens. Garcia, Molino and Berto (2009) recommend the addition of up to 0.89% of annatto seeds in sorghum-based diets, while Garcia *et al*. (2015) reported that it is possible to add up to 2.0% of ground seeds in commercial diets for laying hens, and Nunes Júnior *et al*. (2020) found that 0.5% of annatto seeds can be included in corn-based diets.

Leucaena hay can be added to corn- or sorghum-based hen diets at a level of 2% or up to 10% without affecting performance (Lopes *et al*., 2014). While for moringa hay, Kakengi *et al*. (2007), Olugbemi, Mutayoba and Lekule (2010), Abou-Elezz *et al*. (2011), and Valdivié, Mesa and Rodríguez (2016) found that levels of up to 10% had no negative effect on the productive performance of laying hens.

When evaluating egg quality (Table 4), there was no interaction between the factors (levels x pigments); there was, however, a significant difference between the results of the control group and the other treatments for yolk color and lipid oxidation of the yolks evaluated using the TBARS method. The birds fed the control diet had less intensely colored yolks and greater lipid oxidation of the yolks than the birds in the other treatments.

When the effect of the type and level of pigment was evaluated, there was no significant interaction between the factors; there was also no effect from the type of pigment on the variables. However, for the level of pigment, there was a significant difference for yolk color and TBARS value. With the addition of 1% pigment, the yolks showed more pigmentation and a reduction in lipid oxidation.

The increased intensity of the yolk color in the eggs of birds fed with added pigments may be related to a higher carotenoid content from the annatto and the leucaena and moringa hay, as these were added proportionally to replace the inert material in the control diet, ensuring that all the diets had the same proportion of corn, which is the main source of yolk pigment in the diets of laying hens. The different levels of added pigment can therefore increase the pigmentation of the egg yolks compared to yolks from birds fed the control diet, with the best result when adding 1%, regardless of whether the hay used was leucaena, moringa or a mixture of the two.

Table 3 - Performance of commercial laying hens fed diets containing different natural pigments

Treatment	Intake (g/bird/day)	Eggs laid (%/bird/day)	Egg weight (g)	Egg mass (g/bird/day)	Feed conversion by egg mass (g/g)
Control	98.94	66.82	65.61	43.78	2.28
0.5% P1[1]	100.15	71.25	65.61	46.81	2.19
0.5% P2[2]	102.87	70.58	65.22	46.07	2.29
0.5% P3[3]	101.87	68.90	65.08	42.66	2.54
1.0% P1	100.50	68.04	63.95	43.65	2.38
1.0% P2	99.46	72.10	64.53	46.55	2.18
1.0% P3	101.39	69.03	64.51	43.75	2.41
SEM[4]	0.4836	1.2400	0.2748	0.9870	0.0605
ANOVA			*P-value*		
Treatment	0.3294	0.9405	0.6584	0.8958	0.7140
			Level		
0.5%	101.63	70.24	65.30	45.18	2.34
1.0%	100.45	69.73	64.33	44.65	2.32
			Pigment		
P1	100.32	69.65	64.78	45.23	2.28
P2	101.17	71.34	64.87	46.31	2.23
P3	101.63	68.96	64.80	43.21	2.48
SEM	0.5345	1.3864	0.3105	1.1267	0.0696

Continuation Table 3

ANOVA	*P-value*				
Levels	0.2853	0.8657	0.1462	0.8245	0.9159
Pigments	0.6135	0.8017	0.9922	0.5672	0.3580
Lvl × Pig	0.3481	0.8036	0.7553	0.7375	0.5933

[1]50% annatto + 50% leucaena hay; [2]50% annatto + 50% moringa hay; [3]50% annatto + 25% leucaena hay + 25% moringa hay; [4]Standard error of the mean

Table 4 - Characteristics and quality of eggs from commercial laying hens fed diets containing different natural pigments

Treatment	Yolk (%)	Albumen (%)	Shell (%)	Yolk color	SG[1] (g/cm³)	HU[2]	ST[3] (mm)	TBARS (g MDA /kg)
Control	27.27	64.26	8.47	6.10	1.076	90.06	0.33	1.26
0.5% P1[4]	26.95	64.66	8.39	7.12*	1.075	88.12	0.32	1.16*
0.5% P2[5]	26.86	64.56	8.58	7.11*	1.076	88.46	0.34	1.15*
0.5% P3[6]	26.64	64.73	8.63	7.10*	1.076	87.94	0.33	1.13*
1.0% P1	27.55	64.06	8.39	7.78*	1.074	89.11	0.32	1.09*
1.0% P2	26.59	65.07	8.34	7.98*	1.074	88.10	0.33	1.08*
1.0% P3	26.50	65.15	8.35	7.88*	1.074	90.16	0.32	1.07*
SEM[7]	0.1439	0.1608	0.0442	0.1096	0.0003	0.3271	0.0016	0.0165
ANOVA				P-value				
Treatment	0.4246	0.5512	0.4733	< 0.0001	0.4817	0.3209	0.1811	< 0.0176
				Level				
0.5%	26.81	64.65	8.54	7.11 b	1.076	88.17	0.33	1.15 a
1.0%	26.88	64.76	8.36	7.88 a	1.074	89.12	0.32	1.08 b
				Pigment				
P1	27.25	64.36	8.39	7.45	1.075	86.62	0.32	1.11
P2	26.72	64.82	8.46	7.55	1.075	88.28	0.33	1.12
P3	26.57	64.94	8.49	7.49	1.075	89.05	0.33	1.11
SEM	0.1640	0.1838	0.0512	0.0809	0.0004	0.3428	0.0018	0.0124
ANOVA				P-value				
Levels	0.8530	0.7729	0.1064	< 0001	0.0560	0.1797	0.0639	< 0.0068
Pigments	0.2304	0.4313	0.6978	0.6314	0.9198	0.6603	0.2495	0.8339
Lvl × Pig	0.5230	0.4234	0.4917	0.5398	0.8852	0.3205	0.4033	0.7281

[1]Specific gravity; [2]Haugh unit; [3]Shell thickness; [4]50% annatto +50% leucaena hay; [5]50% annatto + 50% moringa hay; [6]50% annatto + 25% leucaena hay + 25% moringa hay; [7]Standard error of the mean. *Significant statistical effect by Dunnett's test (P < 0,05). Mean values followed by different letters in a column differ by F-test (P < 0.05)

The effects of the pigments on the intensity of yolk color are consistent with reports in the literature on the individual use of pigment components in the diets of laying hens. According to Garcia *et al*. (2015), the addition of ground annatto seeds to a commercial hen diet increased yolk pigmentation – from 0.5% onwards, pigmentation was significantly higher than in the diet with no annatto. Similar results were found by Lopes *et al*. (2014) when adding 2% hay from leucaena leaves to corn-based diets for laying hens. Using hay from moringa leaves, Kakengi *et al*. (2007), Olugbemi, Mutayoba and Lekule (2010), and Abou-Elezz *et al*. (2011), Valdivié, Mesa and Rodríguez (2016) saw an increase in yolk pigmentation; however, the level of hay is limited to 10% due to the adverse effects on egg production at higher levels.

The addition of 1% pigment promoted a reduction in the value of TBARS regardless of whether the hay was from moringa or leucaena, or a mixture of the two. The reduction in the lipid oxidation of egg yolks from birds fed with added pigments in relation to the control diet, may be related to polyphenolic compounds present in annatto and in the hays from leucaena and moringa. These compounds act synergistically, to protect against lipid oxidation of the yolk and increase the shelf life of stored eggs, with a beneficial effect on human health (Xu *et al*., 2018).

The protective effect from increasing the level of added pigments to 1% is mainly due to the flavonoids present in the plants, especially quercetin in leucaena, which accounts for 11.2 g/kg DM, and has a high antioxidant capacity (Xu *et al*., 2018). Among other polyphenolic constituents, moringa includes phenolic acids (gallic acid, chlorogenic acid, ellagic acid and ferulic acid) and flavonoids (kaempferol, quercetin and rutin), substances with important antioxidant activity, attributed to the protective effect against lipid oxidation, and which are transferred to the egg (Verma; Vijayakumar; Mathela, 2009). Ahmad *et al*. (2018) found that adding 0%, 0.5%, 1.0%, and 1.5% moringa leaf powder to the diets of laying hens affords a linear increase in quercetin deposition in the egg yolks.

In a proportionate mixture with annatto seeds, hay from the leaves of leucaena and moringa acts as a pigment and protects against the lipid oxidation of egg yolks, and can be used as a natural product to intensify egg yolk pigmentation in corn-based feed.

CONCLUSION

The use of pigments made up of a proportionate mixture (50%/50%) of annatto seeds and hay, whether from leucaena or moringa leaves, or from a mixture of both, has no effect on the performance or quality of the albumen or shell of the eggs of laying hens at 115 weeks of age. However, the yolk color intensifies and there is a reduction in the TBARS value of the egg yolks with the addition of the pigment, the best results being obtained at a level of 1%.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analysed during the current study are available in the [http://www.repositorio.ufc.br/handle/riufc/69129].

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