Effects of duration and supplementation dose with astaxanthin on egg fortification

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ABSTRACT Long-term and graded dose of astaxanthin supplementation in laying hen's diet was assessed for egg fortification. Five groups of laying hens with 8 replications each were fed for 24 wk with diet supplemented astaxanthin at 0 mg/kg (control), 7.1 mg/kg, 14.2 mg/kg, 21.3 mg/kg, and 42.6 mg/kg (**Basal, A7, A14, A21,** and **A42,** respectively). The performance of laying hens, egg quality, astaxanthin concentration as well as conversion efficiency and geometric isomers proportion in yolks were assessed on wk 8 and 24. One-way analysis of variance (**ANOVA**) and linear and quadratic regression analyses were used to evaluate the dose effect. In parallel, the Student's *t* test compared the values between wk 8 and wk 24 of test within a group. Overall, the results revealed that neither production performance nor egg physical quality was affected by astaxanthin dose level and feeding duration. Following the supplementation dose, the redness of yolks (**a***) increased ($P < 0.001$). But, the a* score in A42 (23.48) was just 3-folds the a* score in A7 (8.89). Concentration of astaxanthin in eggs was dose-level dependent showing a linear relationship ($P < 0.001$) with a slight declination observed in all groups on wk 24 compared to wk 8. The deposition rate of astaxanthin into egg yolk was higher in A21 and A42. The proportion of geometric isomers in egg yolk were not affected by the feeding duration. As the supplementation dose increased, all-trans isomer proportion gradually decreased in the egg yolk, while 13-cis isomer proportion rose. It was concluded that astaxanthin is an efficient carotenoid for egg fortification, which can be supplemented in diet up to 42.6 mg/kg for 24 wk without compromising the performance of laying hens or physical quality of eggs. This appreciably affects the egg yolk color and confers a better accumulation of total astaxanthin and cis isomers into eggs as the supplementation dose increases.

Key words: astaxanthin, laying hen, long-term supplementation, fortification, geometric isomer

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INTRODUCTION

Among the numerous macro and micro-nutrients found in egg, carotenoids are sort of fat-soluble compounds present in the yolk. Various roles, especially, the antioxidant activity played by these compounds can protect humans from degradative processes and cardiovascular disease as well as improve the immune system (Kovacs-Nolan et al., 2005; Miranda et al., 2015). Interestingly, advances in nutritional research lately leading to "designer egg" approach allows further egg enrichment with desired nutrients including carotenoids by acting on the formulation of a layer's diet. Carotenoid-enriched eggs can increase human intake of carotenoids without changing the diet. In this regard, different carotenoids have been incorporated into eggs and depending on the country, they are recognized as egg-enriching products (Seuss-Baum, 2007; Nimalaratne et al., 2013).

"Astaxanthin, the king of carotenoids" as called by Nguyen (2013), is a xanthophyll carotenoid naturally found in yeasts, microalgae (specifically the *Haematococcus pluvialis)*, and some wild species of fishes, crustaceans, and birds (Ambati et al., 2014; Visioli and Artaria, 2017). Since the 1970s, astaxanthin was used as a dye and pigment factor for fishes and crustaceans (European Council directive, 1970). In 1995, it was approved as feed additive to salmonid fish by the United States Food and Drug Administration in response to a petition filed by Hoffman-La Roche, Inc., earlier

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(Department of Health and Human Services, Food and Drug Administration, 1995). In the *Haematococcus pluvialis*, astaxanthin is present in optical stereoisomer (3S,3'S) and geometrical isomers (trans and cis) which after consumption are selectively scattered in tissues and have different potency in the treatment of diseases (Hussein et al., 2006; Dhankhar et al., 2012). In vitro and in vivo, the cis isomers were found more efficient in fighting oxidative stress than all-trans isomer (Liu and Osawa, 2007; Yang et al., 2017a,b).

From diet to egg, the amount of carotenoid consumed is one of the factors that influence its transport (Moreno et al., 2016). Other factors such as food matrix, genetic factors, interaction with other nutrients, and species of carotenoids, could be responsible of a lower or higher bioavailability and bioconversion of carotenoids (Castenmiller and West, 1998; Moran et al., 2018). Besides, studies showed that the isomers of astaxanthin were absorbed in different proportions (Bjerkeng et al., 1997; Rüfer et al., 2008). A similar finding was reported on lycopene and β-carotene as well (Yeum and Russell, 2002).

Researchers have tested astaxanthin efficacy for improving the egg yolk color (Johnson et al., 1980; Akiba et al., 2000; Yang et al., 2006; Anderson et al., 2008) and the nutritional quality of eggs (Walker et al., 2012). Otherwise, next to previous researches, astaxanthin accumulation in yolk and deposition of its isomers following a graded supplementation in diet are important criteria to be considered for egg fortification. To the best of our knowledge, there is no study regarding the long-term supplementation of astaxanthin in the diet of laying hens. Therefore, this study aimed to assess the production performance of laying hens and physical quality of eggs. More importantly, the yolk color, deposition of astaxanthin, and geometrical isomers in eggs following a graded dose alongside a long-term supplementation of astaxanthin in the diet of laying hens.

MATERIALS AND METHODS

Animals and Diets

Six hundred Hy-Line Brown laying hens, aged 18-week-old, were involved in a 24-week experiment. All the experimental procedures followed the standard regulation of animal welfare and ethical of Institute of Animal Sciences of Chinese Academy of Agricultural Sciences (IAS 2019-26). The poultry house was permanently ventilated and cleaned twice a day. Room temperature was maintained around 21°C, the relative humidity was about 55% and CO_2 concentration was 0.06%. Throughout the experiment, a photoperiod of 16 h was maintained on a daily basis and the light intensity was 20 Lx. The hens were reared in a battery system comprising 3 tiers per heap where a nipple drinker was installed. The layers were randomly divided into 5 groups of 8 replications each. A replication consisted of 15 laying hens having free access to feeds. All nutrients and energy in the basal diet were formulated to meet or exceed the National Research Council requirements for laying hens (NRC, 1994) and the feed ingredients were mashed. In order to prepare the laying hens to the experimental diets, they were all fed with the basal diet for one wk. Afterward, at 19-week-old, the different groups were fed with the basal diet supplemented with Astaxanthin microcapsules powder AstALPHY (Yunnan Erkang Biotechnology Co., Ltd., Kunming, China) at 0% (control group), 0.025%, 0.05%, 0.075%, and 0.15% of diet to provide 0 mg/kg, 7.1 mg/kg, 14.2 mg/kg, 21.3 mg/kg, and 42.6 mg/kg of astaxanthin respectively. The control and astaxanthin-fortified groups (0 mg/kg, 7.1 mg/kg, 14.2 mg/kg, 21.3 mg/kg, and 42.6 mg/kg) were named Basal, A7, A14, A21, and A42 respectively. Astaxanthin microcapsules powder AstALPHY (byproduct from *Haematococcus pluvialis*) contained 2.84% of astaxanthin. The experimental diet composition and nutritional component are presented in Table 1.

Production Performance of Laying Hens

During the trial, monitoring of the total egg weight, number of laying hens, broken eggs, and abnormal eggs were done day by day. Fortnightly, feed consumption was calculated. Egg production (**EP**), average egg weight (**EW**), daily egg mass (**DEM**), average daily feed intake (**ADFI**), and feed conversion ratio (**FCR**) were determined as described by Liu et al. (2020).

Egg Physical Quality Analysis and Yolk Color Test

Different analyses were schemed in order to assess the quality of eggs from the replications and groups. Then, on wk 8 and 24, 3 fresh eggs were collected from each replication. The egg shape was calculated as length/width using an electronic digital vernier caliper (Qingdao Wepro Tool Co., Ltd., Qingdao, China) for the measurement. The eggshell thickness and eggshell strength were determined respectively with eggshell thickness gauge and egg force reader (ORKA Food Technology Ltd., Ramat HaSharon, Israel). Sonova egg quality analyzer (ORKA Food Technology Ltd., Ramat HaSharon, Israel) was used for egg weighting and Haugh Unit determination. In addition, precision colorimeter analyzer CR-400 (Konica Minolta Inc., Chiyoda, Japan) was used for the International Commission on Illumination L*a*b* model (CIE L*a*b*) values determination of egg yolk color. **L*, a*,** and **b*** corresponded to lightness, redness, and yellowness respectively. The component of each egg (yolk and eggshell) was weighted with analytical balance Sartorius BSA224S-CW (Nona Technologies Pvt. Ltd., Kartanaka, India). Therefore, the proportion of each component in the whole egg was calculated as component weight/corresponding egg weight. The 3 egg yolks per replication were thoroughly blended and kept at -20°C temperature for further analysis.

Table 1. Experimental diet composition and nutritional component of the control and treatment groups.

Items	Basal[1]	A7[1]	A14[1]	A21[1]	A42[1]
Composition (%)					
Corn	60.92	60.92	60.92	60.92	60.92
Soybean meal	26.65	26.65	26.65	26.65	26.65
Soybean oil	0.60	0.60	0.60	0.60	0.60
Limestone	9.00	9.00	9.00	9.00	9.00
$CaHPO_4$	1.00	1.00	1.00	1.00	1.00
Premix[2]	0.66	0.66	0.66	0.66	0.66
DL-Met	0.17	0.17	0.17	0.17	0.17
Zeolite powder	1.00	0.975	0.950	0.925	0.850
Astaxanthin microcapsules powder[3]	0	0.025	0.050	0.075	0.150
Total	100.00	100.00	100.00	100.00	100.00
Nutritional components[4]					
Metabolisable Energy (kcal/kg)[5]	2,665	2,665	2,665	2,665	2,665
Crude Protein (%)[6]	16.50	16.50	16.50	16.50	16.50
Calcium (%)[6]	3.40	3.40	3.40	3.40	3.40
Non-phytate phosphorus (%)[5]	0.34	0.34	0.34	0.34	0.34
Lysine (%)[6]	0.86	0.86	0.86	0.86	0.86
Methionine (%)[6]	0.43	0.43	0.43	0.43	0.43
Methionine + Cystine (%)[6]	0.73	0.73	0.73	0.73	0.73
Lysine / Methionine	1.98	1.98	1.98	1.98	1.98

[1]Basal, A7, A14, A21, and A42 correspond to the different groups of laying hens fed diet supplemented with astaxanthin at 0 mg/kg (control), 7.1 mg/kg, 14.2 mg/kg, 21.3 mg/kg and 42.6 mg/kg respectively.

[2]Premix provided per kilogram of diet: vitamin A (vitamin A acetate): 12 500 IU; vitamin D_3: 425 IU; vitamin E (*DL*-α-Tocopherol acetate): 15 IU; vitamin K: 2 mg; vitamin B_1: 0.98 mg; vitamin B_2: 8.5 mg; vitamin B_6: 8 mg; D-pantothenic acid: 50 mg; niacin: 32.5 mg; biotin: 2 mg; folic acid: 5 mg; vitamin B_{12}: 5 mg; Cu (copper sulfate): 8 mg; I (potassium iodide): 1 mg; Fe (ferrous sulfate): 60 mg; Se (sodium selenite): 0.3 mg; Mn (manganese sulfate): 65 mg; Zn (as zinc sulfate): 66 mg; phytase: 500 mg; NaCl: 3 g; choline 500 g; ethoxyquin: 100 mg.

[3]Astaxanthin microcapsules powder, AstALPHY™, is a bioproduct from *Haematococcus pluvialis*, manufactured by Yunnan Erkang Biotechnology Co., Ltd., containing 2.84% of astaxanthin. Astaxanthin microcapsules powder was incorporated to the basal diet by replacing zeolite powder at 0.025%, 0.050%, 0.075%, and 0.150% in order to make 7.1 mg, 14.2 mg, 21.3 mg and 42.6 mg of astaxanthin per kilogram of feed respectively.

[4]The nutritional components were measured and calculated for the control group diet (Basal) and then extended to other groups.

[5]Metabolizable Energy (11.16 MJ/kg) and non-phytate phosphorus are calculated values.

[6]Crude protein, calcium, lysine, methionine, and cystine are measured values.

Analysis of Astaxanthin Concentration

Astaxanthin was determined in feeds and egg yolk with reference to Bjerkeng et al. (1997) and Du et al. (2016) with modification. For the determination in egg yolk, 1 g aliquot of yolk from the blend of 3 egg yolks per replication was weighed. Thereafter, 5 mL of a solution of tetrahydrofuran/methyl alcohol 50% v/v was added and the whole was vortexed for 2 min to form a homogeneous mixture. After that, the mixture was heated in water bath at 60°C for 20 min and vortexed for 2 min. Then, 5 mL of ethyl acetate was added to the mixture which was vortexed again. A separation ensued with centrifuge at 4000 rpm for 5 min. After that, 1 mL of supernatant was transferred into 1.5 mL microcentrifuge tube and centrifugated at 10,000 rpm for 10 min. Finally, the substance was filtered through 0.45 μm strainer into HPLC vials.

The HPLC phase was performed with Shimadzu Prominence LC-20A (Shimadzu Corp., Kyoto, Japan). The temperature of the column (C30, 250 mm × 4.6 nm, 5μm) was 25°C. The emission wavelength of the detector was 474 nm. The column velocity was set at 1.0 mL/min, the injection volume at 10 μL, and the test duration at 12 min. The mobile phase was solvent A: 92% (methanol: tert-Butyl methyl ether, 81:15) and solvent B: 8% (ultrapure water). The HPLC ran 2% solvent A and 98% solvent B isocratically.

Astaxanthin isomer standards, all-trans (41659-1MG, Merck KGaA, Darmstadt, Germany), 9-cis (51881-1MG, Merck KGaA, Darmstadt, Germany), and 13-cis (52991-1MG, Merck KGaA, Darmstadt, Germany) were analyzed at different concentrations such as 0.1, 0.5, 1, 2, 5, and 10 μg/mL. The calibration curves of the standards were considered reliable and used for astaxanthin determination in samples when the coefficient of determination (R^2) equal to 0.995 or higher. Astaxanthin content in a sample was determined on the basis of the sample weight, sample dilution rate, sample curve shape, and the curve equations of the different isomer standards. Approximation was made on the deposition rate of astaxanthin in egg yolk and calculated as:

$$\text{Astaxanthin deposition rate} = \frac{\text{astaxanthin content} \times \text{yolk weight} \times \text{egg production}}{\text{average feed intake} \times \text{supplementation dose}} \times 100$$

14 d of feed consumption before each test was considered for calculating the average feed intake and the egg production.

Analysis of astaxanthin in diets was extremely lower than the detection limit of the standards used for the test. Only the highest dose supplementation (42.6 mg/kg) was detected in feed. Astaxanthin might have interfered with zeaxanthin or formed complex bounds with proteins in feeds which limited the detection as reported by Du et al. (2016). However, the darkening of the color of the diet and the change of the color of the egg yolks

indicated that the test results did not match with the astaxanthin analysis in diets. Therefore, the calculated values in the diet formulation were considered to perform the statistical analysis.

Statistical Analysis

All analysis were made with R version 3.6.1 (The R Foundation for Statistical Computing). First, normality of data was verified using Shapiro-Wilk test and then followed the Levene's test for homogeneity of variances checking. Therefore, one-way analysis of variance (ANOVA) was conducted. Tukey post-hoc test was used for performing multiple comparisons of means. Linear and quadratic regression analysis were carried out for evaluating the relationship existing between the measured parameters and expository doses. The Student's t test was used to compare data on wk 8 and 24 within a group. GraphPad Prism (version 7.00) was used for plotting graphs. Differences were declared significant at probability level $P < 0.05$ and results presented as mean ± SEM.

RESULTS AND DISCUSSION

Production Performance of Laying Hens

The production performance of laying hens during the experiment is summarized in Table 2. Overall, EP, EW, and DEM did not statistically vary among groups. Similar results were found in previous studies for astaxanthin as well as canthaxanthin (Anderson et al., 2008; Weber et al., 2013). However, ADFI showed significant difference ($P = 0.009$) between groups. Basal and A42 groups exhibited the highest values (124.07 g/hen/d and 123.16 g/hen/d respectively), while A7 group showed the lowest value (112.87 g/hen/d). This was followed by A14 group (114.80 g/hen/d), and A21 group (115.38 g/hen/d) sequentially. The Tukey test revealed that A7 group differed from Basal and there was no significant difference between the astaxanthin-supplemented groups for ADFI. The regression analysis presented a quadratic relationship ($P = 0.007$). ADFI in the present study especially decreased in the lowest dose increment of astaxanthin. Our results are not similar to those reported by Ao and Kim (2019) in feeding Pekin duck with *Phaffia rhodozyma* derived astaxanthin. It was observed that astaxanthin contributed to a numerical increase of ADFI of birds in astaxanthin supplemented groups. These differences demonstrate the inconsistency of the effect of astaxanthin on feed intake. Thus, the slight variations observed in our study might be related to personal physiology status of the laying hens. The reduction of ADFI in the groups did not affect the productive performance of layers so that the FCR values followed the same trends as ADFI.

Egg Physical Quality

The results of the physical quality of eggs collected on wk 8 and 24 of the experiment are presented in Table 3. Egg shape, eggshell strength, egg yolk proportion, eggshell proportion, and Haugh Unit values appeared to not be affected by the dose supplementation of astaxanthin. Similar findings were reported by Yang et al. (2006), who did not observe changes in eggs after feeding laying hens with astaxanthin supplemented diet for 14 d. Unlike eggshell strength and eggshell proportion, eggshell thickness on wk 24 showed a difference ($P = 0.028$). However, a consideration of the Tukey's test showed no difference between pairs of group means. Regression analysis revealed both linear ($P = 0.007$) and quadratic ($P = 0.012$) relationships. Englmaierová and Skřivan (2013) did not find correlation between eggshell strength increase and eggshell thickness following a supplementation of lutein to laying hens diets. Likewise, shell thickness did not present similar trend as eggshell strength nor the shell proportion in egg in our study. With regard to albumen proportion, the slight discrepancy observed on wk 8 followed a quadratic relationship ($P = 0.012$) with A21 showing the lowest content (65.59%). That trend appeared to assume normality on wk 24. Moreover, Haugh Units were not statistically different either on wk 8 or 24. This translated into the albumen quality not being affected by astaxanthin supplementation. Overall,

Table 2. Production performance of laying hens during the trial[1].

							P value		
Items	Basal[2]	A7[2]	A14[2]	A21[2]	A42[2]	SEM[3]	ANOVA	Linear	Quadratic
EP (%)[4]	88.10	89.78	88.29	86.74	87.49	1.29	0.610	0.386	0.666
EW (g)[5]	59.83	59.59	59.77	59.91	59.58	0.33	0.954	0.757	0.906
DEM (g/hen/d)[6]	52.77	53.68	52.96	52.04	52.10	0.75	0.575	0.248	0.517
ADFI (g/hen/d)[7]	124.07[a]	112.87[b]	114.80[ab]	115.38[ab]	123.16[ab]	2.53	0.009	0.429	0.007
FCR (g/g)[8]	2.38[a]	2.13[b]	2.20[ab]	2.25[ab]	2.39[a]	0.06	0.011	0.193	0.019

[a,b]Means within a row with no common superscript indicate significant difference between groups ($P < 0.05$).
[1]Data are presented as means ± SEM (n = 8; a replication consisted of 15 laying hens).
[2]Basal, A7, A14, A21 and A42 correspond to the different groups of laying hens fed diet supplemented with astaxanthin at 0 mg/kg (control), 7.1 mg/kg, 14.2 mg/kg, 21.3 mg/kg, and 42.6 mg/kg, respectively.
[3]SEM: pooled SEM.
[4]EP: egg production.
[5]EW: average egg weight.
[6]DEM: daily egg mass.
[7]ADFI: average daily feed intake.
[8]FCR: feed conversion ratio.

Table 3. Egg physical quality tested on wk 8 and wk 24[1].

Items	Basal[2]	A7[2]	A14[2]	A21[2]	A42[2]	SEM[3]	*P* value		
							ANOVA	Linear	Quadratic
Egg shape (mm)									
Wk 8	1.28	1.27	1.28	1.28	1.28	0.01	0.901	0.839	0.975
Wk 24	1.28	1.29	1.29	1.30	1.29	0.01	0.318	0.136	0.123
Shell thickness (mm)									
Wk 8	0.44	0.41	0.44	0.44	0.46	0.01	0.121	0.068	0.162
Wk 24	0.36	0.36	0.37	0.38	0.38	0.01	0.028	0.007	0.012
Shell strength (kg/cm^2)									
Wk 8	4.20	4.59	4.48	4.22	4.35	0.17	0.453	0.893	0.904
Wk 24	4.57	4.60	4.65	4.72	4.62	0.14	0.961	0.764	0.769
Yolk proportion (%)									
Wk 8	23.68	23.74	24.36	24.61	23.76	0.30	0.124	0.748	0.050
Wk 24	26.44	25.74	25.82	26.46	26.50	0.37	0.149	0.401	0.529
Albumen proportion (%)									
Wk 8	66.81	66.67	65.75	65.59	66.58	0.32	0.036	0.590	0.012
Wk 24	63.44	64.18	64.22	63.48	63.48	0.35	0.345	0.512	0.529
Shell proportion (%)									
Wk 8	9.51	9.59	9.89	9.80	9.66	0.10	0.090	0.401	0.050
Wk 24	10.13	10.08	9.96	10.06	10.01	0.11	0.843	0.498	0.680
Haugh Unit									
Wk 8	93.03	91.59	93.36	90.30	90.73	1.19	0.291	0.153	0.339
Wk 24	87.75	88.38	88.82	84.58	87.60	1.18	0.161	0.539	0.537
Wk 8 × wk 24 *t* test									
Egg shape	0.750	0.107	0.085	0.066	0.153				
Shell thickness	<0.001	<0.001	0.003	<0.001	<0.001				
Shell strength	0.190	0.962	0.418	0.017	0.155				
Yolk proportion	<0.001	0.008	0.012	0.006	<0.001				
Albumen proportion	<0.001	0.001	0.018	0.006	<0.001				
Shell proportion	0.031	0.004	0.483	0.137	0.034				
Haugh Unit	0.004	0.078	0.070	0.058	0.156				

[1]Data are presented as means ± SEM (n = 8; a replication consisted of 3 eggs from different laying hens). Differences are significant at $P < 0.05$.
[2]Basal, A7, A14, A21 and A42 correspond to the different groups of laying hens fed diet supplemented with astaxanthin at 0 mg/kg (control), 7.1 mg/kg, 14.2 mg/kg, 21.3 mg/kg, and 42.6 mg/kg respectively.
[3]SEM: pooled SEM.

variations observed between data on wk 8 and 24 for A7, A14, A21 and A42 were also noted for Basal; suggesting that these differences are not dependent on the supplementation duration of astaxanthin.

Egg Yolk Color

The 3 parameters L*, a*, and b* were different between groups ($P < 0.001$) during the trial (Figure 1). Egg yolk color changes from one group to another appeared to vary with the astaxanthin supplementation dose in diet. A similar result was reported by Gernat (2001) in supplementing diet of laying hens with shrimp meal containing astaxanthin. In fact, astaxanthin like other carotenoids is a fat-soluble compound that accumulates in egg yolk with lipid metabolism and confers the latter a dark reddish coloration (Surai et al., 2001). While a* scores critically increased ($P < 0.001$) with the supplementation dose (from 0.48 to 23.87 on wk 8 and 0.66 to 23.48 on wk 24), L* and b* scores decreased ($P < 0.001$) but less considerable with comparison to a* score progression. This is in agreement with the findings of Akiba et al. (2000). Astaxanthin significantly increased the redness of egg yolks resulting in a slight reduction of lightness and yellowness. Besides, a* score in A42 (23.87 and 23.48 on wk 8 and 24, respectively) is just about 3-folds a* score in A7 (8.28 and 8.89 on wk 8 and 24 respectively). This consideration suggests that astaxanthin adequately impacts the redness of egg yolk and might meet consumers' preference for egg yolks color that vary worldwide as reported by Grashorn (2016).

Experiments conducted by Nelson et al. (1990) have shown that egg yolk color change under effect of canthaxanthin started to stabilize from d 10 to 12 with a very slight change appearing after the d 13. Comparison of data on d 13 and 42 showed similar results. Furthermore, Walker et al. (2012) during an 8 wk experiment found that color change following astaxanthin supplementation reached the peak after 8 d of feeding and became stable overtime. This may explain the similarity between data on wk 8 and 24 in our study. Egg yolk color change following astaxanthin supplementation does not vary much with the long duration feeding.

Astaxanthin Concentration in Egg Yolk

As shown in Table 4, the concentration of astaxanthin in egg yolk was closely dependent on the supplementation dose in feed as determined on wk 8 and 24. That evolution followed both linear and quadratic regressions ($P < 0.001$). However, the data plotting showed that linear regression was more suitable to describe these variations (Figure 2A and 2B). Previous studies have demonstrated the dose-related accumulation of astaxanthin in eggs (Akiba et al., 2000; Walker et al., 2012). Our results are in agreement with these studies.

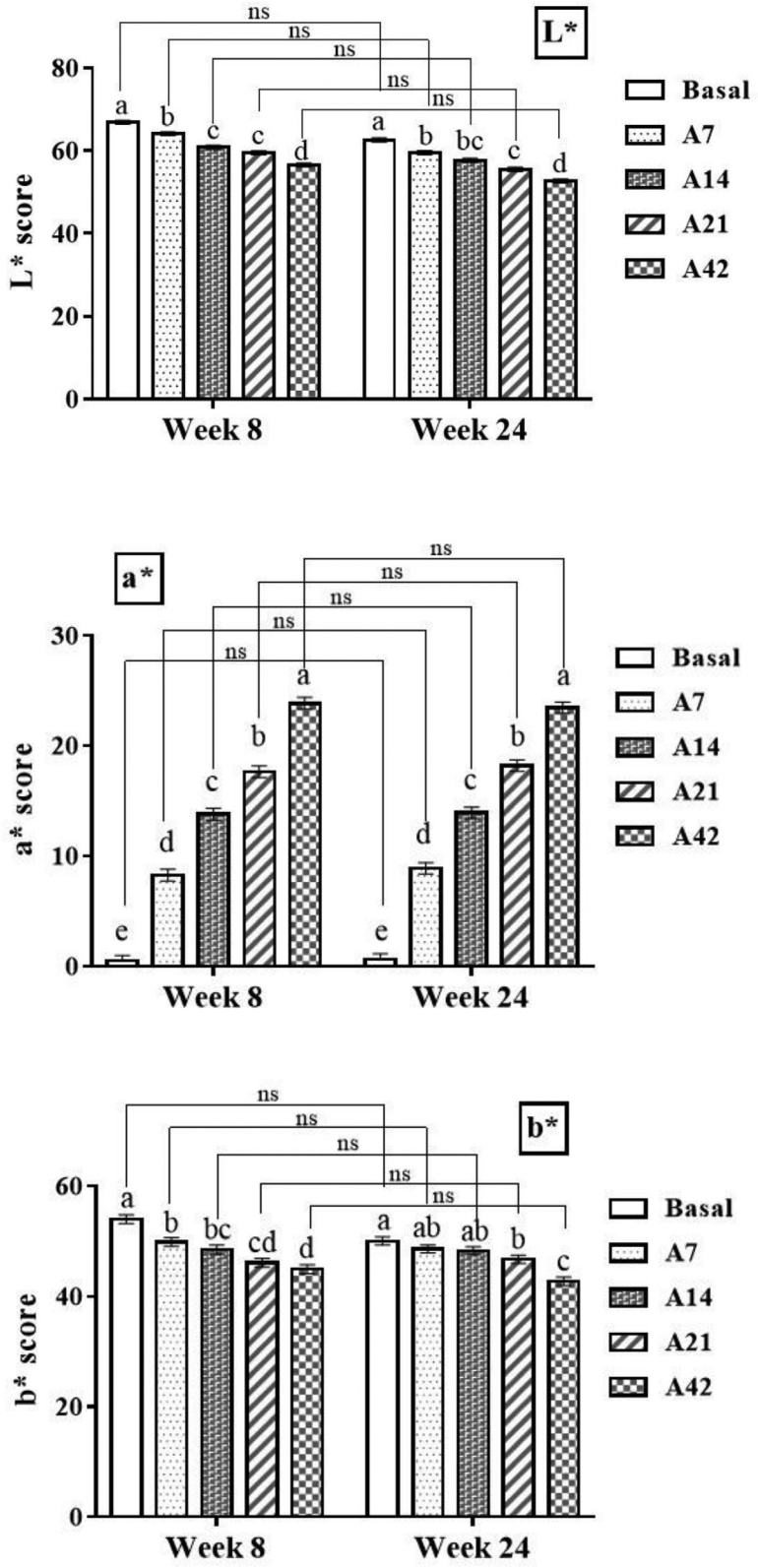

Figure 1. Color test values of egg yolks analyzed on wk 8 and wk 24. Data are presented as means ± SEM (n = 8). a−e: different letters within a time of test indicate significant difference between groups ($P < 0.05$). ns: no significant difference with t test between wk 8 and wk 24. L*: lightness, a*: redness, b*: yellowness. Basal, A7, A14, A21, and A42 correspond to the different groups of laying hens fed diet supplemented with astaxanthin at 0 mg/kg (control), 7.1 mg/kg, 14.2 mg/kg, 21.3 mg/kg, and 42.6 mg/kg respectively.

Despite the non-significant difference revealed by the t test analysis on the whole, astaxanthin numerical content in egg yolk in the groups slightly decreased from wk 8 to 24, except in A7. A14 content decreased from 6.65 to 5.20 μg/g, A21 from 10.67 to 8.77 μg/g, and A42 from 22.13 to 20.23 μg/g on wk 8 and 24 respectively. The study conducted by Torrissen et al. (1995) on salmon fish demonstrated that astaxanthin concentration in muscle

Table 4. Astaxanthin concentration in egg yolk analyzed on wk 8 and wk 24[1].

							P value		
Items	Basal[2]	A7[2]	A14[2]	A21[2]	A42[2]	SEM[3]	ANOVA	Linear	Quadratic
All-trans (μg/g)									
Wk 8	ND[4]	1.63[d]	3.64[c]	5.54[b]	10.82[a]	0.28	<0.001	<0.001	<0.001
Wk 24	ND	1.67[d]	2.92[c]	4.65[b]	9.99[a]	0.28	<0.001	<0.001	<0.001
9-cis (μg/g)									
Wk 8	ND	0.07[d]	0.26c	0.48[b]	1.10[a]	0.03	<0.001	<0.001	<0.001
Wk 24	ND	0.05[c]	0.19[c]	0.40[b]	0.85[a]	0.04	<0.001	<0.001	<0.001
13-cis (μg/g)									
Wk 8	ND	0.72[d]	2.75[c]	4.65[b]	10.20[a]	0.32	<0.001	<0.001	<0.001
Wk 24	ND	0.83[c]	2.09[c]	3.72[b]	9.39[a]	0.30	<0.001	<0.001	<0.001
Total (μg/g)[5]									
Wk 8	ND	2.43[d]	6.65[c]	10.67[b]	22.13[a]	0.62	<0.001	<0.001	<0.001
Wk 24	ND	2.54[d]	5.20 [c]	8.77[b]	20.23[a]	0.60	<0.001	<0.001	<0.001
Total-egg (mg/egg)[6]									
Wk 8	ND	0.04[d]	0.10[c]	0.16[b]	0.32[a]	0.01	<0.001	<0.001	<0.001
Wk 24	ND	0.04[c]	0.09[c]	0.15[b]	0.35[a]	0.01	<0.001	<0.001	<0.001
wk 8 × wk 24 *t* test									
All-trans	-	0.849	0.169	0.003	0.170				
9-cis	-	0.233	0.114	0.215	0.007				
13-cis	-	0.382	0.210	0.005	0.246				
Total	-	0.695	0.181	0.005	0.164				
Total-egg	-	0.178	0.449	0.245	0.193				

[a–d]Means within a row with no common superscript indicate significant difference between groups ($P < 0.05$).

[1]Data are presented as means ± SEM (n = 7; inversely to production performance and egg physical analysis data, one outlier replication value was deleted per group; a replication consisted of a mixed of 3 eggs analyzed in duplicate).

[2]Basal, A7, A14, A21 and A42 correspond to the different groups of laying hens fed diet supplemented with astaxanthin at 0 mg/kg (control), 7.1 mg/kg, 14.2 mg/kg, 21.3 mg/kg, and 42.6 mg/kg, respectively.

[3]SEM: pooled SEM.

[4]ND: not detected.

[5]Total: astaxanthin concentration in egg yolk = all-trans + 9-cis + 13-cis.

[6]Total-egg: total astaxanthin in egg = astaxanthin concentration in egg yolk × egg yolk weight.

was negatively affected by the supplementation period and the final weight of these fishes. In addition, Walker et al. (2012) found that the accumulation of astaxanthin in eggs rose until 10th d and started to decrease after that. The shallow concentration depletion observed between wk 8 and 24 of the actual test might be due to a saturation of astaxanthin absorption accompanied by an increase of the egg yolks on wk 24. Astaxanthin was not detected in the control group of eggs.

Deposition Rate of Astaxanthin into Egg

Figure 2C presents the deposition rate of astaxanthin into eggs during the test period. The results were 4.19%, 5.54%, 6.07%, and 5.76% for A7, A14, A21, and A42 respectively on wk 8; 4.44%, 4.56%, 5.19%, and 5.17% for A7, A14, A21, and A42 respectively on wk 24. These values are slightly superior to data reported by Johnson et al. (1980) (about 4% by feeding laying quail with *Phaffia rhodozyma*) and Akiba et al. (2000) (3.6% with laying hens fed yeast *Phaffia rhodozyma*). Indeed, *Haematococcus pluvialis* is known to accumulate more astaxanthin than *Phaffia rhodozyma* (Shah et al., 2016; López-Cervantes and Sánchez-Machado, 2018). However, our results are lower to those reported by Moreno et al. (2020) (14.4% by feeding hens without vitamin A and biofortified maize containing a set of carotenoids in which astaxanthin formed the major part). Moreno et al. (2016) previously demonstrated that the deposition of non-provitamin carotenoids into eggs was affected by the addition of vitamin A (retinol) into feeds which competed with the carotenoids and reduced their absorption. Vitamin A in our study accounted for 12 500 IU/kg of diet. This might be responsible for the reduction in our values compared to that of Moreno et al. (2016). Similarly, the slight difference observed between astaxanthin transfer ratio in A7 and A21 might be ascribed to the content of astaxanthin in feed. The proportion of vitamin A/astaxanthin in feed was probably slightly higher at very low dose and astaxanthin amount slightly lower in order to enhance the competitions between astaxanthin, vitamin A and other carotenoids present in the diets. It may also be suspected that at very low supplementation dose, astaxanthin is metabolized and transferred to different tissues in laying hens rather than accumulation into eggs. Nevertheless, considering the numerical values in A21 and A42 on either wk 8 and 24, the deposition rates were maximal in A21. A42 presented a little depletion on wk 8 and tend to stabilize with A21 on wk 24. Lutein supplementation from 125 to 1000 ppm (0.125 to 1 g/kg) in laying hens diet resulted on a depletion of transfer efficiency, as the dose increased (Leeson and Caston, 2004). Thenceforth, the dose-related increase of deposition rate observed from A7 to A21 may be limited and could decrease with further increase of supplementation dose. Further studies involving higher supplementation dose could enlighten the reverse effect of high supplementation dose on the deposition rate of astaxanthin in egg.

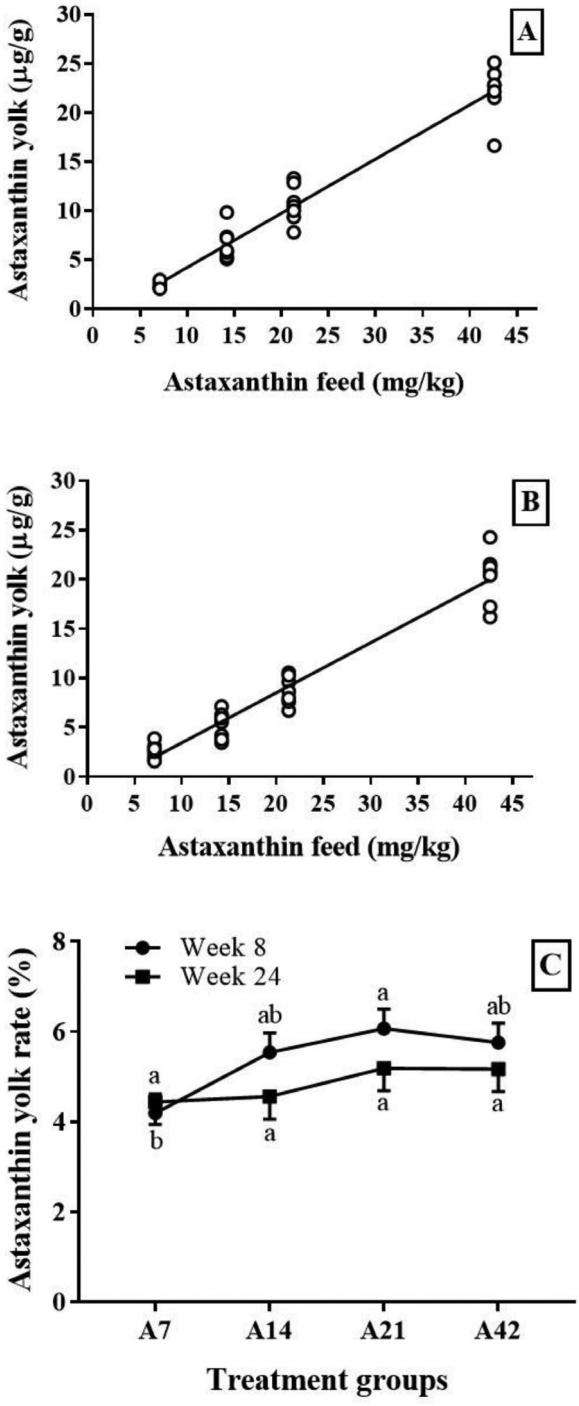

Figure 2. Linear regression plotting of astaxanthin concentration in egg yolk according to the dose supplementation in feed analyzed on wk 8 (A) and wk 24 (B) and deposition rate of astaxanthin into eggs from different groups calculated on wk 8 and 24 (C). Data in (C) are presented as means ± SEM. n = 7. a–b: different superscript letters within a time of test indicate significant difference between groups ($P < 0.05$). Astaxanthin feed: astaxanthin supplementation dose in feed (mg/kg). Astaxanthin yolk: astaxanthin concentration in egg yolk (μg/g). Astaxanthin yolk rate: deposition rate of astaxanthin into egg (%). A7, A14, A21, and A42 correspond to the different groups of laying hens fed diet supplemented with astaxanthin at 7.1 mg/kg, 14.2 mg/kg, 21.3 mg/kg, and 42.6 mg/kg respectively.

Astaxanthin Geometric Isomers Study

It is of great importance to consider the isomers distribution in eggs due to their different effects as

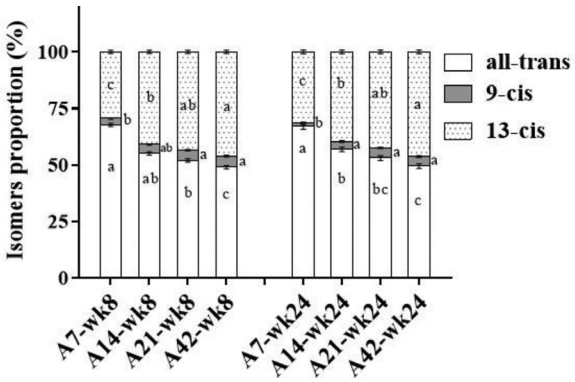

Figure 3. Astaxanthin isomers proportion in total astaxanthin analyzed in egg yolks on wk 8 (wk8) and wk 24 (wk24). n = 7. A7, A14, A21, and A42 correspond to the different groups of laying hens fed diet supplemented with astaxanthin at 7.1 mg/kg, 14.2 mg/kg, 21.3 mg/kg, and 42.6 mg/kg respectively. Bars correspond to SEM. Different letters show significant differences between groups for same isomer ($P < 0.05$).

antioxidants. All the isomers (all-trans, 9-cis, and 13-cis) were numerically and statistically different based on the ANOVA and regression analyses (Table 4). The overall values of the isomers slightly declined with regard to the contents on wk 8 and wk 24 respectively. The *t* test revealed no significant differences. This suggests that the distribution of isomers is not dependent to the feeding duration.

All-trans isomer is reported to be the most prevalent in *Haematococcus pluvialis* in nature (Dhankhar et al., 2012). Previous studies on fishes and crustaceans have shown that all-trans isomer is prevalent in these animals as well (Yu and Liu, 2020). In our study, the proportion of isomers in eggs (Figure 3) seems different from those previously reported. With the supplementation dose, 13-cis isomer gradually increased (29.41%, 40.90%, 43.42%, and 46.00% for A7, A14, A21, and A42 respectively on wk 8 and 31.32%, 39.56%, 42.39%, and 46.31% for A7, A14, A21, and A42 respectively on wk 24). Nevertheless, all-trans isomers were still predominant in all groups (67.67%, 55.04%, 52.09%, and 48.99% for A7, A14, A21, and A42 respectively on wk 8 and 66.92%, 56.92%, 53.10%, and 49.48% for A7, A14, A21, and A42 respectively on wk 24). Studies on fishes and crustaceans have given rise to possible different and preferential uptake mechanisms of isomer into organs and tissues (Osterlie et al., 2000; Su et al., 2020). In vitro study conducted by Yang et al. (2017b) revealed that both all-trans to cis and cis to all-trans isomerization are possible. It was remarkable for 9-cis which markedly isomerized into all-trans followed by a little fraction of 13-cis in the gastric and intestinal steps. On the other hand, all-trans was more isomerized into 13-cis than 9-cis and the bio-accessibility of 13-cis was higher than those of 9-cis and all-trans. We assume that all these factors are conceivable in our study. A selective uptake of different isomers into different tissues and egg yolk as well as isomerization in the digestive tract and during the metabolism may generate the increase of cis isomers as observed in egg yolks. Interestingly, we found that the dose supplementation has a great impact on the distribution of

isomers. In fact, the more the supplementation dose, the more the proportion of cis isomers in eggs. The deposition of cis isomers is facilitated by the amount of total astaxanthin added to the feed by then. Thus, we came to the inference that the selective intake of cis isomers or trans-cis isomerization might occur depending on the disposable amount of astaxanthin resulting in a lower or better deposition of cis isomer into eggs.

CONCLUSION

This study gives further insights on astaxanthin fortified eggs enlightening the concentration of astaxanthin and the geometric isomers in eggs. Based on our results, long-term supplementation of astaxanthin in diet up to 42.6 mg/kg has no adverse consequences on the performance of laying hen and the physical quality of egg. Astaxanthin is well deposited into egg yolk of laying hen with a slight decrease of the content in egg after the long-term supplementation. The redness of the egg yolk is moderately affected by astaxanthin, and a better accumulation of total astaxanthin and cis isomers is perceptible as the supplementation dose increases.

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DISCLOSURES

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2021.101304.

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