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Effect of the Source and Level of Carotenoids in Diets on Their Retention in Eggs

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ABSTRACT

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The lutein and zeaxanthin deposition in egg yolks of hens was evaluated. The effects of various levels of extracts from Mexican marigold flowers in hen diets were compared in Experiments 1 (from 0 to 350 mg/kg) and 2 (from 0 to 950 mg/kg). In Experiment 3, the sources of carotenoids such as lutein (250 mg/kg) and *Chlorella* (12.5 g/kg) were examined. All three experiments were conducted using brown egg layers housed in enriched cages. The lutein concentrations in yolks were increased ($P < 0.05$) from 0.141 to 0.232 mg/60 g of egg (Experiment 1) and from 0.096 to 0.283 mg/60 g of egg (Experiment 2). A similar trend was observed for zeaxanthin. In Experiments 1 and 2, the zeaxanthin content increased from 0.096 to 0.150 mg/60 g of egg and from 0.046 to 0.200 mg/60 g of egg, respectively. However, the retention of lutein and zeaxanthin decreased in a dose-dependent manner from 55.8 to 33.0% and 49.1 to 29.3%, respectively, in Experiment 1 and from 81.2 to 23.3% and 57.0 to 21.6%, respectively, in Experiment 2. In Experiment 3, both the treated groups had greater lutein and zeaxanthin contents in the yolks. The ratio of lutein in the yolks from hens fed lutein to those from hens fed algae was 2.7 : 1 (1.044 and 0.382 mg/60 g of egg). Overall, 14.0 and 16.4% of lutein and 13.7 and 15.3% of zeaxanthin was retained in the eggs of hens fed lutein and algae, respectively. The concentrations of carotenoids in hen egg yolks depend on dietary intake, and the retention of carotenoids decreases with increasing dose. A higher carotenoid retention in the yolks was found when the hen diets were supplemented with the Mexican marigold extract than when *Chlorella* or pure lutein were used as supplements.

Keywords: hen; lutein; zeaxanthin; yolk; *Chlorella*; Mexican marigold

Carotenoids pigments are common ingredients in the feed of egg-laying hens used for commercial breeding. Due to their low price synthetic xanthophylls, such as canthaxanthin and ethyl ester of β-apo-8'-carotenoic acid, are exclusively used. But the amount of these carotenoids in the mixed feed for laying hens is limited, because of the known negative effect on human health (Breithaupt 2007). Canthaxanthin should not exceed 8 mg/kg (EFSA 2014) and the safe dose of ethyl ester of β-apo-8'-carotenoic acid for the laying hens is also 8 mg/kg (EFSA 2016). Promising alternatives to synthetic carotenoids are natural sources of lutein and zeaxanthin (Breithaupt 2007). Mexican marigold (*Tagetes erecta*) and *Chlorella* algae contain high concentrations of lutein and zeaxanthin. Both of these natural sources of carotenoids are prospective feed supplements that are suitable for the

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production of eggs containing high concentrations of lutein and zeaxanthin, so-called functional foods (Englmaierova et al. 2013; Kotrbacek et al. 2013; Skrivan et al. 2015, 2016). The positive effects of carotenoids on human and animal health are evident from many studies (Cermak et al. 2015; Karaskova et al. 2015; Meza-Herrera et al. 2017).

There are reports on the enrichment of egg yolks with lutein when diets containing commercially available lutein (Leeson and Caston 2004; Leeson et al. 2007; Golzar Adabi et al. 2010), algae (Fredriksson et al. 2006), or carrots (Hammershoj et al. 2010) were used. Dietary lutein supplementation at 250 mg/kg increased the lutein concentration in the egg yolks from 0.12 mg to 1.35 mg/57 g of egg (Golzar Adabi et al. 2010). The plasma lutein was significantly increased in men, with a mean age of 60 years, who consumed one lutein-enriched egg each day. Leeson and Caston (2004) showed that dietary lutein at 375 mg/kg increased the lutein contents in the yolks from 0.3 mg to 1.5 mg/60 g of egg. There was no significant increase in the yolk lutein content with diet supplementation above 375 mg/kg. In subsequent experiments by the same authors, the lutein contents in egg yolks plateaued at 125 mg/kg diet, with 1.67 mg/egg (Leeson et al. 2007). Fredriksson et al. (2006) tested the addition of marine microalgae *Nannochloropsis oculata* at 20% of dry matter in the diet of hens. The lutein and zeaxanthin content in the eggs after 4 weeks of feeding was 22 mg/kg, i.e. 1.32 mg/60 g of egg. Hammershoj et al. (2010) examined the effects of three carrot varieties on the deposition of carotenoids in yolks. Hens were fed a standard feed supplemented with either orange, yellow or purple carrots at 70 g/hen/day. Carrot supplementation increased the lutein concentration in the yolks from 7.5 mg/kg to 8.2–12.2 mg/kg. The total carotenoids concentration increased from 10.8 mg/kg to 14.0–20.2 mg/kg. The deposition efficiency of lutein and zeaxanthin from the feed to egg yolk was approximately 25%, while the deposition efficiency of β-carotene was only 0.5%.

A review of the literature provides information about the transfer of carotenoids from mixed feed into eggs. We assume the differences in the transfer efficiency of various sources and levels of carotenoids in mixtures for laying hens. Therefore, the aim of this work was to measure the ratio of lutein and zeaxanthin deposited in an egg yolk to the daily intake of both carotenoids by a hen in three experiments with different levels and sources of carotenoids.

MATERIAL AND METHODS

Primary data obtained from three previous experiments published by Englmaierova et al. (2013) and Skrivan et al. (2015, 2016) were used in this study. The ratios of carotenoid intake and carotenoid deposition in yolks were not included in these publications. In the present work, data from the three experiments are processed in a different way to obtain new findings. The daily dietary feed intake of the carotenoids lutein and zeaxanthin and their retention in egg yolks (%) were evaluated.

Experiment 1. This experiment was started with 66-week-old hens and lasted for 12 weeks. One hundred and sixty ISA Brown hens were housed in enriched cages, with 10 hens per cage, in an air-conditioned facility. The cages were 7560 cm^2 in area. Inside each cage there were a nest, a feeder (120 cm), and 3 nipple drinkers. The cages were furnished with a nest box, perch (150 cm), dust bath, and equipment for abrasion of the claws, which conformed to the European Council Directive 1999/74 EC (1999). Room temperature was maintained at 20–22°C, and the light cycle was 16 h of light and 8 h of darkness. The light intensity was approximately 10 lx in the central storey. The microclimatic factors, experimental design, data collection, and data analyses were the same in the three experiments.

The hens were randomly distributed into 16 cages with four dietary treatments and four replicates of each treatment. The hens were fed diets without synthetic carotenoids. The ingredients and nutrient composition of the control diet are shown in Table 1. The three experimental diets were supplemented with 150, 250 or 350 mg/kg of Avizant® Yellow 20 HS (Lohmann Animal Health GmbH, Germany), an extract from Mexican marigold flowers (*Tagetes erecta* L.) containing 21.263 g/kg of lutein and 9.649 g/kg of zeaxanthin. The major component of Avizant® Yellow 20 HS is calcium carbonate. Therefore, it was dosed at the expense of limestone. All diets were stored in a dark, air-conditioned room at 18–20°C and relative humidity 50–60% for 12 weeks. Feed and fresh water were supplied to the animals *ad libitum*. Feed intake was registered weekly per cage. Hen egg pro-

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duction and health were determined daily. Each week, the eggs were weighed on three consecutive days. Ninety-six eggs were used to determine the carotenoid contents in the yolks once during the experiment (3 eggs per sample; n = 8).

Experiment 2. In this experiment, 240 egg laying Lohmann Brown hens aged 30 weeks were used. The hens were housed in cages, with 10 hens per cage. The hens were randomly assigned to 6 dietary treatments, each with 4 replicate cages. Experimental feed mixtures were supplemented with 150, 350, 550, 750, and 950 mg/kg of Avizant® Yellow 20 HS, as in Experiment 1. Table 1 presents the composition of the basal diet. Two hundred and sixteen eggs were used to determine the carotenoid contents in the yolks once during the experiment (3 eggs per sample; n = 12).

Experiment 3. One hundred and eighty ISA Brown hens aged 27–39 weeks were randomly assigned to 3 dietary treatments with 6 replicate cages. The control group was fed the basal diet without carotenoid supplementation. Lutein was added to the diet of the second treatment group at 250 mg/kg in the form of commercially available lutein (Alchimica, Prague, Czech Republic). The third treatment group (Chlorella) was fed a diet supplemented with 12.5 g/kg of spray-dried algae, *Chlorella* sp. (autotrophic cultivation) (Institute

Table 1. Ingredients and analyzed composition of the basal diet (g/kg)

Item	Experiment 1[b]	Experiment 2[c]	Experiment 3[d]
Wheat	265	280	243.2
Maize	350	350	355
Soybean meal	211.5	210.5	215
Rapeseed oil	30	30	30
Lucerne meal	20	20	20
Wheat bran	15	–	15
Fish meal	–	–	15
Dicalcium phosphate	13	9	18
Sodium chloride	2.0	2.0	2.0
Crushed limestone (1–2 mm)	86	91	81
L-Lysine hydrochloride	1.0	1.0	–
DL-Methionine	1.5	1.5	0.8
Vitamin-mineral premix[a]	5.0	5.0	5.0
Analyzed nutrient content			
Dry matter	901.2	901.2	885
Crude protein	171.7	171.7	176.9
Ether extract	49.4	49.5	49.6
Crude fibre	34.2	34.2	34.3
Ash	118.5	118.6	118.2
AME_N (by calculation; MJ/kg)	11.57	11.40	11.3
Methionine (by calculation)	4.78	4.76	4.79
Calcium	37.3	37.3	37.4
Phosphorus	6.2	5.6	5.6

AME_N = apparent metabolizable energy corrected for nitrogen

[a]vitamin-mineral premix provided per kg of diet: 3.0 mg retinylacetate, 3000 IU vitamin D_3, 30 mg vitamin E, 25 mg niacin, 8 mg Ca pantothenate, 2.0 mg thiamine, 5 mg riboflavin, 4 mg pyridoxine, 0.5 mg folic acid, 0.075 mg biotin, 0.01 mg cobalamine, 250 mg choline Cl, 2.0 mg menadione, 100 mg betaine, 7.5 mg butylated hydroxytoluene, 5.6 mg ethoxyquin, 1 mg butylhydroxyanisole, 0.7 g DL-methionine, 70 mg Mn, 50 mg Zn, 40 mg Fe, 6 mg Cu, 1 mg I, 0.3 mg Co, 0.2 mg Se

[b]supplement 0, 150, 250 or 350 mg/kg of Avizant® Yellow 20 HS (Mexican marigold flower extract containing 980 g of dry matter, 21.263 g/kg of lutein, and 9.649 g/kg of zeaxanthin) at the expense of wheat

[c]supplement 0, 150, 350, 550, 750 or 950 mg/kg of Avizant® Yellow 20 HS at the expense of wheat

[d]supplement 0, 0.250 mg/kg of lutein and 12.5 g/kg *Chlorella* (autotrophic cultivation) at the expense of wheat

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of Microbiology, Třeboň, Czech Republic). This *Chlorella* algae contained lutein and zeaxanthin at 619 and 613 mg/kg, respectively (analytically determined). The ingredients and nutrient composition of the basal diet are shown in Table 1. Two hundred and sixteen eggs were used to determine the carotenoid contents in the yolks twice during the experiment (3 eggs per sample; n = 24).

Laboratory analyses and calculation of carotenoids retention. The lutein and zeaxanthin contents in the yolks, feed, and Avizant® Yellow 20 HS were measured by using high performance liquid chromatography (HPLC) based on the method described by Froescheis et al. (2000), with the exception that the extracts were evaporated at 50°C and methanol/tetrahydrofuran was used instead of hexane; dichloromethane was used to dissolve the residues in a vacuum evaporator. A 60 µl aliquot was subjected to HPLC (VP series; Shimadzu, Japan) analysis. A Kinetex C18 column (100 × 4.6 mm; 2.6 µm; Phenomenex, USA) was used. A gradient system was established with acetonitrile : water : ethylacetate (88 : 10 : 2) as eluent A and acetonitrile : water : ethylacetate (88 : 0 : 15) as eluent B. The values of carotenoids concentrations in the yolks were recalculated to a 60 g egg with a 26% portion of yolk in egg which is 15.6 g.

The basic units for the calculation of daily carotenoid intake per hen and carotenoid retention in one yolk were repeatedly measured within a group (cage). The calculations were based on the feed intake, concentrations of carotenoids in the feed, egg mass, and a 26% portion of yolks in eggs. The ratios of individual carotenoids deposited in the yolks to their intake via feed, expressed in %, represent the retention of carotenoids. The retention of carotenoids (RC) was calculated as follows:

$$RC = (EM \times 26 \times CCY)/CIF$$

where:
EM = egg mass (g/day/hen)
CCY = carotenoid content in yolk (mg/g)
CIF = carotenoid intake via feed (mg/day/hen)

The mixed feed and Avizant® Yellow 20 HS dry matter were determined by oven drying at 105°C to a constant weight, and the crude protein content of the feed was measured using a Kjeltec Auto 1030 analyzer (Tecator, Sweden). The fat content in the diet was determined by extraction with petroleum ether using a Tecator Extraction System 1045 Soxtec. Analyses of the P and Ca content of the diets were conducted. Dry homogenized diets were ashed in a muffle furnace at 550°C, and the ash was dissolved in 3 M hydrochloric acid and quantitatively transferred into a volumetric flask. The total P concentration in the solution was determined using a vanadate-molybdate reagent (AOAC 2005; method 965.17), and the Ca concentration in the hydrochloric acid extract was measured by atomic absorption spectrometry using a Solaar M6 instrument (TJA Solutions, UK).

Statistical analysis. The concentrations of the deposited carotenoids in the egg yolk (mg/60 g of egg) were analyzed by the analysis of variance (ANOVA) using the General Linear Models (GLM) procedure of the SAS software (Statistical Analysis System, Version 9.2, 2003). Differences were considered significant at $P < 0.05$. The results in the graphs are presented as the means. The procedure was identical for the three experiments.

RESULTS

Experiment 1. The intake of lutein increased in a dose-dependent manner from 0.235 to 0.678 mg/hen/day. Consequently, the lutein concentration in the yolks was increased ($P < 0.05$) from 0.141 to 0.232 mg/60 g of egg. However, the lutein retention percentage was decreased from 55.8% (control) to 41.4, 36.5, and 33.0% in the yolks of hens fed diets supplemented with 150, 250, and 350 mg of Avizant® Yellow 20HS, respectively (Figure 1). The differences between the groups were significant ($P < 0.05$). The efficiency of the zeaxanthin retention was 49.1% in the control group and it gradually decreased to 29.3% at the highest dose of Avizant (Figure 1).

Experiment 2. The daily feed intake of lutein gradually increased from 0.105 to 1.002 mg/hen due to the increased dose of Avizant. The effect of Avizant supplementation on lutein concentration in yolks was statistically significant ($P < 0.05$). The highest lutein content in the yolks (at the highest Avizant dose) was 0.283 mg/60 g of egg. The highest efficiency of lutein utilization was in the control group (81.2%) and decreased to 23.3% in the hens fed the diet supplemented with 750 mg Avizant/kg ($P < 0.05$). When Avizant was added at 950 mg/kg, lutein utilization was higher than when Avizant was supplemented at 750 mg/kg

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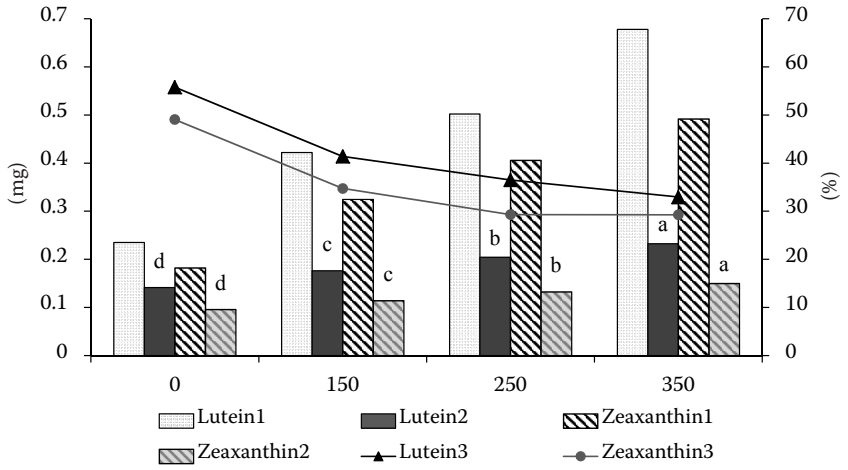

Figure 1. Carotenoids contents and their retention in egg yolks (Experiment 1)
1 = intake of carotenoids (mg/hen/day), 2 = concentration of carotenoids in egg yolks (mg/60 g of egg), 3 = retention of carotenoids in egg yolks (%)
[a–d]means in the same marked columns with different superscripts differ significantly ($P < 0.05$)

(Figure 2). Likewise, the daily zeaxanthin intake gradually increased from 0.072 to 0.754 mg/hen. The content of zeaxanthin in the egg yolks increased from 0.046 to 0.200 mg/60 g of egg at the Avizant dose of 950 mg/kg. Similar to lutein, zeaxanthin utilization decreased from 57.0 to 21.6% for Avizant doses of 0 and 750 mg/kg, respectively. At 950 mg/kg Avizant, the zeaxanthin utilization efficacy was 25.0% (Figure 2).

Experiment 3. The highest daily lutein intake was in the hens of the Lutein group, at 6.958 mg/hen. In the hens of the Chlorella group, the daily intake of lutein was three-times lower (2.216 mg/hen). In both the treated groups, the lutein content in the yolks increased. The ratio of lutein content in the yolks was 2.7 : 1 (1.044 and 0.382 mg/60 g of egg in hens of the Lutein and Chlorella groups, respectively). The efficiency of lutein utilization decreased from 73.7% (control group) to 14.0% and 16.4% in the Lutein and Chlorella groups, respectively (Figure 3). The highest daily intake of zeaxanthin was in the Lutein group, at 6.596 mg/hen, which was almost identical to the

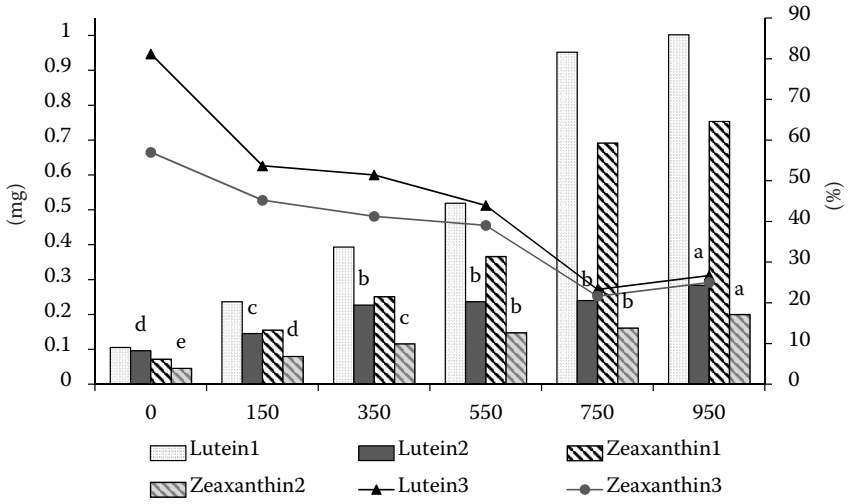

Figure 2. Carotenoids contents and their retention in egg yolks (Experiment 2)
1 = intake of carotenoids (mg/hen/day), 2 = concentration of carotenoids in egg yolks (mg/60 g of egg), 3 = retention of carotenoids in egg yolks (%)
[a–e]means in the same marked columns with different superscripts differ significantly ($P < 0.05$)

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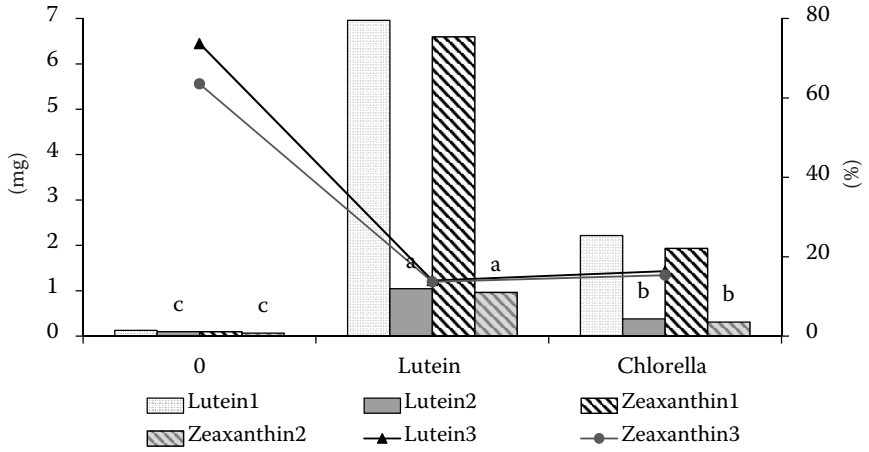

Figure 3. Carotenoids contents and their retention in egg yolks (Experiment 3)
1 = intake of carotenoids (mg/hen/day), 2 = concentration of carotenoids in egg yolks (mg/60 g of egg), 3 = retention of carotenoids in egg yolks (%)
[a–c]means in the same marked columns with different superscripts differ significantly ($P < 0.05$)

daily intake of lutein in the same group. In hens fed *Chlorella* alga, the daily intake of zeaxanthin was 1.936 mg/hen. In the treated hens, the concentrations of carotenoids in yolks increased significantly ($P < 0.05$). The zeaxanthin in the egg yolks of hens fed lutein represented 13.7% of the hens' dietary intake of zeaxanthin. The corresponding value in the eggs of hens fed *Chlorella* was 15.3%, and the value in the hens fed the basal diet was 63.6% (Figure 3). The lutein and zeaxanthin concentrations in the yolks of hens fed *Chlorella* were significantly lower than those in the yolks of hens fed lutein ($P < 0.05$). The lowest carotenoid content was observed in the egg yolks of hens in the control group.

DISCUSSION

Commercial hen eggs are a good source of carotenoids, mainly lutein and zeaxanthin. It is feasible to increase the content of carotenoids in eggs using feed supplements. Unfortunately, natural sources of carotenoids for hens kept in indoor cages without outdoor access are more expensive than synthetic carotenoids. The exception to this is the rearing of hens in pastures, which may increase the content of carotenoids in eggs. One 60 g egg of pastured hens contains 0.48 mg and 0.36 mg of lutein and zeaxanthin, respectively (Skrivan and Englmaierova 2013), which are greater concentrations than those observed in Experiments 1 and 2 with the highest Avizant dose or in Experiment 3 with 1.25% of dried autotrophic *Chlorella* supplementation. In the study of Leeson and Caston (2004), 375 mg/kg of lutein in the diet increased the lutein contents in the egg yolks from 0.3 to 1.5 mg per egg (60 g). In addition, the lutein content in the egg yolks plateaued when 375 mg/kg (Leeson and Caston 2004) or 125 mg/kg of lutein was present in the diet (Leeson et al. 2007). The supplementation of feed mixtures with carotenoids may be efficient if the relationship between carotenoid dosing and deposition in yolks is known.

In Experiment 3, lutein added at 250 mg/kg to the diet increased the lutein and zeaxanthin contents in the yolks to 1.044 and 0.966 mg/60 g of egg, respectively. Using the same lutein dose (250 mg/kg), Golzar Adabi et al. (2010) observed a lutein content of 1.35 mg in the resulting egg yolks. Dietary *Chlorella* in Experiment 3 cannot be precisely compared with the sea algae *Nannochloropsis oculata* (Fredriksson et al. 2006). Fredriksson et al. (2006) added algae at 20% of dry matter in the diet, which resulted in 1.32 mg of lutein and zeaxanthin per egg. In the present study, both carotenoids were present at a concentration of 0.70 mg/60 g of egg, with 1.25% *Chlorella* supplementation. *Chlorella* increased yolk lutein more than the daily intake of 70 g of carrots. According to Hammershoj et al. (2010), carrots combined with the feed mixture increased egg lutein to 8.2–12.2 mg/kg (10.2 mg/kg on average).

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This increase was equivalent to 0.17 mg in a 60-g egg containing 28% yolk, which is 2.2 times less than in the *Chlorella*-fed hens (0.382 mg/60 g of egg). Carotenoid deposition in yolks, expressed as the percentage of the carotenoid intake, depends on the source and dietary concentrations of the carotenoids. Avizant supplementation at 350 mg/kg increased lutein and zeaxanthin retention in the yolk by 18.5% and 12%, respectively, compared to the same supplementation of Avizant in Experiment 1. These increases were higher than those obtained after more than two-fold dosing of the carotenoid supplementation to the basal diets in Experiments 1 and 2. In all the experiments presented, the higher carotenoid intake in the hens was connected to lower retention for all carotenoid sources. The lowest retention of lutein and zeaxanthin in the yolks was observed in Experiment 3, when lutein was supplemented at 250 mg/kg. The low retention of both carotenoids in the yolks is consistent with the data from Leeson et al. (2007). According to these authors, a pure lutein dose of 125 mg/kg is sufficient. Carotenoids present in *Chlorella* algae were deposited in the yolks less efficiently than those present in the Mexican marigold extract. Further investigation is needed to determine why this result was observed.

CONCLUSION

The concentrations of the main carotenoids present in egg yolks, lutein and zeaxanthin, strongly depend on their dietary intake. High lutein and zeaxanthin intake in hens, or their ascending intake, reduces the lutein and zeaxanthin contents in the eggs when expressed as a percentage of the dietary intake. The retention of carotenoids in the yolks was higher when the basal diet was supplemented with the Mexican marigold extract than with *Chlorella* or pure lutein.

Further work is necessary to elucidate the absorption of carotenoids in the intestinal tract when the various sources presented here are used, along with the losses of carotenoids and the deposition in the liver and adipose tissue. Any future research should investigate if the carotenoid concentrations in egg yolks are limited and if there is a genetic influence warranting the optimum ratio of nutrients for the developing embryo.

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