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Supplementation of Laying-Hen Feed with Annatto Tocotrienols and Impact of α-Tocopherol on Tocotrienol Transfer to Egg Yolk

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Supporting Information

ABSTRACT: Hens can efficiently transfer nutrients from their feed to the eggs. Tocotrienols (T3s) have various health benefits including lowering cholesterol. Annatto is the only known source of T3s without the presence of α-tocopherol; hence it can be used to study T3 transfer without the interference of α-tocopherol. In this study, hens were fed diets for 7 weeks containing annatto at 100, 500, or 2000 ppm (by weight) and also 2000 ppm annatto with 200, 600, or 1000 ppm of added α-tocopherol to study the effect of α-tocopherol on transfer of T3s. No significant differences were found in egg production or properties. Significant differences ($p < 0.05$) were found in transfer efficiencies of tocopherol and T3s to the yolks. α-Tocopherol was transferred more efficiently (21.19−49.17%) than γ-T3 (0.50−0.96%) or δ-T3 (0.74−0.93%). Addition of 1000 ppm of α-tocopherol decreased the amount of γ-T3 but did not impact the transfer of δ-T3 to the egg. These feeding treatments did not impact the cholesterol content of the eggs.

KEYWORDS: *α-tocopherol, annatto, cholesterol, egg, tocotrienols (T3s), transfer efficiency*

INTRODUCTION

Feeding different diets to laying hens can dramatically change the nutrient composition[1] and appearance[2] of the resulting eggs with minimal changes to functionality, making feed supplementation a means of nutrient enrichment or modification for the human diet. Vitamin E is a fat-soluble vitamin with eight major forms that are classified into two major groups, tocopherols (TCs) and tocotrienols (T3s), differing in three double bonds on the phytyl tail in the T3s.[3] Each of these groups has an α, β, γ, and δ form varying in the number and placement of methyl groups on the chromanol ring.[3]

Of these forms, α-tocopherol is the most bioavailable due to the presence of an α-tocopherol transfer protein in the liver that has specific affinity for the α-tocopherol form of vitamin E.[4] More recently, interest has grown in T3s, which have been shown to have many health benefits.[5] One significant benefit is lowering cholesterol by downregulating the hepatic enzyme 3-hydroxymethylglutaryl coenzyme A reductase, a key enzyme in cholesterol synthesis.[5−12] Walde et al.[13] showed that the addition of barley or palm oils to laying-hen feed decreased egg yolk cholesterol by 4% and 6%, respectively, but both of these sources have α-tocopherol naturally present, limiting the ability to determine the impact of the presence or absence of α-tocopherol on transfer of T3s.

Annatto is the only known natural source of T3s (δ ∼90% and γ ∼10%) without α-tocopherol present,[14] making it possible to observe T3 transfer efficiency in both the presence and absence of added α-tocopherol. The absence of α-tocopherol may help improve the transfer of the T3s to the resulting egg yolks. Annatto is naturally derived from the *Bixa orellana* rainforest plant and is commonly used by the food industry to give certain dairy products the characteristic yellow color from carotenoids (bixin and norbixin) present in the annatto.[14] Annatto has been used in laying-hen feed by Harder et al.[15] to study cholesterol reduction in eggs, and a reduction was observed when annatto was supplemented at above 1.5% in feed. However, McGonigle et al.[16] did not observe a significant difference in cholesterol content in the egg when the laying-hen feed was supplemented with annatto at 200−600 parts per million (ppm) levels compared to control.

In order to provide health benefits to laying hens or to egg consumers, we wanted to evaluate the transfer efficiency of T3s from feed to egg in both the presence and absence of α-tocopherol, as well as their effect on egg cholesterol content. The hypotheses of this study were (a) supplementation of laying-hen feed with annatto T3s would increase the level of T3s in the egg yolk, (b) the presence of T3s would lower the cholesterol levels in laying hens and consequently lower cholesterol in the egg yolk, and (c) added α-tocopherol would interfere and reduce the transfer of T3s, thus attenuating the effects of the T3s on egg cholesterol. It was also hypothesized that supplements would result in minimal changes in hen production performance and egg's physical and sensory qualities.

MATERIALS AND METHODS

Feeding Experiments. For 7 weeks, 84 laying hens (Hy-Line W-36 breed, 30 weeks of age) were fed base diet (Table S1, Supporting Information) and diets supplemented with annatto tocotrienols (DeltaGold 70, American River Nutrition, Hadley, MA) and α-tocopherol (ADM, Decatur, IL) in a treatment scheme described in

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Table 1. The feeding experiment was approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC).

Table 1. Concentrations of Annatto and α-Tocopherol in Supplemented Diets

diet	annatto[a] (ppm)	α-tocopherol[b] (TC, ppm)
control	0	0
T3 100	100	0
T3 500	500	0
T3 2000	2000	0
TC 200	2000	200
TC 600	2000	600
TC 1000	2000	1000

[a]Annatto tocotrienols: total tocotrienols, 74.5% (δ-tocotrienol 89.2%, γ-tocotrienol 10.8%, and other tocotrienols/tocopherols <1%). [b]α-Tocopherol: 96.6%

The base diet met the NRC nutrient recommendations.[17] The hens were randomly assigned to a treatment and were kept in an environment similar to that of most modern industry laying facilities. The average temperature was 25 °C and the average relative humidity was 40%. The lighting was on for 16 h and off the remaining 8 h of the day. Three hens were placed in each cage (experimental unit, EU) with 30 cages (10 stacks with 3 tiers per stack) total. Only 28 cages were needed to house the 84 laying hens; two cages were not used due to air velocity difference and failure of eggs to roll to the front, causing breakage. There were seven treatments diets (Table 1) with four replicates (4 EUs) for each treatment, thus 12 hens per treatment diet. Upon arrival to the new environment, the hens were given a week of acclimation. No mortalities occurred during this study.

Feed Mixing. A horizontal mixer (Precision horizontal batch mixer; H.C. Davis Sons Mfg Co., Inc., Bonner Springs, KS) combined the base diet for 30 min. To mix the supplements into the base diet, the appropriate amount of annatto and/or α-tocopherol was weighed and then was mixed into a small amount of the base diet to ensure it was well incorporated. This mixture was then added to more feed and mixed well with a Hobart mixer (model H-600; Hobart, Troy, OH). This portion was then incorporated into the entire diet portion with the same horizontal mixer that was used to mix the base diet. Feed was stored at refrigeration temperatures (4 °C) away from light and taken to the experimental site daily, where hens were fed and watered ad libitum throughout the entire study. Daily measurements taken precisely every 24 h were feed disappearance and egg production. Hen body weight was taken weekly as an indicator if the hens were eating properly.

Egg Sampling and Storage. The eggs were collected daily for the first 2 weeks and weekly for the remaining 5 weeks. Eggs were taken from 2 days and pooled (i.e., day 1 and day 2 eggs are referred to day 2 eggs in this study). The sampling days were 2 (1−2 days), 4 (3−4 days), 6 (5−6 days), 8 (7−8 days), 10 (9−10 days), 12 (11−12 days), 14 (13−14 days), 21 (20−21 days), 28 (27−28 days), 35 (34−35 days), 42 (41−42 days), and 49 (48−49 days). For sample preparation, the egg yolks were carefully separated from the albumen and rolled on a paper towel to ensure only yolk was taken. The yolks from one cage over 2 days were homogenized manually, and a sample was taken and stored at −4 °C until further analyses were carried out. The frozen egg yolks were then freeze-dried (Virtis Genesis 25LE) for at least 72 h to remove the moisture by sublimation to preserve the lipids.

Physical and Sensory Properties of Eggs and Egg Yolks. The quality of the eggs collected was measured at every sampling time by using a high-accuracy digital egg tester DET6000 (Nabel, Japan). The measurements taken included whole egg weight (grams), eggshell strength (newtons), egg yolk color (yolk color fan, YCF), Haugh unit (calculated from albumen height), and eggshell thickness (millimeters). The YCF taken objectively by this instrument is based on tristimulus values standardized by the 1931 CIE colormetric according to the instrument manual.

Trained Sensory Panel Evaluation on Egg Yolk. To determine whether and how different the treatment eggs (days 35 and 36) were from the control, nine panelists were trained in four training sessions to evaluate the egg yolk for yolk color (less to more yellow), moistness (low to high), smoothness (lumpy to smooth), chalkiness or mouth drying (none to intense), savory egg yolk flavor (none to intense), bitterness (none to intense), and off-flavor (none to intense) on a 15 cm line scale. To calibrate the panelists to these attributes, anchors were used and included egg yolks from the extreme diets (control, T3 2000, and TC 1000) for each scale, YCF for yolk color, and quinine solution for bitterness. For sample preparation, eggs were hard-boiled and consistently cooled by refrigeration, followed by cutting in half and serving to the panelists, cut side down, on a plate labeled with a random 3-digit code in a random order to minimize bias. The four replicates were evaluated on four separate days with panelists' scores averaged on each day.

For all remaining analyses, the egg yolk was carefully separated from the albumen and analysis was conducted on only the egg yolk. Viscosity was measured on the raw separated egg yolks collected from day 47 by use of a Haake RS 150 rheometer (ThermoOrion, Karlsruhe, Germany). This measurement was taken as explained in Walker et al.[2]

Chemical Properties of Egg Yolks. *Total Egg Yolk Moisture.* Total moisture was measured by drying 2 g of yolk in an aluminum dish for 4−5 h at 105 °C as stated in AOAC Standard Method 922.06. This measurement was done on day 28 yolks after they were homogenized.

Egg Yolk Lipid Extraction. Freeze-dried egg yolk samples were ground with a mortar and pestle to reduce particle size, and the yolk was a fine powder. About 0.5 g of the ground freeze-dried yolk powder was accurately weighed into a glass vial and mixed with 5.0 mL of chloroform/methanol (2:1 v/v), similar to the procedure of Folch et al.[18] The vials were capped and manually shaken for 30 s to ensure full dispersion of the solid in the solvent. The vials were placed in a shaker at ambient temperature overnight and then centrifuged to obtain a clear layer. Approximately 2 mL of the supernatant was filtered through a 0.45 μm filter [polytetrafluoroethylene, PTFE] with a glass syringe. The filtrate (1.0 mL) was taken precisely and placed in a preweighed glass vial, and the solvent was evaporated with a nitrogen evaporator to prevent lipid oxidation. To ensure all of the residual solvent was removed, the samples were placed in a vacuum oven at ambient temperature overnight. The vials were weighed to calculate total oil extracted. The oil was then dissolved in HPLC-grade hexanes and stored in an explosion-proof −20 °C freezer until further analyses were carried out, including HPLC quantification of tocopherols and tocotrienols.

Egg Yolk Phospholipid Composition. ^{31}P NMR was used to determine the phospholipid profile for day 21 egg yolk samples. After the egg yolk oil was extracted, a small sample (80−120 mg) of oil was taken and prepared as explained by Yao and Jung.[19] These samples were analyzed for phospholipid content and class composition in the egg yolk oil.

Egg Yolk Fatty Acid Composition. A small sample of egg yolk oil from day 49 was converted to fatty acid methyl esters (FAME) by mixing the lipid with 1 mL of 1 M sodium methoxide at ambient temperature for 5 h. The reaction was stopped by adding 3 mL of deionized (DI) water. Hexanes (2 mL) were added to extract the FAME to the top layer. The FAME samples were then analyzed by gas chromatography (GC; Hewlett-Packard 5890 series II) with a FID detector and a capillary column from Supelco (Bellefonte, PA; SP-2340, 60 m long × 0.25 mm internal diameter ×0.2 μm film thickness). Parameters used for GC quantification were injector and detector temperature of 250 °C and oven temperature-programmed to start at 100 °C and rise 4 °C/min to finish at 240 °C. The flow of gases was set as in Walker et al.[2] A standard solution of FAME (Nu-Check Prep Inc. GC reference standard 566) was used to identify fatty acid peaks in the egg yolk lipid samples.

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Table 2. Hen Performance and Laying Rate for Diets with Different Levels of Annatto Tocotrienol and α-Tocopherol Supplementation[a]

diet[b]	daily feed disappearance (g·day^{-1}·hen^{-1})	laying rate (%)	weekly hen weight[c] (kg·hen^{-1})
control	103.16 ± 6.28 B	94.56 ± 0.96	1.50 ± 0.02
T3 100	107.85 ± 5.05 A	94.90 ± 2.86	1.49 ± 0.03
T3 500	107.43 ± 4.75 A	97.62 ± 3.26	1.50 ± 0.03
T3 2000	108.28 ± 4.87 A	94.73 ± 2.96	1.48 ± 0.04
TC 200	107.14 ± 6.49 A	96.94 ± 2.39	1.48 ± 0.03
TC 600	107.54 ± 5.20 A	94.39 ± 3.66	1.50 ± 0.03
TC 1000	106.76 ± 6.39 A	96.43 ± 1.70	1.49 ± 0.02
significance[d]	*	NS	NS

[a]Values are means ± standard deviations for $n = 4$. Different letters (comparing all treatments) in the same column indicate significant differences at the 95% confidence level. [b]Diets are detailed in Table 1. Tocotrienols (T3) = ppm of annatto; α-tocopherol (TC) = ppm of α-tocopherol with constant 2000 ppm of annatto. [c]Hen weights were taken weekly and averaged over 7 weeks. [d]NS, not significant at 5%.

Table 3. Egg and Egg Yolk Quality Properties and Sensory Attributes of Boiled Eggs from Laying-Hen Diets with Different Levels of Annatto Tocotrienol and α-Tocopherol Supplementation[a]

diet[b]	whole egg weight (g)	egg yolk weight (g)	egg shell strength (N)	yolk color, YFC	Haugh unit, HU	egg shell thickness (mm)	yolk viscosity (kg/s·m)
control	59.71 ± 2.03	14.47 ± 0.22	37.37 ± 1.84	4.9 ± 0.07	89.44 ± 1.09	0.36 ± 0.01	0.93 ± 0.19 A
T3 100	59.87 ± 1.33	14.71 ± 0.31	39.87 ± 3.00	4.9 ± 0.19	89.47 ± 1.42	0.36 ± 0.01	0.74 ± 0.12 A,B
T3 500	59.14 ± 1.43	14.49 ± 0.38	37.44 ± 3.45	5.0 ± 0.14	89.70 ± 2.28	0.35 ± 0.01	0.59 ± 0.10 B
T3 2000	59.74 ± 0.65	14.70 ± 0.16	37.48 ± 3.17	5.1 ± 0.18	87.35 ± 1.45	0.35 ± 0.01	0.65 ± 0.12 B
TC 200	59.44 ± 1.07	14.64 ± 0.14	40.69 ± 3.06	5.2 ± 0.14	86.96 ± 2.10	0.37 ± 0.01	0.50 ± 0.14 B
TC 600	60.32 ± 1.16	14.89 ± 0.14	36.39 ± 1.38	5.1 ± 0.15	87.88 ± 1.13	0.35 ± 0.01	0.63 ± 0.07 B
TC 1000	59.58 ± 0.84	14.42 ± 0.48	38.92 ± 1.52	5.0 ± 0.16	88.84 ± 1.57	0.35 ± 0.01	0.69 ± 0.06 A,B
significance[c]	NS	NS	NS	NS	NS	NS	*

diet[b]	yellow color	moistness	smoothness	chalk/mouth drying	savory yolk flavor	bitterness	off-flavor
control	6.3 ± 0.6 B	5.2 ± 0.9	8.6 ± 1.0	5.5 ± 1.2	5.7 ± 0.2 A,B	0.3 ± 0.4	0.2 ± 0.3
T3 100	6.8 ± 0.7 A,B	5.6 ± 1.4	9.5 ± 1.2	4.1 ± 0.4	5.5 ± 0.3 A,B	0.1 ± 0.1	0.2 ± 0.2
T3 500	6.9 ± 0.3 A,B	6.3 ± 1.4	8.9 ± 0.6	4.4 ± 0.4	5.3 ± 1.1 A,B	0.6 ± 0.6	0.5 ± 0.5
T3 2000	7.5 ± 0.2 A,B	6.2 ± 1.2	8.2 ± 1.2	4.0 ± 1.5	6.5 ± 1.0 A	0.4 ± 0.3	0.1 ± 0.1
TC 200	8.3 ± 1.0 A	5.7 ± 0.4	8.9 ± 0.9	4.5 ± 0.4	6.4 ± 0.5 A	0.6 ± 0.9	0.1 ± 0.1
TC 600	8.3 ± 0.9 A	5.4 ± 0.9	9.1 ± 0.9	4.6 ± 0.4	6.7 ± 0.5 A	0.8 ± 0.9	0.1 ± 0.1
TC 1000	6.7 ± 0.4 A,B	5.2 ± 1.0	8.6 ± 0.6	5.2 ± 0.5	4.4 ± 0.5 B	0.1 ± 0.1	0.5 ± 0.6
significance[c]	**	NS	NS	NS	**	NS	NS

[a]Values are means ± standard deviations for $n = 4$. Different letters (comparing all treatments) in the same column indicate significant differences at the 99% confidence level. [b]Diets are detailed in Table 1. Tocotrienols (T3) = ppm of annatto; α-tocopherol (TC) = ppm of α-tocopherol with constant 2000 ppm of annatto. [c]NS, not significant at 1% for **, and at 5% for *.

Vitamin E Content in Egg Yolk. Egg oil was dissolved in an appropriate volume of HPLC-grade hexanes to obtain vitamin E isomers in the quantifiable region. HPLC was run as explained in Walker et al.[2] Concentrations of tocopherols and tocotrienols were determined by normal-phase HPLC with a Luna 3 μm NH_2 100 Å, 150 mm × 3.0 mm column (Phenomenex, Torrance, CA) and fluorescence detection. The separation was achieved isocratically with a mobile phase of 98% hexanes and 2% 2-propanol.

Vitamin E Transfer Efficiency to Egg Yolk. Transfer efficiency was calculated on the basis of the maximum concentration reached for each treatment (all of these occurred at approximately day 10). For this calculation, the amount of supplement that theoretically could have been completely or 100% transferred (i.e., the amount of supplement provided from feed daily) was calculated from supplement composition (Table 1), daily feed consumption, laying rate, and egg yolk weight. The ratio of the value quantified to the theoretical value calculated on the basis of complete transfer is the transfer efficiency, expressed as a percentage.

Egg Yolk Cholesterol Quantification. To quantify total cholesterol in the egg yolks, a Wako Cholesterol E (Mountain View, CA) kit was used. The procedure was carried out as instructed, with 2 mg of freeze-dried egg yolk (weighed with an analytical balance) mixed with the color reagent provided by the kit. This mixture was shaken and allowed to react for 15 min at 37 °C, ensuring that all of the yolk was uniformly dispersed in solution for blue pigment formation. The samples were centrifuged to settle the egg yolk particle, and the liquid was carefully pipetted into a cuvette. The cuvette was read on a spectrophotometer (Beckman Coulter DU 720 UV/vis spectrophotometer) at 600 nm, with DI water as a blank. A standard curve was constructed each time samples were measured, by use of the standard cholesterol provided in the kit, to quantify the cholesterol in the egg yolks.

Statistical Analysis. Statistical analysis was done with JMP Pro (version 10, SAS Institute Inc., Cary, NC). One-way analysis of variance (ANOVA) was used for mean comparisons, and all-pairwise comparisons were done by use of Tukey's honest significant difference (HSD) at $p = 0.01$ (sensory and fatty acid data) or $p = 0.05$ (all other data).

RESULTS AND DISCUSSION

Hen Production Performance. The daily feed disappearance, weekly laying rate (100 × eggs produced/hens in cage), and weekly averaged hen body weight are shown in Table 2. No significant differences were found among treatments in the laying rate or hen body weight. The laying rate was over 90% for all diets, similar to that reported in literature.[2,13,20] All hens on treatment diets consumed significantly more feed than those

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on the control diet, with no differences among treatments, indicating that the annatto additive caused the hens to consume more feed even at the lowest concentration.

Annatto has a potent red-orange color from bixin and norbixin carotenoids present and is traditionally used in many industries for coloring various products. But due to the low lighting in the environment, broad-spectrum sight of the laying hens,[21] and low concentration of supplement in the feed with very subtle visual change in color of feed, the cause for the higher feed disappearance is not believed to be associated with the color of the additive. The pure annatto supplement used was tasted by the researchers, and it had a bitter and fruity taste. Its addition to the feed may have made the feed more palatable to the laying hens, causing an increase in the average amount of feed consumed. Chickens have been shown to be sensitive to salty or bitter substances.[22] Not knowing how the laying hens perceive the annatto supplement, it is difficult to know exactly what caused this increase. The hens consuming more feed but not producing more eggs would lead to higher production costs. The egg is considered to be the gold standard for protein;[23] thus increasing the cost of production is not desirable for the industry or egg consumers. A potential application of this supplementation may be for broiler chickens for more rapid muscle growth. Kudo et al.[24] found that broiler chickens had the highest number of taste buds over layer type and Rhode Island Red type. The higher feed consumption likely can lead to faster rate of growth and meat production.

Physical and Sensory Properties of Eggs and Egg Yolks. No significant differences were found in whole egg weight, egg yolk weight, egg shell strength, yolk color, Haugh unit (HU), or egg shell thickness averaged over time (Table 3). It was expected that there would be minimal changes in the physical and quality properties due to the supplements. All HUs on average were greater than 72, indicating that the eggs produced in this feeding study were AA eggs by USDA grading standards,[25] and the HUs were similar to those found in literature.[2,26] Significant differences were found in yolk viscosity, with many of the treatment diets (T3 500, T3 2000, TC 200, and TC 600) being significantly lower than the control.

Kirunda et al.[27] also reported inconsistent and lower measurements for viscosity of yolk from diets with vitamin E supplementation. Environmental or storage conditions may cause changes in viscosity, that is, increasing storage time decreases viscosity,[28] but all eggs were exposed to the same environmental and storage conditions. In the study of Walker et al.,[2] the only significant difference in yolks from hens supplemented with palm toco concentrate and astaxanthin was the emulsification capacity, with a significant decrease upon increased level of supplementation. It is uncertain why a decrease in emulsification capacity was seen in the egg yolks, but it was thought to be due to changes in egg yolk protein and lipid interactions. A similar change could have occurred in this study due to feed supplementation. Egg yolk is a complex system with a large amount of water, lipoproteins, and free proteins. Understanding the changes in viscosity will require further compositional and microstructural studies of the yolk, which are beyond the scope of this work. Nonetheless, such changes in viscosity may impact the egg yolk functionality and its applications.

Trained Sensory Panel Evaluation on Egg Yolk. A trained sensory panel was carried out to determine what changes were perceived by a trained sensory panel. No significant differences were perceived in moistness, smoothness, chalkiness/mouth drying, bitterness, or off-flavor (Table 3). It is interesting to note that a general trend is seen that as the moistness increased, the viscosity decreased, although not significantly. Although the annatto supplement had bitter, astringent, and fruity flavors, these flavors of the pure annatto extract were not transferred to the eggs based on the trained sensory panelist's evaluation. Panelists did perceive the yolks of the TC 200 and TC 600 diets to be significantly more yellow than those of the control. The egg yolk color measured instrumentally (Table 3) did not show any significant differences in the color of the raw egg, indicating that cooking of the eggs may have enhanced the difference in yellow color of the yolk. The scale set by panelists was a narrow range in yellow color with the extremes not being unacceptable to a consumer, indicating although a change in yolk color in some of the supplemented diets was perceived, it was not seen as a negative quality. Some consumers prefer a more intense yellow or paler yellow egg yolk. Preferences in egg yolk color also vary with geographical and cultural differences, with many consumers associating a certain color of egg yolk with level of quality and safety of the eggs.[29,30] Other studies had shown an increase in the yellow color of egg yolks with annatto supplementation,[20,29] but the levels of supplementation in this study were much lower, thus showing much less of an effect on yolk color. The savory yolk flavor was perceived to be lowest in the TC 1000 diet, but the level of change is not expected to be unacceptable to consumers.

Chemical Properties of Egg Yolks. *Total Egg Yolk Moisture.* There was no significant difference among the treatment diets for moisture content (48.30−48.84%). Moisture content of an egg yolk can vary with breeds of chicken, but 48−49% moisture is consistent with the dry solids content of 52−53% reported by Varadarajulu and Cunningham.[31] Generally, 50% moisture content is accepted for an egg yolk,[32] and the values found in this study were comparable to this.

Because significant differences were found in viscosity, it was expected that there would be differences in moisture content. Many of the treatments had lower viscosity values than the control, indicating a thinner yolk, which would be attributed to higher moisture; but this was not observed.

Egg Yolk Lipid Content. There was no significant difference among the treatment diets for egg yolk lipid content (63.43−64.34% dry basis, db). The total lipid found in the egg yolks was slightly higher than that found in literature of 62.5% dry basis.[32] It was assumed that the modification of the Folch[16] lipid extraction procedure of not using a water wash step would leave extractable proteins in the lipid extract, thus causing the increase. However, when the traditional Folch wash was carried out for the same egg yolk samples, the results were similar to those measured with the modified method.

Egg Yolk Phospholipid Composition. ^{31}P NMR was performed to determine the weight percent of phospholipids for egg yolks from day 21. Peaks in the NMR spectrum were identified by methodology from Yao and Jung.[19] No significant differences were found among treatment diets in any of the phospholipids. The major phospholipids detected were phosphatidylcholine (74.74−75.54%) and phosphatidylethanolamine (19.00−20.25%), which are expected in egg yolk. The total phospholipid in the yolk lipid (21.11−24.94%) was comparable with that reported in literature.[2,32] Other minor phospholipids in the egg yolk samples were phosphatidylinositol (0.82−1.48%), lysophosphatidylcholine (1.22−1.44%),

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sphingomyelin (1.90−2.47%), and lysophosphatidylethanolamine (0.59−0.80%). Phospholipids have many applications in the food industry[33] and are important biological molecules in maintaining a healthy body.[34]

Egg Yolk Fatty Acid Composition. The fatty acids detected in day 49 yolk samples were 16:0 (24.37−26.26%), 16:1 (2.04−2.57%), 18:0 (9.29−10.05%), 18:1 (39.53−40.92%), 18:2 (15.91−17.05%), 20:4 (1.88−2.19%), and 22:6 (0.48−0.60). There were no significant differences in any of the fatty acids reported across the treatment diets, and the percentages found are similar to those reported in literature.[2,32,35]

Vitamin E Content in Egg Yolk. After 10 days of feeding the supplement diets, a steady state of nutrient transfer of vitamin E to the egg yolk was reached, as shown in Figure 1, the same as reported by Walker et al.[2] The forms of vitamin E reported in the figure include α-tocopherol, γ-T3, and δ-T3 because these are the major forms of vitamin E in the supplements added to the feed. It is evident from the kinetics of transfer shown in Figure 1 that α-tocopherol was transferred at a much higher rate than either γ- or δ-T3, even when supplemented in similar amounts.

To understand how the additives were transferred to the yolk, measurements after steady state (day 10 and after) were averaged and compared, as shown in Table 4. Diets without α-tocopherol supplementation (control, T3 100, T3 500, and T3 2000) were not significantly different, with similar amounts of α-tocopherol as reported by the USDA Nutrient Database.[36] There is α-tocopherol in the base diet, and the amount found and transfer efficiencies are the same across these treatments. As expected, the amount of α-tocopherol in the egg yolks significantly increased as the amount of α-tocopherol added to the feed increased. At steady state, γ-T3 was the highest in T3 2000, showing that α-tocopherol did decrease the amount of γ-T3 transferred, but this was not significant until the use of highest level of α-tocopherol (TC 1000). This outcome indicates that when α-tocopherol is present at a high concentration, it lowers the amount of γ-T3 transferred to the egg yolk from the feed.

δ-T3 was significantly higher in diets supplemented with 2000 ppm of annatto, with none of the diets supplemented with α-tocopherol being significantly different from T3 2000. Therefore, α-tocopherol did not impact the transfer of δ-T3 as it did for γ-T3, and this is believed to be due to the differences and similarities of structure between the various forms of vitamin E. γ-T3 is more structurally similar to α-tocopherol (three methyl groups) with two methyl groups on the ring structure, but δ-T3 has only one methyl group on the phenolic ring.[3] α-Tocopherol is reported to interfere with absorption of T3s with a dose−response relationship,[37] and this is believed to be due to how the various forms of vitamin E are inherently transported and distributed.

When the supplement diets without α-tocopherol were compared, T3 500 and T3 2000 diets were significantly higher in γ- and δ-T3 than the control, and they were different from each other. Thus, it is possible to increase γ- and δ-T3 in the egg yolks with increasing annatto supplementation, although this was not proportional to its concentration in the feed and the quantity transferred is not very high.

Vitamin E Transfer Efficiency to Egg Yolks. α-Tocopherol was transferred much more efficiently (21.19−49.17%) to the egg yolk than either γ-T3 (0.50−0.96%) or δ-T3 (0.74−0.93%), as reported in Table 5. Significant decreases in the transfer efficiency of α-tocopherol were observed when it was supplemented at higher concentration. Decreases in γ-T3 transfer efficiencies were observed after supplementation passed

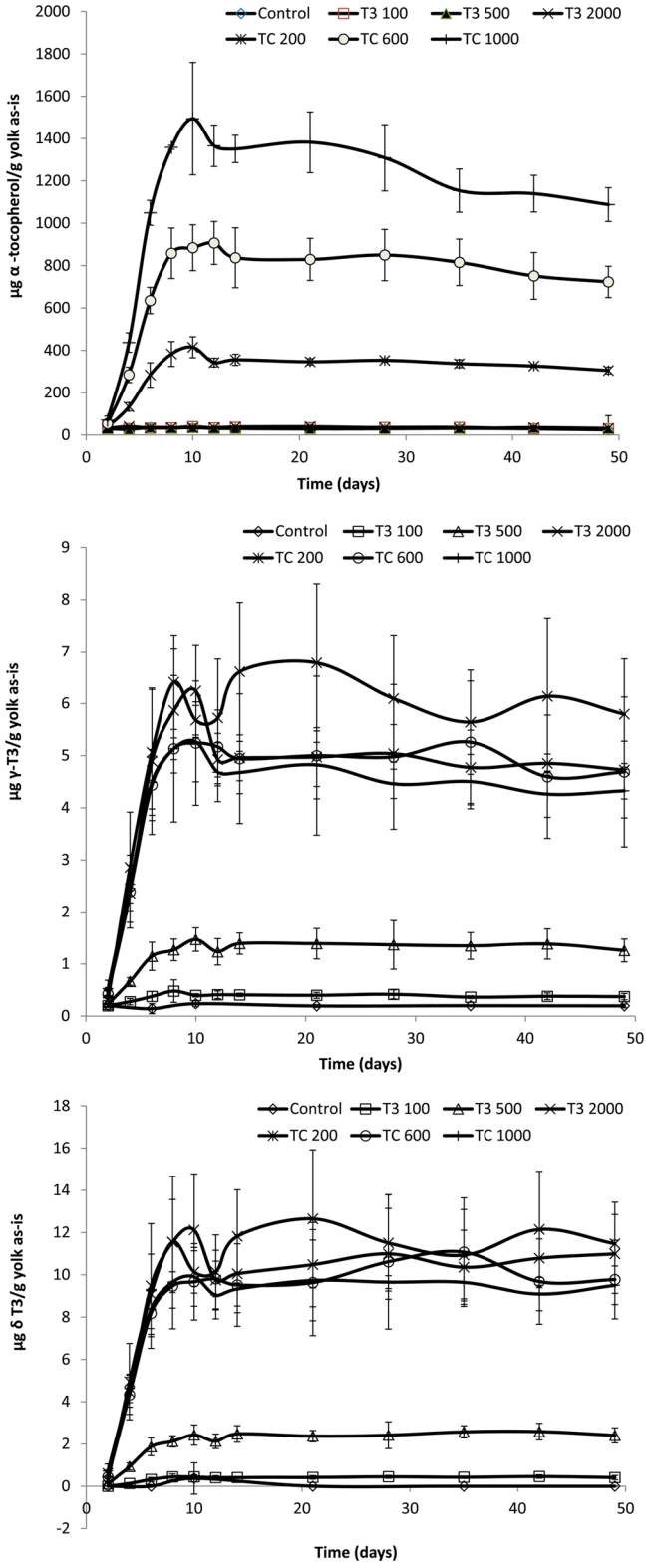

Figure 1. Kinetics of (top) α-tocopherol, (middle) γ-T3, and (bottom) δ-T3 accumulation in egg yolk over 7-week feeding period, showing means and standard deviation bars (n = 4). Diets are detailed in Table 1. T3 = ppm of annatto; TC = ppm of α-tocopherol with constant 2000 ppm of annatto.

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Table 4. Average Concentration of Vitamin E Isomers in Egg Yolks after Reaching Steady State (Days 10−49) from Diets with Different Levels of Annatto Tocotrienol and α-Tocopherol Supplementation[a]

diet[b]	α-TC (μg/g of yolk as-is)	γ-T3 (μg/g of yolk as-is)	δ-T3 (μg/g of yolk as-is)
control	29.15 ± 6.39 D	0.21 ± 0.01 C, c	0.09 ± 0.18 B, c
T3 100	33.64 ± 2.62 D	0.39 ± 0.04 C, bc	0.43 ± 0.06 B, c
T3 500	30.71 ± 3.04 D	1.35 ± 0.23 C, b	2.43 ± 0.32 B, b
T3 2000	34.21 ± 3.66 D, δ	6.06 ± 0.95 A, a	11.35 ± 1.84 A, a
TC 200	347.49 ± 4.71 C, γ	5.06 ± 0.49 AB	10.69 ± 1.81 A
TC 600	824.63 ± 94.56 B, β	4.98 ± 1.16 AB	9.97 ± 1.91 A
TC 1000	1285.40 ± 94.96 A, α	4.62 ± 0.36 B	9.49 ± 0.56 A
significance	*	*	*

[a]Values are means of values from day 10, 12,14, 21, 28, 35, 42, and 49 ± standard deviations for $n = 4$. Different uppercase letters (comparing all treatments), lowercase letters (comparing control, T3 100, T3 500, and T3 2000), and Greek letters (comparing T3 2000, TC 200, TC 600, and TC 1000) in the same column indicate significant differences at the 95% confidence level. [b]Diets are detailed in Table 1. Tocotrienols (T3) = ppm of annatto; α-tocopherol (TC) = ppm of α-tocopherol with constant 2000 ppm of annatto.

Table 5. Transfer Efficiency of Vitamin E Isomers into Egg Yolks from Feed with Different Levels of Annatto Tocotrienol and α-Tocopherol Supplementation[a]

diet[b]	α-TC	γ-T3	δ-T3
control	39.50 ± 5.01 A	N/A	N/A
T3 100	46.89 ± 7.82 A	0.96 ± 0.32 A	0.74 ± 0.45
T3 500	41.60 ± 5.71 A	0.60 ± 0.12 A,B	0.81 ± 0.49
T3 2000	49.17 ± 7.62 A, α	0.67 ± 0.12 A,B	0.93 ± 0.54
TC 200	26.91 ± 2.19 B, β	0.56 ± 0.08 B	0.90 ± 056
TC 600	21.19 ± 2.52 B, β	0.53 ± 0.10 B	0.79 ± 0.44
TC 1000	21.42 ± 3.16 B, β	0.50 ± 0.06 B	0.80 ± 0.47
significance[c]	*	*	NS

[a]Values are means at maximum value ± standard deviations for $n = 4$. Different uppercase letters (comparing all treatments), and Greek letters (comparing T3 2000, TC 200, TC 600, and TC 1000) in the same column indicate significant differences at the 95% confidence level. [b]Diets are detailed in Table 1. Tocotrienols (T3) = ppm of annatto; α-tocopherol (TC) = ppm of α-tocopherol with constant 2000 ppm of annatto. [c]NS, not significant at 5%.

100 ppm, but the efficiency was not impacted by added α-tocopherol.

The transfer efficiency for α-tocopherol was much higher than that reported by Walker et al.[2] (9.9%) and very similar to that reported by Walde et al.[13] (39.59−44.78%). Efficiency decreased as more α-tocopherol was added. This decrease indicates that there was a leveling off, possibly due to saturation of the α-tocopherol transfer protein (αTTP) in the liver that is an important step in the uptake of vitamin E, especially α-tocopherol.

Tocotrienols had been shown to have poor absorption and transfer to egg yolks in the literature previously;[2,13] thus low transfer efficiencies were not unexpected. The transfer efficiency of γ-T3 was higher than those reported by both Walker et al.[2] (<about 0.1%) and Walde et al.[13] (0.37−0.40%). δ-T3 was transferred at a level higher than that reported by Walker et al. and Walde et al. (about 0%).

α-Tocopherol was more efficiently transferred to the yolk and this was expected due to the presence of an αTTP in the liver, which preferentially binds α-tocopherol, making it more bioavailable.[4] When the highest T3 diet, T3 2000, is compared with the TC diets, no significant differences are found, indicating that adding more α-tocopherol did not impact the transfer efficiency of γ-T3. This can also be seen in δ-T3, with no significant differences among the treatment diets. This trend was expected because α-tocopherol dominated the αTTP, causing the T3s to be mostly excreted. Overall, it was observed that annatto T3s were not well absorbed, with high concentration detected in the manure (manuscript under review), showing a need for a better carrier system or matrix to transport T3s into the body.

Egg Yolk Cholesterol Quantification. The amount of cholesterol in day 2, 21, and 49 yolks was determined spectrophotometrically, and the results are shown in Table 6.

Table 6. Cholesterol Content of Day 2, 21, and 49 Egg Yolks from Diets with Different Levels of Annatto Tocotrienol and α-Tocopherol Supplementation[a]

	cholesterol (mg/g of yolk as-is)		
diet[b]	day 2	day 21	day 49
control	14.62 ± 1.64	14.61 ± 1.22	14.87 ± 0.49
T3 100	14.71 ± 1.74	15.80 ± 2.93	15.21 ± 1.71
T3 500	14.09 ± 1.32	14.60 ± 1.13	14.37 ± 1.03
T3 2000	15.18 ± 1.41	14.37 ± 1.03	14.46 ± 0.25
TC 200	13.68 ± 0.96	13.20 ± 1.50	13.49 ± 0.92
TC 600	14.90 ± 0.68	15.52 ± 1.43	13.52 ± 0.45
TC 1000	14.71 ± 1.43	13.24 ± 1.21	13.94 ± 1.42
significance[c]	NS	NS	NS

[a]Values are means ± standard deviations for $n = 4$. [b]Diets are detailed in Table 1. Tocotrienols (T3) = ppm of annatto; α-tocopherol (TC) = ppm of α-tocopherol with constant 2000 ppm of annatto. [c]NS, not significant at 5%.

This method was a modification that used a cholesterol quantification kit designed for biological samples, especially blood serum. To validate this method, various tests were performed to ensure it was accurate and appropriate. With the freeze-dried egg yolk being a mass of solid particles, the concern was length of time for the reaction to reach completion. A time-lapse study was done to ensure that all cholesterol present in the egg yolk powder reacted fully. It was observed that at 15 min (3 times longer than required by kit), the ratio of absorbance to mass of freeze-dried egg yolk reached a constant, and it did not change significantly after 15 min.

When an average of the egg yolk weights (14.61 g/yolk) is used, the range of cholesterol in the yolks was 192−231 mg/yolk. This amount is in general consistent with values reported in the literature.[39−41] Kazmierska et al.[41] discussed how different techniques for quantifying cholesterol gave slightly different results. For this reason, AOCS saponification and then GC method (Ca 6b-53) was done on selected treatments

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(control and T3 2000) to compare the amount of cholesterol quantified. No significant differences were detected among treatments. However, it was noted that the kit used in this study resulted in 18.37−20.63% higher values than the AOCS saponification and GC method, possibly due to complete quantification of all cholesterol forms. The kit method in this study allowed for detection of both free cholesterol and cholesterol ester, with the enzyme cholesterol ester hydrolase freeing cholesterol esters to allow for total quantification.

It is well-known that cholesterol ester takes longer to free the cholesterol during saponification compared to the hydrolysis of other lipid ester bonds.[42] It is not well understood how the amount of cholesterol changes (i.e., hydrolysis of ester or degradation of the free sterol) under conditions of heat and high pH during saponification. Busch and King[43] reported that cholesterol was heat-labile at 45 °C in the presence of 1 M KOH, which are conditions typically milder than that used in various cholesterol quantification procedures. Egg yolks were reported to have 10−15% of the cholesterol present as esters.[44] If the ester hydrolysis was incomplete or there was free cholesterol degradation during the saponification, the standard AOCS method would lead to an underestimation of the total cholesterol content.

In reporting the amount of cholesterol in an egg, it is important to specify the weight size class of the egg (peewee, small, medium, large, extra-large, or jumbo).[25] The average egg weight found in this study was 59.69 ± 0.37 g (with shell). According to the USDA Nutrient Database,[36] a large egg is 50 g and an extra-large egg is 56 g at a minimum without shell, so it is most appropriate to use the USDA grading manual[25] (weights with shells included) to categorize the eggs with shell in this study. According to these standards, the eggs in this study are large eggs. USDA reports the yolk of a large egg to be 17 g, much larger than the average in this study (14.61 g). Our yolk weight is very accurate because any attached egg albumen outside the yolk was removed with paper towels. It is evident that, based on weight for the large egg class and its yolk size as determined in this study, the amount of cholesterol reported by the USDA nutrient database, 184 mg/large yolk or 12.6 mg cholesterol/g of yolk as-is, is lower than that found in this study, 14.4 mg cholesterol/g of yolk as-is average across all treatments and days. The reasons for this difference are discussed above.

No significant cholesterol reductions over the feeding study were seen across treatment diets. Walde et al.[13] found a 4% or 6% reduction in diets supplemented with barley or palm T3s, respectively, but this trend was not seen in our study. A key difference in our study and the study by Walde et al.[13] is the form and amount of T3s present in the sources. Barley had mixed tocopherols and T3s, with α-T3 being the dominant form. Palm also contained mixed tocopherols, with γ-T3 being the dominant molecule. Even though egg from this study can contain up to a similar level of total T3 (~100 μg/egg) as that of Walde et al., we did not observe cholesterol reduction. Harder et al.[15] also observed lower cholesterol content in eggs with higher levels of supplemented annatto in the feed, particularly above 1.5% supplementation. However, there was no information on the T3 content in the annatto. Nonetheless, McGonigle et al.[16] observed no significant difference in the cholesterol content in eggs when feed was supplemented with annatto, similar to the observation in this study.

T3s have been shown to lower cholesterol levels in many studies.[5−12] Yu et al.[12] showed that supplementation with 50−2000 ppm of δ- and γ-T3 in young female chickens caused decreases in total serum cholesterol (32%), LDL levels (66%), and triacylglycerols (TAGs), with minimal changes to HDL cholesterol. In our study, blood samples were taken from the hens at day 49 of our feeding trial (37 weeks old), and they also showed no significant changes in lipid chemistry (total cholesterol, HDL, and TAGs; data reported in Hansen et al., manuscript under review). We do not know the cause of the different outcomes, but we thought that the use of young female birds versus adult laying hens may play a role in these differences.

In summary, annatto and α-tocopherol supplements did not significantly impact most of the hen performance indicators and quality parameters of the resulting egg yolks. Significant differences were found in daily feed disappearance, egg yolk viscosity, sensory yolk color, and savory yolk flavor and in the amount and transfer efficiency of various forms of vitamin E in the egg yolks. On the basis of both data over time and transfer efficiencies at the maximum value for α-tocopherol, γ-T3, and δ-T3, it is evident that α-tocopherol is much better transferred to hen egg yolks and does play some role in how the other forms of vitamin E are absorbed. This study confirms that the T3s cannot be substantially transferred in hen's body and yolk, even when there is no significant amount of α-tocopherol present to compete with the binding protein. This study illustrates that by changing what is in laying-hen feed, it is possible to change the nutrients in an egg, but only when the physiology of the animals allows. Novel approaches have to be designed to overcome the physiology barrier in order to make the hens more efficiently transfer the T3s to the egg. With a better delivery system, it may be possible to observe a cholesterol reduction effect of T3s in egg.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

One table listing composition of base diet fed to laying hens for 7 weeks. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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