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Supplementation of laying-hen feed with annatto tocotrienols and impact of α-tocopherol on tocotrienol transfer to egg yolk and tocopherol and annatto tocotrienol distribution analysis in laying-hen body

by

Hannah E. Hansen

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Food Science and Technology

Program of Study Committee:
Tong Wang, Major Professor
Kenneth Prusa
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Iowa State University

Ames, Iowa

2015

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NOMENCLATURE AND ABBREVIATIONS

α-TTP	α-Tocopherol Transfer Protein
AEB	American Egg Board
AOCS	American Oil Chemists' Society
ARA	Arachidonic acid
CVD	Cardiovascular Disease
DAOS	3,5-dimethoxy-N-ethyl-N-(2-hydroxy-3-sulfopropyl)-aniline sodium salt
DHA	Docosahexaenoic acid
ENC	Egg Nutrition Center
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl esters
FDA	Food and Drug Administration
GRAS	Generally Recognized As Safe
HDL	High-density lipoproteins
IEC	Iowa Egg Council
LDL	Low-density lipoproteins
MUFA	Monounsaturated fatty acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PUFA	Polyunsaturated fatty acid
RDA	Recommended Daily Allowance
SFA	Saturated fatty acid
SM	Sphingomyelin
T3(s)	Tocotrienol (s)
TAG	Triglycerides
TC(s)	Tocopherol (s)
TPP	Triphenol phosphate
USDA	United States Department of Agriculture

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ABSTRACT

Laying-hens efficiently transfer nutrients from their feed to the resulting eggs, and nutrients from feed are distributed in many organs and tissues for various uses. Annatto is the only known source of tocotrienols (T3s) (gamma-T3 ~10% and delta-T3 ~90%) without alpha-tocopherol present. The T3s have many health benefits including lowering cholesterol. The objective of this research was to study the effect of alpha-tocopherol on the transfer of T3s to eggs and various organs and tissues in the body of the laying-hen. To do so, laying-hens were fed treatment diets for 7 weeks supplemented with annatto T3s in the presence and absence of alpha-tocopherol. The diet regimens were a control diet with not supplementation and three diets with 2000 ppm annatto extract and added alpha-tocopherol at 200 and 1000 ppm.

No significant differences were found in egg production or egg yolk properties (moisture content of moisture, lipid, phospholipids, fatty acids, or cholesterol). Significant differences ($p<0.05$) were found in feed intake, yolk viscosity, sensory yolk flavor and color, and transfer efficiency of tocopherols and T3s to the egg yolks.

Alpha-tocopherol was transferred more efficiently (21.19-49.17%) than gamma-T3 (0.50-0.96%) or delta-T3 (0.74-0.93%) to the egg yolks. Addition of 1000 ppm of alpha-tocopherol decreased the transfer of gamma-T3 (by 23.76%) but it did not impact the transfer of delta-T3 to the egg yolk. The addition of annatto T3s did not significantly impact the cholesterol content of the laid eggs.

A total of 18 organs or tissues (skin, fat pad, liver and gall bladder, heart, oviduct, forming yolk, laid yolk, lungs, spleen, kidney, pancreas, gizzard, digestive tract, brain, thigh, breast, manure, and blood) were collected after 7 weeks of feeding the diets.

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Tissue weights, moisture content (except for manure), lipid, alpha-tocopherol, gamma-T3, delta-T3, cholesterol, and fatty acid composition of extracted lipids in the collected organs and tissues (except for blood) were determined.

Tissue weights, moisture content, and lipid content did not change significantly with feed supplementation across treatments, except that the liver became heavier with increased supplementation. Minimal changes were found in the fatty acid composition, except in the fat pad, oviduct, brain, and manure.

Overall, the main organs that accumulated the supplemented forms of vitamin E were fat pad, liver and gall bladder, oviduct, forming yolks, laid yolks, kidney, brain, thigh, and breast. Much of annatto supplement (gamma-T3 and delta-T3) was detected in the manure (>90%), indicating that most was excreted and not used by the hen. In some tissues (brain and oviduct) a significant increase in polyunsaturated fatty acids was seen with increased supplementation. Alpha-tocopherol impacted the transfer of gamma-T3 to the forming and laid yolks, but did not impact delta-T3 transfer. No significant differences were found in the cholesterol in the liver, kidney, laid yolks, breast meat, oviduct, or thigh meat, except for cholesterol reduction in the heart based on as-is tissue weight. Blood samples showed large variation among individual hens with no significant differences in total cholesterol, HDL, or total triacylglycerols.

The results indicate that supplementing hen-laying feed with annatto T3s and alpha-tocopherol can alter the vitamin E profile and its distribution among the laid eggs and laying-hen organ and tissues (especially in the liver and egg yolks). Therefore it is possible to enhance the nutritional profile of the egg to further benefit the consumers and to increase oxidative stability of various organs and tissues.

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CHAPTER 1

GENERAL INTRODUCTION

Egg Nutrition

Eggs are the gold standard for protein (complete amino acid profile), source for many micronutrients, including choline, lutein, zeaxanthin, and vitamin D in the diet (Egg Nutrition Center, 2014) , and low in calories (70 Calories per large egg) and cost. Eggs are the most affordable complete protein (Iowa Egg Council, 2014). Eggs are believed to be part of a healthy and well-balanced diet (American Egg Board, 2011). Moreover, eggs possess many functional properties that are useful to many industries, such as medical, pharmaceutical, cosmetic, nutraceutical, and biotechnological (Anton et al., 2007 and Aro et al., 2009).

Laying-hens are very efficient at transferring nutrients from their feed to the resulting eggs (fat soluble nutrients transfer to egg yolk). This fact provides a method to alter the nutritional composition of eggs (Walker et al., 2012 and Yao et al., 2013) as a means for human nutrition. Along with supplementation, genetics, environment, and age also play roles in affecting the egg composition (Frediksson et al., 2006) and quality (Koelkebeck, 2014).

Eggs consist of a yolk, albumen (white), and shell. The yolk makes up approximately one third (36%) by weight of the egg. The egg yolk is approximately 50-52% solids depending upon breed, age, storage conditions, and storage time (Anton et al., 2007). The majority of the dry solids in the egg yolk are lipids (62.5%) and proteins (33%), with minor carbohydrates (1-2%) and minerals (3-4%).

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The lipids are composed of triglycerides (62%), phospholipids (33%), cholesterol (<5%), and carotenoids (<1%) (Anton et al., 2007). The fatty acid composition, based on typical commercial diet, of these lipids classes are saturated fatty acids (SFA, 30-35%), monounsaturated fatty acids (MUFA, 40-45%), and polyunsaturated fatty acids (PUFA, 20-25%). Within these lipid classes, the main fatty acids are oleic acid (C18:1, 40-45%), palmitic acid (C16:0, 20-25%), and linoleic acid (C18:2, 15-20%) (Anton et al., 2007). The main phospholipids found in the egg yolk are phosphatidylcholine (PC, 76%) and phosphatidylethanolamne (PE, 22%). Other minor phospholipids found in egg yolk include; phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SM), cardiolipins (CL), lysoPC, and lysoPE (Anton et al., 2007).

Changing laying-hen feed composition can lead to alteration of the composition of the egg. When the composition of an egg and egg yolk is considered, it is important to know that both positive and negative changes can occur with different feeds or environmental conditions.

Rationale for Nutrient Enrichment

Vitamin E is a fat soluble vitamin which has eight major forms classified into two major groups, tocopherols and tocotrienols (T3s). These groups differ in 3 double bonds (unsaturation) on the phytyl tail of the T3s (Sontag et al., 2007). Within each of these groups (tocopherols and T3s), there is an alpha, beta, gamma, and delta form varying in the number and position of the methyl groups on the chromanol ring (Sontag et al., 2007). Alpha-tocopherol is the most bioavailable form due to the presence of an alpha-tocopherol transfer protein in the liver. This protein has higher affinity for the alpha-tocopherol form of vitamin E, causing biodiscrimination against the other forms of

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vitamin E (Traber et al., 1999). Alpha-tocopherol being the most bioavailable could dominate the absorption, causing poor absorption of the T3s.

T3s have been shown to have many health benefits not possessed by the tocopherol counterparts. One significant benefit is lowering cholesterol by down-regulating a key hepatic enzyme in cholesterol synthesis (3-hydroxy-menthyglutaryl coenzyme A reductase) (Qureshi et al., 1986; Pearce et al., 1992; Parker et al., 1993; Qureshi et al., 2000; Qureshi et al., 2001; Qureshi et al., 2001; Yu et al., 2006; and Yang et al., 2013). Yu et al. (2006) showed that supplementation of 50-2000 ppm of delta and gamma T3s to 5-week-old female chickens caused decreases in total serum cholesterol by 32% and decreases of LDL levels by 66%, with minimal changes to HDL cholesterol.

Vitamin E has been supplemented to laying-hen feed in many studies; Walker et al. (2012) showed that fat soluble nutrients (like vitamin E) reach a steady state after 8-10 days of feeding. Previous work has studied how T3s are transferred to eggs from feed (Walker et al., 2012 and Walde et al., 2013), but none examined the efficiency of transfer without alpha-tocopherol present. In the previous studies mentioned, the sources of tocopherols and T3s used (palm and barley) have alpha-tocopherol naturally present, making it impossible to observe T3 transfer without the influence of alpha-tocopherol. Ikeda et al. (2003) showed that alpha-tocopherol at certain levels blocked selected T3 forms (alpha-T3) but not others (gamma-T3), thus alpha-tocopherol must be at the proper concentration to optimize transfer of T3s. Having alpha-tocopherol supplemented at the correct level will allow the benefit of added alpha-tocopherol but not to an extent that it will attenuate the T3s transfer. Alpha-tocopherol being present at certain levels has been shown to block or decrease the benefits of T3s (Qureshi et al, 1996).

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Annatto is the only known natural source of T3s (delta ~90% and gamma ~10%) without alpha-tocopherol naturally present (Frega et al., 1998). Using annatto extract makes it possible to determine T3 transfer to the egg yolk both in the presence and absence of alpha-tocopherol. Walde et al. (2013) showed that the addition of barley or palm to laying-hen feed decreased egg yolk cholesterol by 4 or 6%, respectively. However, the presence of alpha-tocopherol in the source of T3s this may have hindered T3 transfer. Using annatto will allow for T3s to be exposed to the biological system without alpha-tocopherol's interference or influence. This may possibly increase bioaccumulation, and consequently it may lower cholesterol further in the resulting eggs.

Egg yolk is composed of many classes of lipids, but the most notorious lipid is cholesterol. Historically, eggs were thought to be unhealthy due to high concentration of cholesterol in the yolk. High cholesterol in the human body and blood is associated with higher risk of cardiovascular disease. However, as more research has been done, this opinion has shifted to suggest dietary cholesterol is a small part in heart health (Kantor et al., 2012). Nonetheless, the concern of cholesterol has caused many consumers to avoid consuming the highly nutritious eggs routinely. Reducing the amount of cholesterol in eggs (currently 186 mg per large egg according to USDA, 2014), may help encourage more egg consumption.

T3s are unique compounds that have many health benefits. We wanted to determine the effects of supplementing laying-hen feed with annatto T3s and alpha-tocopherol on T3s transfer to egg yolk, the effect of alpha-tocopherol on transfer of T3s to egg yolk, and the impact of T3s on cholesterol content in egg yolk.

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Rationale for Distribution Determination

As stated above, there is biodiscrimination in the uptake of the various forms of vitamin E. Many studies (Nockels et al., 1976; Tengerdy et al., 1977; Surai et al., 2000; and Tres et al., 2012) have shown vitamin E distribution in a select number of organs (liver especially). But a study examining all laying-hen organs and characterizing other components (moisture, lipid, fatty acid, and cholesterol) is lacking in literature.

To obtain a complete understanding of how the alpha-tocopherol, gamma-T3, and delta-T3 from the supplements were distributed throughout the body of the laying-hens, 18 organ/tissue and by-product samples (skin, fat pad, liver and gall bladder, heart, oviduct, forming yolk, laid yolk, lungs, spleen, kidney, pancreas, gizzard, digestive tract, brain, thigh, breast, manure, and blood) were collected and examined. The composition obtained will help better understand the transfer and impact of the nutrients supplemented in the biological system.

If the vitamin E supplement can lead to increased concentration in certain tissues, this could result in a healthier laying-hen and higher quality products (i.e. eggs and meat). Higher levels of T3s in the tissues and food products derived from the laying-hens could improve oxidative stability as well, as alpha-tocopherol has been shown to reduce oxidation in chicken meat products (Brenes et al., 2008).

Thesis Organization

This thesis contains a general introduction, literature review, two manuscripts as prepared for submission to two different journals, and general conclusions.

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References

Anton, M. Composition and Structure of Hen Egg Yolk. In *Bioactive Egg Compounds*; Huopalahti, R., López-Fandiño, R., Anton, M., Schade, R., Eds.; Springer: Berlin, New York, 2007; pp 1−5.

American Egg Board. Incredible Edible Egg: Egg Nutrients. Available at http://www.incredibleegg.org/health-and-nutrition/egg-nutrients (last accessed Dec 2014).

Aro, H.; Jarvenpaa, E.; Konko, K.; Sihvonen, M.; Hietaniemi, V.; Huopalahti, R. Isolation and purification of egg yolk phospholipids using liquid extraction and pilot-scale supercritical fluid techniques. *Eur. Food Res. Technol*. **2009**, 228, 857-863.

Egg Nutrition Center. Research and Nutrition. Available at http://www.eggnutritioncenter.org/nutrition-research/ (last accessed Dec 2014).

Brenes, A.; Viveros, A.; Goni, I.; Centeno, .; Sayago-Ayerdy, S.; Arija, I.; Saura-Calixto, F. Effect of grape pomace concentrate and vitamin E on digestibility of polyphenols and antioxidant activity in chickens. *Poult. Sci*. **2008**, 87, 307-316.

Fredriksson, S.; Elwinger, K.; Pickova, J. Fatty acid and carotenoid composition of egg yolk as an effect of microalgae addition to feed formula for laying hens. *Food Chem.* **2006,** 99, 530-537.

Frega, N.; Mozzon, M.; , F. Identification and estimation of tocotrienols in the annatto lipid fraction by gas chromatography-mass spectrometry. *J. Am. Oil Chem. Soc.* **1998**, 75 (12), 1723-1727.

Ikeda, S.; Tohyama, T.; Yoshimura, H.; Hamamura, K.; Abe, K.; Yanmashita, K. Dietary alpha-tocopherol decreases alpha tocotrienol but not gamma tocotrienol concentration in rats. *J. Nutr.* **2003**, 133, 428–434.

Iowa Egg Council. Available at http://iowaegg.org/#sthash.HH5porln.wxzK2AeQ.dpbs (last accessed Oct 2014).

Kantor, M.; Kris-Etherton, P.; Fernandez, M.; Vickers, K. Katz, D. Exploring the factors that affect blood cholesterol and heart disease: is dietary cholesterol as bad for you as history leads us to believe?. *Adv. Nutr*. **2012**, 3, 711-717.

Koelkebeck, K. What is egg quality and conserving it. Available at http://livestocktrail.illinois.edu/poultrynet/paperDisplay.cfm?ContentID=522 (last accessed Dec. 2014).

المنارة للاستشارات
www.manaraa.com

Nockels, C.F.; Menge, D.L.; Kienholz, W. Effect of excessive dietary vitamin E on the chick. *Poult Sci.* **1976**, 55, 649-652.

Parker, R.; Pearce, B.; Clark, R.; Gardon, D.; Wright, J. Tocotrienols regulate cholesterol production in mammalian cells by post-transcriptional suppression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *J. Biol. Chem.* **1993**, 268 (15), 11230-11238.

Pearce, B.; Parker, R.; Deason, M.; Qureshi, A.; Wright, J. Hypocholesteolemic activity of synthetic and natural tocotrienols. *J. Med. Chem.* **1992**, 35, 3595-3606.

Qureshi, A.; Burger, W.; Peterson, D.; Elson, C. The structure of an inhibitor of cholesterol biosynthesis isolated from barley. *J. Biol. Chem.* **1986**, 261 (23), 10544-10550.

Qureshi, A.; Pearce, B.; Nor, R.; Gapor, A.; Peterson, D.; Elson, C. Dietary alpha-tocopherol attenuates the impact of gamma-tocotrienol on hepatic 3-hydroxy-3-methylglutaryl coenzyme A reducase activity in chickens. *J. Nutr.* **1996**, 126, 389-394.

Qureshi, A.; Mo, H.; Packer, L.; Peterson, D. Isolation and identification of novel tocotrienols from rice bran with hypocholesterolemic, antioxidant, and antitumor properties. *J. Agric. Food Chem.* **2000**, 48, 3130-3140.

Qureshi, A.; Peterson, D. The combined effects of novel tocotrienols and lovastatin on lipid metabolism in chickens. *Atherosclerosis*. **2001**, 156, 39-47.

Qureshi, A.; Peterson, D.; Hasler-Rapacz, J.; Rapacz, J. Novel tocotrienols of rice bran suppress cholesterogenesis in hereditary hypercholesterolemic swine. *J. Nutr.* **2001**, 131, 223-230.

Sontag, T.; Parker, R. Influence of major structural features of tocopherols and tocotrienols on their ω-oxidation by tocopherol-ω-hydroxylase. *J. Lipid Res.* **2007**, 48, 1090-1098.

Surai, P.F.; Sparks, N.C.H.Tissue-specific fatty acid and alpha-tocopherol profiles in male chickens depending on dietary tuna oil and vitamin E providion. *Poult. Sci*. **2000**, 79, 1132-1142.

Tengerdy, R.; Brown, J. Effect of Vitamin E and A on Humoral Immunity and Phagocytosis in E. *coli* infected chicken. *Poult. Sci.* **1976**, 56, 957-963.

Traber, M. Molecular mechanisms of vitamin E transport. *Annu. Rev. Nutr.* **1999**, 19, 343-355.

المنارة للاستشارات

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Tres, A.; Nuchi, C.D.; Magrinya, N.; Guardiola, F.; Bou, R.; Codony, R. Use of palm-oil by-products in chicken and rabbit feeds: effect on the fatty acid and tocol composition of meat, liver, and plasma. *Animal*. **2012**, 6(6), 1005-1017.

USDA Nutrient Database Agricultural Research Service. United States Department of Agriculture: Full Report (All Nutrients): 01123, Egg, whole, raw, fresh. Available at http://ndb.nal.usda.gov/ndb/foods/show/112?fg=&man=&lfacet=&count=&max=&sort=&qlookup=&offset=&format=Full&new=&measureby= (last accessed Sept 2014).

Walde, C.; Drotleff, A.; Ternes, W. Comparison of dietary tocotrienols from barley and palm oils in hen's egg yolk: transfer efficiency, influence of emulsification, and effect on egg cholesterol. *J. Sci. Food Agric*. **2013**, 94 (4), 810-818.

Walker, L.; Wang, T.; Xin, H.; Dolde, D. Supplementation of laying-hen feed with palm tocos and algae astaxanthin for egg yolk nutrient enrichment. *J. Agric. Food Chem.* **2012**, 60, 1989-1999.

Yang, Z.; Lee, M.; Sang, S.; Yang, C. Bioavailability and metabolism of tocotrienols. In *Tocotrienols Vitamin E Beyond Tocopherols*, 2nd ed.; Tan, B.; Watson, R.; Preedy, V., Eds.; CRC Press: Boca Raton, FL, **2013**, 37-51.

Yao, L.; Wang, T.; Persia, M.; Horst, R.; Higgins, M. Effects of vitamin D_3-enriched diet on egg yolk vitamin D_3 content and yolk quality. *J. of Food Sci.* **2013**, 78 (2), 178-183.

Yu, S.; Thomas, A.; Gapor, A.; Tan, B.; Qureshi, N.; Qureshi, A. Dose-response impact of various tocotrienols on serum lipid parameters in 5-week-old-female-chickens. *Lipids*. **2006**, 41, 453-461.

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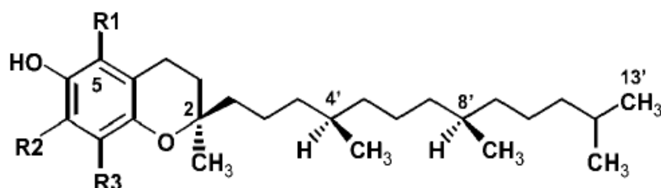
CHAPTER 2

LITERATURE REVIEW

Vitamin E: Tocopherols and Tocotrienols

Vitamin E is a fat soluble vitamin, and is grouped into tocopherols and tocotrienols (T3s). The major difference between these two classes (tocopherols and T3s) is the three double bonds (unsaturation) in the hydrophobic tail (phytyl tail) at the 3’, 7’, and 11’ positions in the T3s. These unsaturations cause the structure of T3s to be more rigid (lack ability to spin and move) giving unique properties not had by the tocopherol counterparts. Within tocopherol and T3 classes there are four forms alpha, beta, gamma, and delta, differing in the number and position of methyl groups on the chroman ring (Figure 1). Of these eight forms, alpha-tocopherol with three methyl groups on the chroman ring is the most bioavailable.



Form	R1	R2	R3
Alpha	$-CH_3$	$-CH_3$	$-CH_3$
Beta	$-CH_3$	$-H$	$-CH_3$
Gamma	$-H$	$-CH_3$	$-CH_3$
Delta	$-H$	$-H$	$-CH_3$

Tocopherols = no unsaturation
Tocotrienols (T3s) = unsaturation/double bonds at 3’, 7’, and 11’

Figure 1. Structure of various vitamin E forms (tocopherols and tocotrienols) modified from Sontag et al. (2007)

Vitamin E is an essential nutrient and cannot be made (or interconverted) by the human body, thus must be consumed in the diet (Gropper et al., 2013). Vitamin E (alpha-tocopherol) status is believed to be good in the United States with sufficient intake of

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vitamin E (National Academy of Science, 2000). Factors to consider that may impact the accuracy of this conclusion are use of supplements and underreporting of fat in diet (National Academy of Science, 2000). There may be minimal cases of deficiency with most cases thought to be caused by genetic defects impacting vitamin E uptake and utilization by the body (Brigelius-Flohe et al., 1999). The recommended daily allowance (RDA) of vitamin E for men and women is 15 mg alpha-tocopherol per day (National Academy of Science, 2000). The RDA has no consideration of the other forms of vitamin E due to high bioactivity of alpha-tocopherol (National Academy of Science, 2000).

The main function of vitamin E in a biological system is preventing oxidation, especially of lipids. It prevents oxidation by breaking the chain of radical reactions by quenching and scavenging free radicals. Terminating lipid oxidation reduces oxidative stress in a system (National Academy of Science, 2000). Oxidative stress is currently the leading theory for aging and many other chronic diseases (Shokolenko et al., 2014).

Antioxidants, like vitamin E, are needed in the human body and other biological systems. This is evident because when vitamin E is deficient symptoms are wide-spread throughout the body. Symptoms of vitamin E deficiency may include; muscle pain, weakness, hemolytic anemia, degenerative neuropathy, fragile membranes, and others (Gropper et al., 2013).

Other studies have shown that vitamin E has various roles in improving many chronic diseases. Some examples include; cardiovascular disease (Stampfer et al., 1993; Stephens et al., 1996; National Academy of Science, 2000; and Gropper et al., 2013),

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cancer (Seifried et al., 2007 and Gropper et al., 2013), and eye health (Mares-Paerlman et al., 2000; Jacques et al., 2005; and Gropper et al., 2013).

Increased intake of vitamin E has helped to prevent the oxidation of LDLs, cholesterol, and other lipid components in the body and blood, thus reducing the risks of cardiovascular disease. The reduced chance of cancer is related to the ability of vitamin E to remove free-radicals that could damage the gene or genetic regulation leading to cancer. Vitamin E may play a role in reducing risk of diabetes (aiding in plasma membrane in glucose uptake), Alzheimer's disease (reduce oxidative stress and possibly reduce protein aggregates), and other diseases (Gropper et al., 2013).

Interest has grown in T3s because of the many unique health benefits they possess. Many T3 benefits are not had or are not as effective in tocopherol counterparts. These benefits include higher potency antioxidant, anti-tumor, lowering cardiovascular disease and hypertension, and neuroprotection (Yang et al., 2013 and Qureshi et al., 2000). A significant benefit of T3s is lowering cholesterol by down-regulating the hepatic enzyme 3-hydroxy-menthyglutaryl coenzyme A reductase, that is the rate limiting enzyme in cholesterol synthesis (Qureshi et al., 1986; Pearch et al., 1992; Parker et al., 1993; Qureshi et al., 2000; Qureshi et al., 2001; Qureshi et al., 2001; Yu et al., 2006; Yang et al., 2013). Walde et al. (2013) showed a 4% to 6% reduction of cholesterol in laid eggs when fed palm and barley, respectively.

Qureshi et al. (1986) first discovered that the structure of the cholesterol lowering compound in barley was a T3 (alpha-T3). In his later work, he continued to build evidence of the ability of the T3s to lower cholesterol by impacting its synthesis. Qureshi et al. (1996) fed 6-week-old female chickens amaranth which showed reductions in the total serum cholesterol of 10-30% and LDL cholesterol of 7-70%, minimal effects to

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HDL cholesterol, higher activity of 10-18% of 7-alpha-hydroxylase that is responsible for converting cholesterol to bile salts, and lower activity of approximately 9% of the rate limiting enzyme in cholesterol synthesis (3-hydroxy-menthyglutaryl coenzyme A reductase). In this work, the authors suggested with such drastic improvements in cholesterol profiles, that another substance may be present that may enhance or mimic the effects of T3s. Qureshi et al. (2001) also showed that T3s from rice bran suppressed cholesterol synthesis and improved blood lipid chemistry in swine by lowering total serum cholesterol by 32-38% and LDL cholesterol by 35-43%. Yu et al. (2006) showed that supplementing 50-2000 ppm in feed of delta and gamma T3s caused decreases in total serum cholesterol (32%), LDL levels (66%), and TAGs, with minimal changes to HDL cholesterol in 5-week-old female chickens. Overall, many of the papers published in this area have used younger animal models.

Sen et al. (2006) and Yang et al. (2013) reviewed many health benefits provided by T3s in detail, analyzed data from many studies to show a wide-spread action and benefit of the T3s throughout the biological system. As more is discovered about T3s and the more roles are found, there is a need to efficiently deliver the T3s to the biological systems.

Absorption and Transport of Vitamin E

Many factors can affect bioavailability of vitamin E to a biological system. Food matrix, formulation, and processing can affect how vitamin E is absorbed and transported within the body or biological system (Reboul et al., 2006). Alpha-tocopherol is the most bioavailable form of vitamin E due to the presence of an alpha-tocopherol transfer protein in the liver that has higher affinity and selectivity for alpha-tocopherol. This affinity

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arises from structural differences in the forms of vitamin E (Figure 1). Selectivity and recognition by the alpha-tocopherol transfer protein relies on three methyl groups on the chromanol ring (especially the methyl at C5), hydroxyl group on the chromanol ring, and phytyl chain structure and orientation (Traber et al., 1999). The methyl groups also impact the special abilities of T3s (Sontag et al., 2007), with some T3 forms being more functional. This preference causes biodiscrimination against non-alpha-tocopherol forms of vitamin E (Traber et al., 1999).

In humans, vitamin E from the diet is first absorbed as a micelle from the small intestine and packaged into a chylomicron with other hydrophobic/lipid constituents from the diet. Vitamin E within the chylomicron is then transported to the liver by the lymph system. In laying-hens the uptake of lipid substances from the diet occurs slightly different. Lipid substances, including vitamin E, are delivered to liver by portomicrons (lipid rich proteins released by intestine) through the portal vein (Surai et al., 2001), due to the lack of a lymph system. In the liver, the alpha-tocopherol transfer protein will selectively and preferentially bind to alpha-tocopherol and be further secreted into the circulation of the body, while other forms are mostly excreted due to longer times in the liver (National Academy of Sciences, 2000).

The alpha-tocopherol transfer protein was thought to be expressed in the liver exclusively (Yoshida et al., 1992; Sato et al., 1993; and Arita et al., 1995), but Hosomi et al. (1998) detected this protein in the brain, spleen, lungs, and kidney of rats. This may signify that other tissues of the body have a preference for certain forms of vitamin E as well. Even though alpha-tocopherol is the most bioavailable form, the other forms of vitamin E are also important in the body, even at low concentrations.

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T3s are thought to be more efficient than tocopherols, with less T3s needed for the same function of alpha-tocopherol. For this reason, less T3s may be needed for the same protection or benefit. Alpha-tocopherol is found in the cellular membrane at one part for approximately every 1,000 lipid molecules (Burton et al., 1990). It is thought that alpha-T3 is three times more efficient at scavenging free radicals than alpha-tocopherol (Lanari et al., 2004). It was suggested that this is because T3s can be more uniform, due to a more rigid structure, within a cellular membrane. T3s have been shown to have a larger impact on the oxidative stress measurements in exercise endurance in rats (Lee et al., 2009). In this study different treatments of similar levels of T3s and alpha-tocopherol (25mg/kg) were compared, with the T3 diet having significantly lower oxidative stress and longer endurance times that was not seen in the alpha-tocopherol diet. Even with poor uptake of T3s, a much lower concentration is thought to be needed for the benefits.

Annatto Tocotrienols

The tocopherol forms of vitamin E is reported to be found in corn, wheat, and soybeans, while the T3 forms are reported to be in annatto, barley, oats, palm, and rice bran (Aggarwal et al., 2010). In these there are various amounts of the eight forms of vitamin E varying with source. With laying-hens it may be possible to supplement feed with the various forms of vitamin E and add eggs as a source of both tocopherols and T3s in the diet.

Annatto is the only known natural source of T3s (delta ~90% and gamma ~10%), without alpha-tocopherol present (Frega et al, 1998). Without the presence of alpha-tocopherol, the transfer of the T3s to a biological system, such as laying-hen egg yolks, may be improved. Annatto is naturally derived from the *Bixa orellana* rainforest plant

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with high purity. Annatto is potent in color, a bright red-orange from its carotenoids (bixin and norbixin) (Frega et al, 1998).

Annatto currently is a GRAS substance and its added into a variety of foods and products for coloring (U.S. Food and Drug Administration, 2014). It is specifically used by the food industry to give certain dairy products the characteristic yellow color (Frega et al, 1998). Annatto's high density of T3s (delta and gamma) make it have other applications and benefits beyond coloring.

Cholesterol Lowering

Cholesterol is a polycyclic amphiphilic compound (Figure 2) that is made by animals, and can be in free or esterified form (cholesterol ester at the hydroxyl group) (American Oil Chemists' Society, 2014). Most cholesterol in the body is found in plasma membrane bilayers, and it facilitates the fluidity and organization of the structure. Cholesterol tends to accumulate (approximately a quarter) in the brain of a biological system (AOCS', 2014). Cholesterol is transported throughout the body in lipoproteins (chylomicron, VLDL, LDL, and HDL), and these are the measurements of cholesterol status in the body.

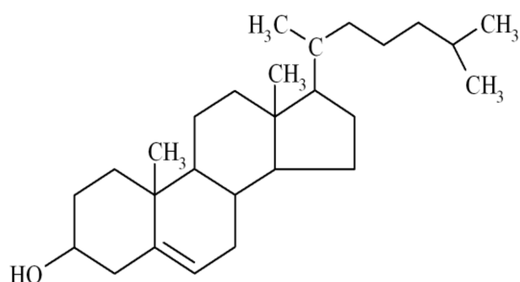

Figure 2. Structure of free cholesterol

Cholesterol is a very important molecule in the body and is a precursor for bile salts, vitamin D, and steroid hormones. It is also an important compound in signal

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transduction, sperm development, embryonic development, and other functions. Cholesterol can be made by most animals (vertebrates mainly), while others (invertebrates mainly) must consume it (AOCS', 2014).

Cholesterol can be made in vivo. The synthesis starts with acetoacetyl-CoA and acetyl-CoA to form 3-hydroxymethyl-glutaryl-CoA. The product is then transformed to mevalonate. This step is the rate limiting step in cholesterol synthesis and it is catalyzed by the hepatic enzyme 3-hydroxy-menthyglutaryl coenzyme A reductase (HMG Co-A reductase). This enzyme is the target of many cholesterol lowing drugs, such as statins, in order to lower cholesterol production. Melvalonate is then converted to isopentylpyrophosphate, which is used to build a larger structure by first making farnesyl pyrophosphate followed by squalene. To finally make the cholesterol structure, three methyl groups are removed, a double bond is reduced in the hydrocarbon tail, and the double bond is moved (Berg et al., 2012).

Once synthesized in the liver, cholesterol is transported throughout the body by lipoproteins (such as chylomicrons, VLDLs, LDLs, and HDLs). Chylomicrons (portomicrons in laying-hens) transport cholesterol from dietary sources. Very low density lipoproteins (VLDLs) are mostly fat, low density lipoproteins (LDL) are known as "bad cholesterol" that deposit cholesterol throughout the body, and high density lipoproteins (HDL) are known as "good cholesterol" that transport cholesterol from the body back to the liver. LDL contributes to atherosclerosis while HDL reverses it, and both of these are important parameters medically. Levels or ratios (i.e. LDL:HDL) of these lipoproteins are used for medical diagnostic purposes that indicate cholesterol status in the body (Berg et al., 2012).

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The body regulates how much cholesterol is produced in many ways. For example, if the diet provides cholesterol, feedback inhibition will lower the activity of HMG Co-A reductase to lower the amount of cholesterol synthesized. The reverse is also true, making dietary cholesterol a minor importance in the level of cholesterol in the body because if it is not in the diet, the body will make what is needed. Overall, short term regulation includes feedback inhibition, and long term regulation includes making less of the rate limiting enzyme (HMG Co-A reductase) (Berg et al., 2012). In some individuals, dietary cholesterol has a larger impact, but this is only a small percent of the population (Fernandez et al., 2010), making genetics a significant factor in determining if an individual has higher than normal level of cholesterol in the body. Other factors also important in consideration of cholesterol and heart health are fat intake especially saturated, and other lifestyle factors (Kanter et al., 2012).

Cholesterol is found in products of animal origin (i.e. meat, dairy, and other animal products). Eggs are characterized as the food source with the highest concentration of cholesterol (currently 186 mg per large egg according to USDA, 2014). The association of cholesterol and cardiovascular disease has made consumers shy away from consuming eggs on a regular basis. Eggs are the gold standard for protein (complete amino acid profile), unique source for many micronutrients (including choline, lutein, zeaxanthin, and vitamin D, Egg Nutrition Center, 2014) in the diet, and they are low in calories and costs (Iowa Egg Council, 2014). Long term consumption of eggs (i.e. dietary cholesterol with low saturated fats) in moderation is not associated with bad heart health and is instead part of a healthy diet (Kanter et al., 2012 and American Egg Board,

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2011). One would hope that the cholesterol concern of eggs would not make people consume less of this nutritious food.

Cholesterol Quantification

The standard for quantification of cholesterol is saponification and then GC (AOCS method Ca 6b-53). This method is very time and solvent intensive and can be cumbersome quantification. There are many methods that are similar to this method but use different conditions and settings, such as the AOAC method (941.09). In all of these methods, saponification is done, which exposes the sample to a harsh heated alkaline condition. This condition could degrade the cholesterol, as it is reported that cholesterol was heat labile at 45°C in the presence of 1M KOH for 3 hours (Busch et al., 2010). These are conditions milder than that used in various cholesterol quantification procedures mentioned. This raises the concern that current methods may create variance and are underestimating the cholesterol content. In addition, cholesterol is present in two forms; free and esterified. It is known that cholesterol esters take longer to hydrolyze during saponification than other lipid esters (Christie et al., 2010). Eggs are reported to have 10-15% of the cholesterol present as esters (Bitman et al., 1980), making this an important consideration in quantification of cholesterol in egg yolks. This is especially important with cholesterol being a hot topic for egg nutrition. More research needs to be done to investigate the stability of cholesterol under the saponification conditions. Overall, the saponification conditions may be too harsh and degrade cholesterol but may also be too mild to free cholesterol esters. Alternatively, a cholesterol kit method can be used. The kit is equipped with enzymes (i.e. cholesterol ester hydrolase) that will rapidly free cholesterol esters to ensure both ester and free forms are quantified.

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The technique used in this study to quantify cholesterol was the kit method in order to quantify total cholesterol (free and esters). Various chemical and enzymatic reactions occur in this kit method. First, cholesterol esters are freed by cholesterol ester hydrolase. Cholesterol oxidase will oxidize the freed cholesterol along with free cholesterol already present in the sample producing hydrogen peroxide. Peroxidase then catalyzes a reaction between hydrogen peroxide, 3,5-dimethoxy-N-ethyl-N-(2-hydroxy-3-sulfopropyl)-aniline sodium salt (DAOS), and 4-aminoantipyrine. The product of this reaction results in a blue pigment that can be spectrophotometrically measured. The absorbance of this measurement is related linearly to the concentration of cholesterol in the sample (Wako Diagnostics, 2014).

Egg Formation

Laying-hens are very efficient birds at producing eggs, laying an egg almost every day when in good health and housing conditions. Approximately 100g of feed is consumed daily by the laying-hen and approximately a 60g egg (size is impacted by genetics, age, clutch order, number of eggs produced, maturity, season, temperature, housing, disease, and other environmental factors) is produced. After the laying cycle has finished (about 23-26 hours), after only 30 minutes, the laying-hen's reproductive system is again starting the cycle to lay another egg (Scanes et al., 2004). Many anatomical features and steps are needed to lay an egg efficiently incorporating nutrients from feed.

Fat soluble nutrients, such as vitamin E, are efficiently taken into the system as described previously. The fat soluble pigment xanthophyll is reported to be transferred very quickly from the digestive tract to the blood stream and finally to the yolk (USDA,

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2000). Overall, all fat soluble nutrients (vitamin E and astaxanthin), Walker et al. (2012) reports that a steady state is reached after 8-10 days, thus there is discrepancy on how the laying-hens handle fat soluble nutrients internally.

Laying-hens upon birth only have one functional ovary (left) that contains a range of 3,600 to 4,000 tiny ova that will develop into the mature yolks of the eggs (Scanes et al., 2004). Upon reaching sexual maturity and release of specific hormones (follicle stimulating hormone and luteinizing hormone) an ova (single female reproductive cell) starts to mature into a yolk, slowly at the beginning and speeding as release (ovulation) is within 8-10 days. The matured yolk is released from the ovary by a rupture of the follicle (stigma). The released yolk is then engulfed by the oviduct. The oviduct is broken into five parts (infundibulum, magnum, isthmus, uterus, and vagina). The infundibulum picks up the released yolk from the body cavity. The magnum (without live sperm present) deposits think albumen around the yolk (which will later form chalaza and inner albumen). When the forming egg arrives in the isthmus, the inner and outer shell membranes are added that give protection from contamination. In the uterus the albumen becomes inflated by added water and minerals and the shell begins to form with calcium deposits. Most of the time in this process is spent in the uterus. The egg is laid just after arrival to the vagina. Overall, the oviduct receives the yolk from the follicles almost daily and forms the albumen network and other various components around the yolk (Scanes et al., 2004; Bell et al., 2002). Abnormalities to this process can produce multiple-yolked eggs, yolkless eggs, blood spots, meat spots, dented eggshells, and soft-shelled eggs.

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Better understanding of how an egg is laid and how various nutrients are deposited into the laying-hen eggs could help further improve the nutritional profile of the egg. Delivery systems could be used to better transport nutrients into the system to be distributed in the various organs, tissues, and eggs of the laying-hen. Having added nutrients in eggs could help encourage consumers to increase their consumption of eggs.

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References

Aggarwal, B.; Sundaran, C.; Prasad, S.; Kannappan, R. Tocotrienols, the vitamin E of the 21st century: its potential against cancer and other chronic diseases. *Biochem. Pharm*. **2010**, 80, 1613-1631.

American Egg Board. Incredible Edible Egg: Egg Nutrients. Available at http://www.incredibleegg.org/health-and-nutrition/egg-nutrients (last accessed Dec 2014).

American Oil Chemists' Society. AOCS Lipid Library. Available at http://lipidlibrary.aocs.org/ (last accessed Dec 2014).

Arita, M.; Sato, Y.; Miyata, A.; Tanabe, T.; Takahashi, E.; Kayden, H.; Arai, H.; Inoue, K. Human alpha-tocopherol transfer protein: cDNA cloning, expression and chromosomal localization. *Biochem. J.* **1995,** 306, 437–43.

Bell, D.; Weaver, W. *Commercial Chicken Meat and Egg Production*. 5th ed.; Kluwer Academic Publishers: Dordrecht, The Netherlands, **2002**.

Berg, J.; Tymoczko, J.; Stryer, L. *Biochemistry*. 7th ed.; W.H. Freeman and Company: New Yolk, NY, **2012**.

Bitman, J.; Wood, D. Cholesterol and Cholesteryl esters of eggs from various avian species. *Poult. Sci*. **1980**, 59, 2014-2023.

Brigelius-Flohe, R.; Traber, M. Vitamin E: function and metabolism. *Faseb. J*. **1999**, 13, 1145-1155.

Burton, G.W., Traber, M. Vitamin E: Antioxidant, activity, biokinetics and bioavailability. *Annu. Rev. Nutr.* **1990**, 10, 357-382.

Busch, T.; King, A. Stability of Cholesterol, 7-ketocholesterol, and beta-sitosterol during saponification: ramifications for artifact monitoring of sterol oxide products. *J. Am. Oil Chem. Soc*. **2010**, 87, 955-962.

Christie, W.; Han, Xiamlin. Preparation of derivatives of fatty acids. *In Lipid Analysis*, 4th ed.; The Oily Press: Bridgewater, England, **2010**; 145-146.

Egg Nutrition Center. Research and Nutrition. Available at http://www.eggnutritioncenter.org/nutrition-research/ (last accessed Dec 2014).

Fernandez M.; Calle M. Revisiting dietary cholesterol recommendations: does the evidence support a limit of 300 mg/d? *Curr. Atheroscler. Rep*. 2010, 12(6), 377-383

المنارة للاستشارات

www.manaraa.com

Frega, N.; Mozzon, M.; Bocci, F. Identification and estimation of tocotrienols in the annatto lipid fraction by gas chromatography-mass spectrometry. *J. Am. Oil Chem. Soc.* **1998**, 75 (12), 1723-1727.

Gropper, S.; Smith, J. Vitamin E. In *Advanced Nutrition and Human Metabolism*, 6th ed.; Wadsworth Cengage Learning, CA, **2013**; 400-409.

Hosomi, A., K. Goto, H. Kondo, T. Iwatsubo, T. Yokota, M. Ogawa, M. Arita, J. Aoki, H. Arai, and K. Inoue. 1998. Localization of alpha-tocopherol transfer protein in rat brain. *Neurosci.Lett*. 256: 159-162.

Iowa Egg Council. Available at http://iowaegg.org/#sthash.HH5porln.wxzK2AeQ.dpbs (last accessed Oct 2014).

Jacques, P.; Taylor, A.; Moeller, S.; Hankinson, S.; Rogers, G.; Tung, W.; Ludovico, J.; Willett, W.; Chylack, L. Long-term nutrient intake and 5-year change in nuclear lens opacities. *Arch Opthalmol*. **2005**, 123, 517-526.

Kantor, M.; Kris-Etherton, P.; Fernandez, M.; Vickers, K. Katz, D. Exploring the factors that affect blood cholesterol and heart disease: is dietary cholesterol as bad for you as history leads us to believe?. *Adv. Nutr*. **2012**, 3, 711-717.

Lanari, M.C.; Hewavitharana, A.; Becu, C.; De Jong, S. Effect of dietary tocopherols and tocotrienols on the antioxidant status and lipid stability of chicken. *Meat Sci*. **2004**, 68, 155-162.

Lee, S.P.; Mar, G.; Ng, L.T. Effects of tocotrienol-rich fraction on exercise endurance capacity and oxidative stress in forced swimming rats. *Eur. J. Appl. Physiol*. **2009**, 107, 587-595.

Mares-Perlman, J.; Lyle, B.; Klein, R.; Fisher, A.; Brady, W.; WandenLangerberg, G.; Trabulsi, J.; Palta, M. Vitamin supplement use and incident cataracts in a population-based study. *Arch Opthalmol*. **2000**, 118, 1556-1563.

National Academy of Sciences. 2000. Vitamin E. Pages 186-283 in *Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids*. Washington D.C.

Parker, R.; Pearce, B.; Clark, R.; Gardon, D.; Wright, J. Tocotrienols regulate cholesterol production in mammalian cells by post-transcriptional suppression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *J. Biol. Chem.* **1993**, 268 (15), 11230-11238.

Pearce, B.; Parker, R.; Deason, M.; Qureshi, A.; Wright, J. Hypocholesteolemic activity of synthetic and natural tocotrienols. *J. Med. Chem.* **1992**, 35, 3595-3606.

المنارة للاستشارات
www.manaraa.com

Qureshi, A.; Burger, W.; Peterson, D.; Elson, C. The structure of an inhibitor of cholesterol biosynthesis isolated from barley. *J. Biol. Chem.* **1986**, 261 (23), 10544-10550.

Qureshi, A.; Mo, H.; Packer, L.; Peterson, D. Isolation and identification of novel tocotrienols from rice bran with hypocholesterolemic, antioxidant, and antitumor properties*. J. Agric. Food Chem.* **2000**, 48, 3130-3140.

Qureshi, A.; Peterson, D. The combined effects of novel tocotrienols and lovastatin on lipid metabolism in chickens. *Atherosclerosis*. **2001**, 156, 39-47.

Qureshi, A.; Peterson, D.; Hasler-Rapacz, J.; Rapacz, J. Novel tocotrienols of rice bran suppress cholesterogenesis in hereditary hypercholesterolemic swine. *J. Nutr*. **2001**, 131, 223-230.

Reboul, E.; Richelle, M.; Perrot, E.; Desmoulins-Malezet, C.; Pirisi, V.; Borel, P. Bioaccessibility of carotenoids and vitamin E from their main dietary sources. *J. Agric. Food Chem.* **2006***,* 54, 8749-8755.

Sato, Y.; Arai, H.; Miyata, A.; Tokita, S.; Yamamoto, K.; Tanabe, T.; Inoue, K. Primary structure of alpha-tocopherol transfer protein from rat liver. Homology with cellular retinaldehyde-binding protein. *J. Biol. Chem.* **1993**, 268, 17705–17710.

Scanes, G.; Brant, G.; Ensminger, M. Poultry Biology. In *Poultry Science*, 4th ed., New Jersey, USA. **2004**, 22-45.

Seifried, H.; Anderson, D.; Fisher, E.; Milner, J. A review of the interaction among dietary antioxidants and reactive oxygen species. *J. Nutr. Biochem.* **2007**, 18, 567-579.

Sen, C.K.; Khanna, S.; Roy, S. Tocotrienols: Vitamin E beyond tocopherols. *Life Sci.***2006**, 78, 2088-2098.

Shokolenko, I.; Wilson, G.; Alexeyev, M. Aging: a mitrochondrial DNA perspective, critical analysis and an update. *World J. Exp. Med*. **2014**, 20(4), 46-57.

Sontag, T.; Parker, R. Influence of major structural features of tocopherols and tocotrienols on their ω-oxidation by tocopherol-ω-hydroxylase. *J. Lipid Res.* **2007**, 48, 1090-1098.

Stampfer, M.J.; Hennekens, C.H.; Manson, J.E.; Colditz, G.A.; Rosner, B.; Willett, W.C. Vitamin E consumption and the risk of disease in women. *New Engl. J. Med*. **1993**, 328, 1444-1449.

المنارة للاستشارات
www.manaraa.com

Stephens, N.G.; Parsons, A.; Schofield, P.M.; Kelly, F.; Cheeseman, K.; Mitchinson, M.J. Randomized controlled trial of vitamin E in patients with coronary disease: Cambridge heart antioxidant study (CHAOS). *Lancet*. **1996**, 347, 781-786.

Surai, P.; Speake, B.; Sparks, N. Carotenoids in avian nutrition and embryonic development. absorption, availability and levels in plasma and egg yolk. *J. Poultry Sci.* **2001***,* 38, 1-27.

Traber, M. Molecular mechanisms of vitamin E transport. *Annu. Rev. Nutr.* **1999**, 19, 343-355.

United States Department of Agriculture. *Egg-Grading Manual*; U.S. Department of Agriculture: Washington DC, **2000**; Vol. 75.

USDA Nutrient Database Agricultural Research Service. United States Department of Agriculture: Full Report (All Nutrients): 01123, Egg, whole, raw, fresh. Available at http://ndb.nal.usda.gov/ndb/foods/show/112?fg=&man=&lfacet=&count=&max=&sort=&qlookup=&offset=&format=Full&new=&measureby= (last accessed Sept 2014)

US FDA. GRAS Notices. Available at http://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices (last accessed Dec 2014).

Wako Diagnostics. Cholesterol E. Available at http://www.wakodiagnostics.com/r_total_cholesterol.html (last accessed Dec. 2014).

Walde, C.; Drotleff, A.; Ternes, W. Comparision of dietary tocotrienols from barley and palm oils in hen's egg yolk: transfer efficiency, influence of emulsification, and effect on egg cholesterol. *J. Sci. Food Agric*. **2013**, 94 (4), 810-818.

Walker, L.; Wang, T.; Xin, H.; Dolde, D. Supplementation of laying-hen feed with palm tocos and algae astaxanthin for egg yolk nutrient enrichment. *J. Agric. Food Chem.* **2012**, 60, 1989-1999.

Yang, Z.; Lee, M.; Sang, S.; Yang, C. Bioavailability and metabolism of tocotrienols. In *Tocotrienols Vitamin E Beyond Tocopherols*, 2nd ed.; Tan, B.; Watson, R.; Preedy, V., Eds.; CRC Press: Boca Raton, FL, **2013**, 37-51.

Yoshinda, H.; Yusin, M.; Ren, I.; Kuhlenkamp, J.; Hirano, T.; Stolz, A.; Kaplowitz, N. Identification, purification and immunochemical characterization of a tocopherol-binding protein in rat liver cytosol. *J. Lipid Res.* 1992, 33, 343–350.

المنارة للاستشارات

www.manaraa.com

Yu, S.; Thomas, A.; Gapor, A.; Tan, B.; Qureshi, N.; Qureshi, A. Dose-response impact of various tocotrienols on serum lipid parameters in 5-week-old-female-chickens. *Lipids*. **2006**, 41, 453-461.

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CHAPTER 3

SUPPLEMENTATION OF LAYING-HEN FEED WITH ANNATTO TOCOTRIENOLS AND IMPACT OF α-TOCOPHEROL ON TOCOTRIENOL TRANSFER TO EGG YOLK

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Abstract: Hens efficiently transfer nutrients from their feed to the eggs. Tocotrienols (T3s) have various health benefits including lowering cholesterol. Annatto is the only 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 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 tocopherols 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 (by 23.76%) but it 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*

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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 which has eight major forms that are classified into two major groups – tocopherols and tocotrienols (T3s), differing in 3 double bonds on the phytyl tail in the T3s.[3] Each of these groups (tocopherols and T3s) has an α, β, γ, and δ form varying in the number and placement of the 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 the interest has grown in T3s which have been shown to have many health benefits.[5] One significant benefit is lowering cholesterol by down-regulating the hepatic enzyme 3-hydroxy-methyglutaryl 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 the presence or absence of α-tocopherol has on the transfer of the 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 both in 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

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from the *Bixa orellana* rainforest plant with high purity and potency 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 significant difference in the cholesterol content in the egg when the laying hen feed was supplemented with annatto at 200-600 ppm levels compared to control.

In order to provide health benefits to the laying-hens or to the egg consumers, we wanted to evaluate the transfer efficiency of the T3s from the feed to the egg both in the presence and absence of α-tocopherol, and their effect on egg cholesterol content. The hypotheses of this study were that: a) the supplementation of annatto T3s to laying-hen feed 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 the cholesterol in the egg yolk, and c) the 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 the supplements would result in minimal changes in the hen production performance and egg quality.

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 1) and diets supplemented with American River Nutrition's DeltaGold® 70 Annatto Tocotrienols and ADM's α-tocopherol (Table 2).

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The feeding experiment was approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC). 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 hours and off the remaining 8 hours 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, 2 cages were not used due to air velocity difference and failure of eggs to roll to the front causing breakage. There were 7 treatments diets (Table 2) with 4 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 minutes. 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 to 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 hours were feed disappearance and

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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 day 2 (1-2d), 4 (3-4d), 6 (5-6d), 8 (7-8d), 10 (9-10d), 12 (11-12d), 14 (13-14d), 21 (20-21d), 28 (27-28d), 35 (34-35d), 42 (41-42d), and 49 (48-49d). 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, NY) for at least 72 hours to remove the moisture by sublimation to preserve the lipid.

Physical and Sensory Properties of Eggs and Egg Yolks. The quality of the eggs collected was measured at every sampling time using a high accuracy Digital Egg Tester DET6000 (Nabel, Japan). The measurements taken included whole egg weight (grams), eggshell strength (Newton), egg yolk color (Yolk Color Fan, YCF), Haugh Unit (calculated from albumin 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 if and how different the treatment eggs (days 35 and 36) were from the control, 9 panelists were trained in 4 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

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intense), savory egg yolk flavor (none to intense), bitterness (none to intense), and off-flavor (none to intense) using a 15 centimeter 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 bitterness solution of quinine for bitterness. For sample preparation, eggs were hard boiled, consistently cooled using refrigeration, followed by preparation 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 4 replicates were evaluated on 4 separate days with panelist's scores averaged on each day.

For all of the 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 using 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.* The total moisture was measured by drying 2 g of yolk in an aluminum dish for 4-5 hours 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 sample 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 by vol), similar to the procedure of Folch et al.[18] The vials were capped and manually shook for 30 seconds to ensure full dispersion of the

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solid in the solvent. The vials were placed in a shaker at ambient temperature overnight, then centrifuged to obtain a clear layer. Approximately 2 mL of the supernatant was filtered through a 0.45μm filter (PTFE) with a glass syringe. The filtrate (1.0 mL) was taken precisely and placed in a pre-weighed 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 the HPLC quantification of the 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 et al.[19] These samples were analyzed for the 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 one milliliter of 1 M sodium methoxide at ambient temperature for 5 hours. The reaction was stopped by adding 3 mL of deionized water (DI water). Two milliliters of hexanes was added to extract the FAMEs to the top layer. The FAMEs samples were then analyzed using 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). The parameters used for the GC quantification were an injector and detector temperature of 250°C, oven temperature programmed to start at 100°C and rise

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4°C/minute to finish at 240°C. The flow of gases was set as in Walker et al.[2] A standard solution of FAMEs (Nu-Check Prep Inc. GC reference standard 566, MN) was used to identify the fatty acid peaks in the egg yolk lipid samples.

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

Vitamin E Transfer Efficiency to Egg Yolks. The transfer efficiency was calculated based on the maximum concentration value 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 based on supplement compositions (Table 2), daily feed consumption, laying rate, and egg yolk weight. The ratio of the value quantified and value calculated based on the complete transfer is the transfer efficiency.

Egg Yolk Cholesterol Quantification. To quantify the total cholesterol in the egg yolks, a Wako Cholesterol E (Mountain View, CA) kit was used. The procedure was carried out as instructed using 2 mg of freeze-dried egg yolk (weighed with an analytical balance) that was 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

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centrifuged to settle the egg yolk particle and the liquid was carefully pipetted into a cuvette. The cuvette was read using a spectrophotometer (Beckman Coulter DU 720 UV/Vis Spectrophotometer, CA) at 600 nm, using DI water as a blank. A standard curve was constructed each time samples were measured using the standard cholesterol provided in the kit to quantify the cholesterol in the egg yolks.

Statistical Analysis. Statistical analysis was done using 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 using Tukey's honest significant difference (HSD) at a $p = 0.01$ (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 ($100 \times$ eggs produced/hens in cage), and weekly averaged hen body weight are shown in Table 3. No significant differences were found among the treatments in the laying rate or hen body weight. The laying rate was over 90% for all diets, and similar to that reported in literature.[2,13,20] All hens on treatment diets consumed significantly more feed than those 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,

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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 upon arrival and it had a bitter and fruity taste. This 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 accordingly would lead to higher production costs. The egg is considered to be the gold standard for protein, being the most affordable complete protein [23], thus increasing the costs of production is not desirable for the industry or egg consumers. A potential application of this supplement may be for broiler chickens for more rapid 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 4). 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 the USDA Grading standards [25], and the HUs were similar to those found in literature.[2, 26] Significant differences were found in the yolk

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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 in diets with vitamin E supplementation. Environmental or storage conditions may cause changes in viscosity, i.e. increasing storage time decreases viscosity [28], but all eggs were exposed to the same environmental and storage conditions. In Walker et al. study [2], the only significant difference in egg yolks supplemented with palm toco concentrate and astaxanthin was the emulsification capacity, with a significant decrease with the increased level of supplementation. It was uncertain why a decrease was seen in emulsification capacity of 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 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. 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, and to determine the magnitude of these changes. No significant differences were perceived in moistness, smoothness, chalkiness/mouth drying, bitterness, or off flavor (Table 4). It is interesting to note that a general trend is seen that as the moistness increased, the viscosity decreased although not significant. 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.

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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 4) did not show any significant differences in the color of the raw egg, indicating that boiling 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 level 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 the lowest in the TC 1000 diet, but the level of change is not expected to be unacceptable to the consumers, and may be more palatable. The 15 cm line scales used to train the panelist were not extremely different when comparing the extremes across all attributes, so the differences noticed were very small in general.

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 et al.[31].

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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 water wash step would leave extractable proteins in the lipid extract, thus causing theincrease. 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 using methodology from Yao et al.[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 phospholipids 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%), lysophophatidylcholine (1.22-1.44%), sphingomyelin (1.90-2.47%), and lysophosphatidylethanolamine (0.59-0.80%).

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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 any of the treatment diets, and the percentages found are similar to that 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 Fig. 1, 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 the transfer as shown in Fig. 1 that α-tocopherol was transferred at a much higher rate than either of the γ or δ -T3s even when supplemented in similar amounts.

To understand how the additives were transferred to the yolk, the measurements after steady state (day 10 and after) were averaged and compared, as shown in Table 5. The diets without α-tocopherol supplemented (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

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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 the 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 (3 methyl groups) with 2 methyl groups on the ring structure but δ-T3 only having 1 methyl group on the phenolic ring.[3] α-Tocopherol is reported to interfere with the 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 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 significantly increase the γ and δ-T3 in the egg yolks with increasing annatto supplementation although this was not proportional to its concentration in the feed.

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 (0.50-0.96%) or δ (0.74-0.93%) T3s, as reported in Table 6. 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 100 ppm, but the efficiency was not impacted by added α-tocopherol.

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The transfer efficiency for α-tocopherol was much higher than that reported by Walker et al. (9.9%) [2] and very similar to that reported by Walde et al.(39.59-44.78%). [13] It 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. (<1%) [2] and Walde et al.(0.37-0.40%).[13] δ-T3 was transferred similarly to that reported by Walker et al. (<1%) [2] but higher than that reported by Walde et al. (0%).[13]

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 the annatto T3s were not well absorbed with high concentration detected in the manure (shown in another paper to be published in Poultry Science), showing a need for a better carrier system or matrix to transport the 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 7. This method was a modification using a cholesterol quantification kit designed for biological

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samples, especially blood serum. To validate this method, various tests were performed to ensure this method 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 preset in the egg yolk powder reacted fully. It was observed that at 15 min (3 times longer than required by kit) the ratio of the absorbance to mass of freeze-dried egg yolk reached a constant and it did not change significantly after 15 min.

Using an average of the egg yolk weights (14.61g/ yolk), the range of cholesterol in the yolks was 192-231 mg per 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 then GC method (Ca 6b-53) was done on selected treatments (control and T3 2000) to compare the amount of cholesterol quantified. No significant differences were detected among treatments. 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 sterol) under conditions of heat and high pH during saponification. Busch et al.[43] reported that cholesterol was heat labile at 45°C in the presence of 1M KOH, which are

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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 yolk as-is, is lower than that found in this study, 14.4 mg cholesterol/g yolk as-is average across all treatments and days. The reasons for this differences is discussed above.

No significant cholesterol reductions over the feeding study were seen across any 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 this study. A key difference in this study and the study by Walde et al. [13] is the form and amount of T3s

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present in the sources. Barley had a mixed tocopherols and T3s, with α-T3 being the dominant form. Whereas, 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 supplementing of 50-2000 ppm of δ and γ T3s in young female chickens caused decreases in total serum cholesterol (32%), LDL levels (66%), and 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 the lipid chemistry (total cholesterol, HDL, and TAGs, data reported in Hansen et al., manuscript under review for publication in Poultry Science). We do not know the cause of the different outcomes, but thought that the use of young female birds *vs.* adult laying hens may play a role in these differences.

Conclusions

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 the daily feed disappearance, egg yolk viscosity,

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sensory yolk color and savory yolk flavor, and in the amount and transfer efficiency of the various forms of vitamin E in the egg yolks. Based on both the 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 this 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 cholesterol reduction effect of the T3s in egg.

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References

(1) Yao, L.; Wang, T.; Persia, M.; Horst, R.; Higgins, M. Effects of vitamin D_3-enriched diet on egg yolk vitamin D_3 content and yolk quality. *J. of Food Sci.* **2013**, 78 (2), 178-183.

(2) Walker, L.; Wang, T.; Xin, H.; Dolde, D. Supplementation of laying-hen feed with palm tocos and algae astaxanthin for egg yolk nutrient enrichment. *J. Agric. Food Chem.* **2012**, 60, 1989-1999.

(3) Sontag, T.; Parker, R. Influence of major structural features of tocopherols and tocotrienols on their ω-oxidation by tocopherol-ω-hydroxylase. *J. Lipid Res.* **2007**, 48, 1090-1098.

(4) Traber, M. Molecular mechanisms of vitamin E transport. *Annu. Rev. Nutr.* **1999**, 19, 343-355.

(5) Yang, Z.; Lee, M.; Sang, S.; Yang, C. Bioavailability and metabolism of tocotrienols. In *Tocotrienols Vitamin E Beyond Tocopherols*, 2nd ed.; Tan, B.; Watson, R.; Preedy, V., Eds.; CRC Press: Boca Raton, FL, **2013**; 37-51.

(6) Qureshi, A.; Burger, W.; Peterson, D.; Elson, C. The structure of an inhibitor of cholesterol biosynthesis isolated from barley. *J. Biol. Chem.* **1986**, 261 (23), 10544-10550.

(7) Pearce, B.; Parker, R.; Deason, M.; Qureshi, A.; Wright, J. Hypocholesteolemic activity of synthetic and natural tocotrienols. *J. Med. Chem.* **1992**, 35, 3595-3606.

المنارة للاستشارات
www.manaraa.com

(8) Parker, R.; Pearce, B.; Clark, R.; Gardon, D.; Wright, J. Tocotrienols regulate cholesterol production in mammalian cells by post-transcriptional suppression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *J. Biol. Chem.* **1993**, 268 (15), 11230-11238.

(9) Qureshi, A.; Mo, H.; Packer, L.; Peterson, D. Isolation and identification of novel tocotrienols from rice bran with hypocholesterolemic, antioxidant, and antitumor properties. *J. Agric. Food Chem.* **2000**, 48, 3130-3140.

(10) Qureshi, A.; Peterson, D. The combined effects of novel tocotrienols and lovastatin on lipid metabolism in chickens. *Atherosclerosis*. **2001**, 156, 39-47.

(11) Qureshi, A.; Peterson, D.; Hasler-Rapacz, J.; Rapacz, J. Novel tocotrienols of rice bran suppress cholesterogenesis in hereditary hypercholesterolemic swine. *J. Nutr*. **2001**, 131, 223-230.

(12) Yu, S.; Thomas, A.; Gapor, A.; Tan, B.; Qureshi, N.; Qureshi, A. Dose-response impact of various tocotrienols on serum lipid parameters in 5-week-old-female-chickens. *Lipids*. **2006**, 41, 453-461.

(13) Walde, C.; Drotleff, A.; Ternes, W. Comparision of dietary tocotrienols from barley and palm oils in hen's egg yolk: transfer efficiency, influence of emulsification, and effect on egg cholesterol. *J. Sci. Food Agric*. **2014**, 94 (4), 810-818.

(14) Frega, N.; Mozzon, M.; Bocci, F. Identification and estimation of tocotrienols in the annatto lipid fraction by gas chromatography-mass spectrometry. *J. Am. Oil Chem. Soc.* **1998**, 75 (12), 1723-1727.

المنارة للاستشارات

www.manaraa.com

(15) Harder, M.; Canniatti-Brazaca, S.; Coelhe, A.; Savino, V.; Franco, C. Cholesterol and iron availability in the yolk of laying hens feed with annatto (Bixa orellana). Animal. 2007, 1, 477-482.

(16) McGonigle, K.; McCracken, K. Study of the effects of Annatto (Bixa orellana) and Yucca schidegera on cholesterol content and yolk colour. Brit Poult Sci. 2002, 43, S41-S42.

(17) NRC, National Research Council. *Nutrient requirements of poultry*, 9th ed.; National Academy Press: Washington DC, 1994.

(18) Folch, J.; Lees, M.; Sloane Stanley, G. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **1957**, 226, 497-509.

(19) Yao, L.; Jung, S. ^{31}P NMR phospholipid profiling og soybean emulsion recover from aqueous extraction. *J. Agric. Food Chem.* **2010**, 58, 4866-4872.

(20) Garcia, E.; Molino, A.; Goncalves, H.; Junqueira, O.; Pelicia, K.; Osera, R.; Duarte, K. Gound annatto seeds (*Bixa Orellana L.*) in Sorghum-based commercial layer diets and their effects on performance, egg quality, and yolk pigmentation. *Brazillian J. Poultry Sci.* **2010**, 12 (4), 259-264.

(21) Wineland, M. Fundamentals of managing light for poultry. In *Commercial Chicken Meat and Egg Production*, 5th ed.,; Bell, D.; Weaver, W., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2002; 129-148.

(22) Webster, B. Behavior of chickens. In *Commercial Chicken Meat and Egg Production*, 5th ed.,; Bell, D.; Weaver, W., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2002; 71-86.

المنارة للاستشارات

www.manaraa.com

(23) Iowa Egg Council. Available at
http://iowaegg.org/#sthash.HH5porln.wxzK2AeQ.dpbs (last accessed Oct 2014).

(24) Kudo, K.; Nishimura, S.; Tabata, S. Distribution of tast buds in layer-type chickens: scanning electron microscopic observations. *Anim. Sci. J*. **2008**, 79, 680-685.

(25) Department of Agriculture. *Egg-Grading Manual*; U.S. Department of Agriculture: Washington DC, 2000; Vol. 75.

(26) Franchini, A.; Sirri, F.; Tallarico, N.; Minelli, G.; Iaffaldano, N.; Meluzzi, A. Oxidative stability and sensory and functional properties of eggs from laying hens fed supranutritional doses of vitamins E and C. *Poult. Sci.* **2002**, 81, 1744−1750.

(27) Kirunda, D.; Scheideler, S.; McKee, S. The efficacy of vitamin E (DL-α-tocopheryl acetate) supplementation in hen diets to alleviate egg quality deterioration associated with high temperature exposure. *Poult. Sci*. **2001**, 80, 1378-1383.

(28) Devera, L.; Nedomova, S.; Buchar, J. Influence of storing tme and temperature on viscosity of an egg yolk. *J. Food Eng*. **2010**, 96, 266-269.

(29) Ofosu, I.; Appiah-Nkansah, E.; Owusu, L.; Apea-Bah, F.; Oduro, I.; Ellis, W. Formulation of annatto feed concentrate for layers and the evaluation of egg yolk color preference of consumers. *J. Food Biochem*. **2010**, 34, 66-77.

(30) Williams, W. Origin and impact of color on consumer preference for food. *Poult. Sci*. **1992,** 71, 744-746.

(31) Varadarajulu, P.; Cunningham, F. A study selected characteristics of hens' egg yolk. Influence of breed, strain, and age of bird. *Poult. Sci*. **1972**, 51, 1327-1331.

المنارة للاستشارات
www.manaraa.com

(32) Anton, M. Composition and Structure of Hen Egg Yolk. In *Bioactive Egg Compounds*; Huopalahti, R., López-Fandiño, R., Anton, M., Schade, R., Eds.; Springer: Berlin, New York, 2007; pp 1−5.

(33) Restuccia, D.; Spizzirri, U.; Puoci, F.; Cirillo, G.; Vinvi, G.; Picci, N. Determination of phospholipids in food samples. *Food Rev. Int*. **2012**, 28, 1-46.

(34) Kullenberg, D.; Taylor, L.; Schneider, M.; Massing, U. Health effects of dietary phospholipids. *Lipids Health Dis*. **2012**, 11 (3), 1-16.

(35) QPFC Kewpie Corporation. Egg yolk lecithin pl-30S egg yolk oil; Kewpie Corporation Fine Chemical Division: Tokyo, Japan; 2010.2

(36) USDA Nutrient Database Agricultural Research Service United States Department of Agriculture: Full Report (All Nutrients): 01123, Egg, whole, raw, fresh. Available at http://ndb.nal.usda.gov/ndb/foods/show/112?fg=&man=&lfacet=&count=&max=&sort=&qlookup=&offset=&format=Full&new=&measureby= (last accessed Sept 2014).

(37) Qureshi, A.; Pearce, B.; Nor, R.; Gapor, A.; Peterson, D.; Elson, C. Dietary α-tocopherol attenuates the impact of γ-tocotrienol on hepatic 3-hydroxy-3-methylglutaryl coenzyme a reductase activity in chickens. *J. Nutr*. **1996**, 126, 389-394.

(38) Campo, J. Comparative yolk cholesterol content in four Spanish breeds of hens, an F_2 cross, and a white leghorn population. *Poult. Sci*. **1995**, 74, 1061-1066.

(39) Bair, W.; Marion, W. Yolk cholesterol in eggs from various avian species. *Poult. Sci*. **1978**, 57, 1260-1265.

المنارة للاستشارات

www.manaraa.com

(40) Golzar, S.; Ahbab, M.; Fani, A.; Hajbaei, A.; Ceylan, N.; Cooper, R. Egg yolk fatty acid profile of avian species – influence on human nutrition. *J. Anim. Physiol. Anim. Nutr* **2013**, 97, 27-38.

(41) Kazmierska, M.; Jarosz, B.; Korzeniowska, M.; Trziszka, T.; Dobrzanski, Z. Comparative analysis of fatty acid profile and cholesterol content of egg yolks of different bird species. *Pol. J. Food Nutr. Sci.* 2005, 55:14, 69-73.

(42) Christie, W.; Han, Xiamlin. Preparation of derivatives of fatty acids. *In Lipid Analysis*, 4th ed.; The Oily Press: Bridgwater, England, 2010; 145-146.

(43) Busch, T.; King, A. Stability of Cholesterol, 7-ketocholesterol, and beta-sitosterol during saponification: ramifications for artifact monitoring of sterol oxide products. *J. Am. Oil Chem. Soc*. **2010**, 87, 955-962.

(44) Bitman, J.; Wood, D. Cholesterol and Cholesteryl esters of eggs from various avian species. Poult. Sci. **1980,** 59, 2014-2023.

المنارة للاستشارات

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Tables and Figures

Table 1. Composition of base diet fed to laying-hens for 7 weeks

Ingredient	Amount (%)
Corn	61.00
Soybean meal	24.43
Calcium carbonate (coarse)	5.84
Calcium carbonate (fine)	3.90
Dicalcium phosphate	2.02
Animal rendered oil	1.57
Vitamin and trace mineral premix [a]	0.68
Sodium chloride	0.38
DL-methionine	0.18
Calculated Composition	%
Crude protein	16.05
Crude fat	3.94
Linoleic acid	1.82
Calcium	4.20
Phosphorus (nonphytate)	0.48
Sodium chloride	0.18
Chloride	0.26
Lysine (digestible)	0.77
Methionine (digestible)	0.41
Methionine + cysteine (digestible)	0.63
Metabolizable Energy (kcal/kg)	2,825

[a] Premix includes (per kilogram of diet): vitamin A, 9000 IU; vitamin D3, 3000 IU; vitamin E, 20 IU; cobalamine, 13 µg; riboflavin, 6 mg; niacin, 45 mg; pantothenic acid, 12 mg; choline, 487 mg; menadione, 1.2 mg; folic acid, 1.5 mg; pyridoxine, 1.2 mg; thiamine, 1.5 mg; biotin, 45 µg; magnesium, 136 mg; manganese, 136 mg; zinc, 136 mg; iron, 140 mg; copper, 14 mg; and selenium, 0.27 mg.

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Table 2. Concentration of annatto and α-tocopherol (TC) 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] DeltaGold® 70 Annatto Tocotrienols: total tocotrienols, 74.5% (δ-tocotrienol 89.2%, γ-tocotrienol 10.8%, and other tocotrienols/tocopherols <1%).
[b]ADM α-Tocopherol: α-tocopherol, 96.6%.

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Table 3. Hen performance and laying rate for diets with different levels of annatto tocotrienols (T3) and α-tocopherol (TC) supplementation (n=4) [a]

Diets [b]	Daily feed disappearance (g/day/hen)	Laying Rate (%)	Weekly Hen Weight [d] (kg/hen)
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 [c]	*	NS	NS

[a] Values are means ± standard deviations. [b] Diets detailed in Table 2. T3 = ppm annatto, TC = ppm α-tocopherol with constant 2000 ppm annatto. [c] Different letters (comparing all treatments) in the same column indicate significant differences at the 95% confidence level. NS, not significant at 5%. [d] Hen weights taken weekly and averaged over 7 weeks.

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Table 4. Egg and egg yolk quality properties and sensory attributes of boiled eggs from laying-hen diets with different levels of annatto tocotrienols (T3) and α-tocopherol (TC) supplementation (n=4) [a]

Diets [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	*

Diets [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. [b] Diets detailed in Table 2. T3 = ppm annatto, TC = ppm α-tocopherol with constant 2000 ppm annatto. [c] Different letters (comparing all treatments) in the same column indicate significant differences at the 99% confidence level. NS, not significant at 1%.

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Table 5. Average concentration of vitamin E isomers in egg yolks after reaching steady state (day 10 to 49) from diets with different levels of annatto tocotrienols (T3) and α-tocopherol (TC) supplementation (n=4) [a]

Diets [b]	α-Tocopherol (μg/g yolk as-is)	γ-T3 (μg/g yolk as-is)	δ-T3 (μg/g 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 [c]	*	*	*

[a] Values are means of values from day 10, 12,14, 21, 28, 35, 42, and 49 ± standard deviations. [b] Diets detailed in Table 2. T3 = ppm annatto, TC = ppm α-tocopherol with constant 2000 ppm annatto. [c] 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.

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Table 6. Nutrient transfer efficiency of vitamin E isomers into egg yolks from feed with different levels of annatto tocotrienols (T3) and α-tocopherol (TC) supplementation (n=4) [a]

Diets [b]	α-Tocopherol	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. [b] Diets detailed in Table 2. T3 = ppm annatto, TC = ppm α-tocopherol with constant 2000 ppm annatto. [c] 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. NS, not significant at 5%.

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Table 7. Cholesterol content of day 2, 21, and 49 egg yolks from diets with different levels of annatto tocotrienols (T3) and α-tocopherol (TC) supplementation (n=4) [a]

Diets [b]	Day 2 (mg Cholesterol/ g yolk as-is)	Day 21 (mg Cholesterol/ g yolk as-is)	Day 49 (mg Cholesterol/ g yolk as-is)
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. [b] Diets detailed in Table 2. T3 = ppm annatto, TC = ppm α-tocopherol with constant 2000 ppm annatto. [c] NS, not significant at 5%.

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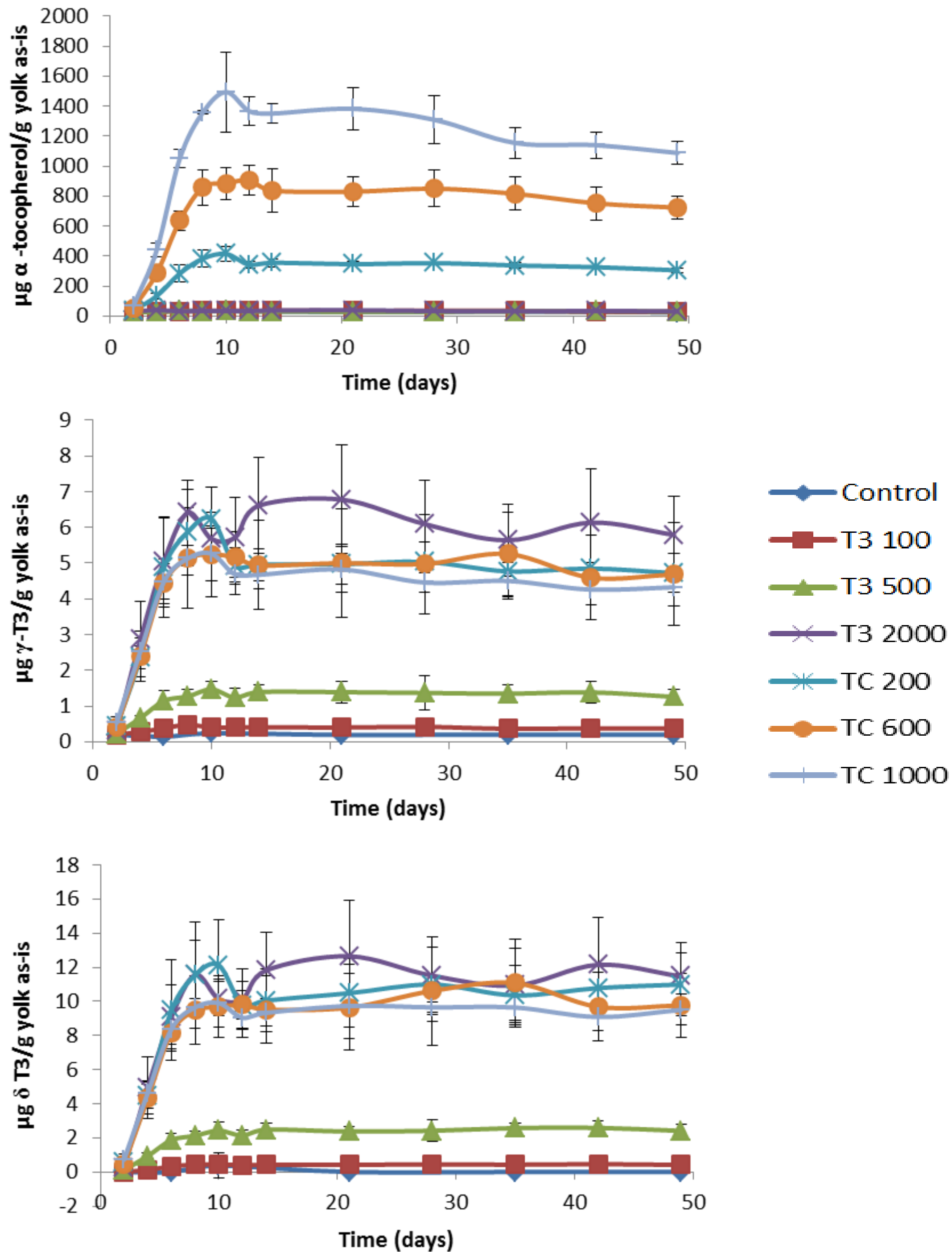

Figure 1. Kinetics of α-tocopherol, γ-T3, and δ-T3 accumulation in egg yolk over 7 week feeding period, showing means and standard deviation bars (n=4). Diets detailed in Table 2. T3 = ppm annatto, TC = ppm α-tocopherol with constant 2000 ppm annatto.

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CHAPTER 4

TOCOPHEROL AND ANNATTO TOCOTRIENOLS DISTRIBUTION IN LAYING HEN BODY

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Abstract

*The impact of supplementing laying hen feed with annatto tocotrienols (**T3s**) and alpha-tocopherol on the distribution of various forms of vitamin E and cholesterol throughout the hen's body was evaluated. A total of 18 organs or tissues (skin, fat pad, liver and gall bladder, heart, oviduct, forming yolk, laid yolk, lungs, spleen, kidney, pancreas, gizzard, digestive tract, brain, thigh, breast, manure, and blood) were collected after 7 weeks of feeding on diets enriched with various levels of alpha-tocopherol and annatto extract that contained gamma-T3 and delta-T3. Tissue weights, contents of moisture, lipid, alpha-tocopherol, gamma-T3, delta-T3, cholesterol, and fatty acid composition of extracted lipids from the collected organs and tissues were determined. Tissue weight, moisture content, and lipid content did not change significantly with feed supplementation treatments, except that the liver became heavier with increased level of supplementation. Minimal changes were found in the fatty acid composition, except in the fat pad, oviduct, brain, and manure. Overall, the main organs that accumulated the supplemented vitamin E were fat pad, liver and gall bladder, oviduct, forming yolks, laid yolks, kidney, brain, thigh, and breast. Much of annatto gamma-T3 and delta-T3 was found in the manure indicating most was excreted (>90%). In some tissues (brain and oviduct,) a significant increase in polyunsaturated fatty acids was seen with increased supplementation. Alpha-tocopherol impacted the transfer of gamma-T3 to forming and laid yolks, but did not impact delta-T3 transfer. No significant differences were found in most of the tissues in cholesterol, except a reduction in heart based on tissue as-is. Blood samples showed large variances in individual hens with no significant differences in total cholesterol, HDL, or total triacylglycerols. Supplementing feed with annatto T3s and*

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alpha-tocopherol showed that the vitamin E profile and distribution of the laying hen body can be altered to different extents depending on tissue.

Key words: Alpha-tocopherol, fatty acid composition, cholesterol, tocotrienols (**T3s**), tissue and organ, vitamin E distribution

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Introduction

Vitamin E is a fat soluble vitamin which has eight major forms that are classified into two major groups, tocopherols and tocotrienols (**T3s**). These two groups differ in 3 double bonds (unsaturation) on the phytyl tail in the T3s, making the T3s structure more rigid (Sontag and Parker, 2007). Each of these groups (tocopherols and T3s) has an alpha, beta, gamma, and delta form varying in the number and placement of the methyl groups on the chromanol ring (Sontag and Parker, 2007). Of these forms, alpha-tocopherol is the most bioavailable form due to the presence of an alpha-tocopherol transfer protein in the liver that has higher affinity for alpha-tocopherol, causing biodiscrimination against other forms of vitamin E (Traber et al., 1999).

Vitamin E is first absorbed from the small intestine and packaged into a chylomicron with other hydrophobic constituents from the diet. It is then transported to the liver. In laying hens the uptake of lipid substances from the diet occurs slightly different than that of humans. Lipids, including vitamin E, are delivered to liver by portomicrons (lipid rich proteins released by intestine) through the portal vein (Surai et al., 2001) due to the lack of a lymph system. In the liver, the alpha-tocopherol transfer protein selectively and preferentially binds to alpha-tocopherol and it is further distributed to other parts of the body, while the majority of the other forms are excreted (National Academy of Sciences, 2000). Even though alpha-tocopherol is the most bioavailable form, the other forms of vitamin E are also important in the body, and they may be absorbed under certain conditions, such as when alpha-tocopherol is absent.

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Interest has grown in T3s which have been shown to have many health benefits. They are higher potency antioxidant than tocopherol counterparts, anti-tumor, and can lower the risk of heart disease and hypertension (Yang et al., 2013; Qureshi et al., 2000). One significant benefit of the T3s is lowering cholesterol by down-regulating the hepatic enzyme (3-hydroxy-menthy-glutaryl coenzyme A reductase), the rate limiting enzyme in cholesterol synthesis (Qureshi et al., 1986; Pearce et al., 1992; Parker et al., 1993; Qureshi et al., 2000; Qureshi and Peterson, 2001; Qureshi et al., 2001; Yu et al., 2006; Yang et al., 2013).

Annatto is naturally derived from a rainforest plant (*Bixa Orellana*). The carotenoids (bixin and norbixin) present in the annatto are commonly used by the food industry to color certain dairy products (Frega et al., 1998). Annatto extract is the only known natural source of T3s (~90% delta and ~10% gamma) without the presence of alpha-tocopherol (Frega et al., 1998), making it possible to observe T3 transfer both in the presence and absence of alpha-tocopherol. We hypothesized that the absence of alpha-tocopherol may help improve the transfer of the T3s.

Previously, we have shown that by supplementing laying hen feed with annatto and alpha-tocopherol, the amount of alpha-tocopherol, gamma-T3, and delta-T3 can be significantly increased in the eggs laid with minimal changes to the egg yolk quality (Hansen et al., 2014). We want to understand further how these compounds are distributed throughout the hen’s body. Knowing how the laying hens accumulate these nutrients, the compositional and nutritional properties of various organs and tissues may be better understood. This may have significant implications for the broiler chicken industry. This work is the continuation of the study of impact of annatto supplementation

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on quality of eggs (Hansen et al., 2014), and it is the first of its kind to examine a complete set of organ and tissue samples (18 total) from birds for their lipid composition profiles.

We hypothesized that organs (especially liver) that are important in vitamin E uptake and absorption will show increases in the amounts of the vitamin E with feed supplementation (alpha-tocopherol, gamma-T3, and delta-T3), and will show decreases in the amount of cholesterol due an increased level of T3s.

Materials and Methods

Feeding Experiment

For 7 weeks, 48 laying hens (Hy-Line W-36, 30 weeks of age) were fed a base diet (Table 1) supplemented with American River Nutrition's (Hadley, MA) DeltaGold® 70 Annatto tocotrienols and ADM's alpha-tocopherol (Table 2). The base diet met the NRC nutrient recommendations (NRC, 1994). The feeding experiment was approved by the Iowa State University Institutional Animal Care and Use Committee (IACUC). The hens were randomly assigned to a treatment and were kept in an environment similar to that experienced in most conventional laying facilities. The average temperature was 25°C and the average relative humidity was 40% over the 7 week feeding period. The lighting was on for 16 hours and off the remaining 8 hours of the day. Three hens were placed in each cage (experimental unit, EU) with 16 cages (10 stacks with 3 tiers per stack) total. There were 4 treatment diets (Table 2) with 4 replicates 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.

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Feed preparation was described by Hansen et al. (2014). Feed was stored at refrigeration temperature (4°C) away from light and taken to the experimental site daily, where hens were fed *ad libitum* and watered daily to ensure both were in excess throughout the entire study. Daily measurements taken precisely every 24 hours were feed intake and egg production. Hen body weight was taken weekly as an indicator of the hens proper feed intake.

Manure and Organ Sampling and Storage

Manure was collected on day 43 from all treatment diets. The manure from each cage was homogenized and a 50g sample was taken and frozen until further analyses were carried out.

Hens were euthanized using injection of pentobarbital sodium (390 mg/ml) by ISU veterinary medical professionals after day 49 of the feeding study. Blood samples were taken just prior to euthanization for lipid chemistry analysis. The hen carcasses were briefly scalded in hot water, de-feathered, and stored at refrigeration temperature (4°C) until the next day when the remaining 15 organ samples (blood, manure, and laid yolks were previous collected) were harvested and weighed (skin, fat pad, liver and gall bladder, heart, oviduct, forming yolk, lungs, spleen, kidney, pancreas, gizzard, digestive tract, brain, thigh, and breast). The organ and tissue samples were frozen until further analyses were carried out. The frozen organ and manure samples were ground using blenders at highest speed and re-frozen.

Blood samples were taken from 2 of the 3 hens at random from each cage from all treatments. The samples were taken to the ISU Veterinary Pathology Services laboratory for quantification of total cholesterol, HDL, and TAGs. A VITROS cholesterol slide

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method was used to determine total cholesterol. A drop of hen blood serum was placed on a slide and spread into an even layer to expose to enzymes for cholesterol esters hydrolysis, cholesterol oxidation, and production of a color pigment. The density of the color pigment formed is proportional to the concentration of cholesterol, and is read colorimetrically at 540 nm. A similar method was used for HDL quantification with the appropriate reagent that selectively dissociates cholesterol from HDL lipoproteins and is quantified colorimetrically at 670 nm. The triacylglycerols (TAGs) were quantified using a slide method that dissociates TAGs from lipoproteins in the blood serum, and then the TAGs are hydrolyzed to glycerol and fatty acids. Glycerol is phosphorylated, oxidized to form a dye that is read colorimetrically at 540 nm. These methods used are explained in more detail by Allain et al (1974) for total cholesterol and HDL and Spayd et al. (1978) for TAG.

Composition of Organs and Tissues

Organ Weights. Upon dissection, organs were collected and weighed to allow for proper calculations and comparisons among treatments. The organs were then frozen at -4°C until further processing was carried out. The frozen organ samples and manure were then freeze-dried (Virtis Genesis 25LE, NY) for at least 5 days to remove the moisture by sublimation to preserve the lipid and facilitate lipid extraction.

Total Moisture. The total moisture was measured by drying 2 g of sample in an aluminum dish for 4-5 hours at 105°C (AOAC method 922.06). This measurement was done for all organs except the manure (75% moisture used for calculations as determined in our previous research).

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Lipid Extraction. Freeze-dried organ and tissue samples were ground using a mortar and pestle to reduce particle size. A small amount of sample (0.5-3g, depending on oil content) of the ground sample was accurately weighed and placed into a glass vial and mixed with chloroform:methanol (2:1 by vol), similar to the procedure of Folch et al (1957). The vials were capped and manually mixed for 30 seconds to ensure full dispersion of the solid in the solvent. The vials were placed in a shaker at ambient temperature overnight, then centrifuged to obtain a clear solvent layer. A large portion of the supernatant was filtered through a 0.45µm filter (PTFE) with a glass syringe. The filtrate was taken precisely (at varying volumes depending on lipid content of each sample) and placed in a pre-weighed glass vial, and the solvent was evaporated with a nitrogen evaporator to prevent lipid oxidation. To ensure all of the residual solvent was removed from the sample, the vials were placed in a vacuum oven at ambient temperature overnight. The vials were weighed to calculate total lipid extracted. The lipid was then dissolved in HPLC grade hexanes and stored in an explosion proof -20°C freezer until further analyses were carried out, including the HPLC quantification of the tocopherols and T3s. Excess supernatant was stored for other analyses.

Fatty Acid Composition. A small sample of the extracted lipid was converted to fatty acid methyl esters (FAME) by mixing the lipid with one milliliter of 1 M sodium methoxide at ambient temperature for 5 hours. Some samples were reacted using acidic conditions (3% sulfuric acid in methanol) at 85°C overnight due to possible high free fatty acid content. The reaction was stopped by adding 3mL of DI water. Two milliliters of hexanes was added to extract the FAMEs to the top layer. The FAME samples were then analyzed using GC (Hewlett-Packard 5890 Series II) with a FID detector and fused

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silica capillary column (SP-2340, 60m long x 0.25mm internal diameter x 0.2µm film thickness) from Supelco (Bellefonte, PA). The parameters used for the GC quantification were: injector and detector temperature of 250°C, oven temperature programmed to start at 100°C and rise 4°C/minute to finish at 240°C. The flow of gases was set as in Walker et al. (2012). A standard solution of FAMEs (Nu-Check Prep Inc. GC reference standard 566, MN) was used to identify the fatty acid peaks.

Vitamin E Quantification

Organ and manure lipids were dissolved in appropriate volume of HPLC grade hexanes to obtain the vitamin E isomers in a quantifiable region. HPLC was run as explained in Walker et al (2012). The concentration of tocopherols and T3s were determined using normal-phase column of Luna 3 µ NH_2 100 Å, 150 mm × 3.0 mm and a fluorescence detector. The separation was achieved isocratically using a mobile phase of 98% hexanes and 2% isopropanol.

Vitamin E Mass Distribution Calculation

In order to understand how the distribution of the various forms of vitamin E is changed due to supplementation, the mass distribution percentage was calculated. The total weight of a particular form of vitamin E was determined in all organs and tissues and each individual weight for each organ was taken as a percentage of the total. Manure was excluded from this distribution calculation because it would skew that data significantly and make the distribution difficult to be seen.

Cholesterol Quantification

To quantify the total cholesterol in the samples, a Wako Cholesterol E (Mountain View, CA) kit was used. The procedure was carried out as instructed using a small

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amount of organ (amount depended on cholesterol content) that was mixed with the color reagent provided by the kit. This mixture was shaken and allowed to react for 15min at 37°C, ensuring that the entire sample was uniformly dispersed for blue pigment formation. The samples were centrifuged to settle the particles and the liquid was carefully pipetted into a cuvette. The absorption was read using a spectrophotometer (Beckman Coulter DU 720 UV/Vis Spectrophotometer, CA) at 600nm, using DI water as a blank. A standard curve was constructed each time samples were measured using the standard cholesterol provided in the kit to quantify the cholesterol in the organ and tissue samples. More detail and discussion on validation of this method can be found in Hansen et al. (2014).

Statistical Analysis

Statistical analysis was done using 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 using Tukey honestly significant difference (HSD) at a $p = 0.01$ (cholesterol and fatty acid) and $p = 0.05$ (all other measurements).

Results and Discussion

In many of the measurements, large variances were observed. These are believed to be mainly due to biological differences naturally occurring among the hens. Each treatment had 4 replicates, with each replicate being a cage of 3 hens. The cage is the experimental unit (**EU**), and combining the organs and tissues from one EU should have reduced the variance. However, large individual differences still existed as shown in individual bird's blood chemistry done by the certified laboratory. For all of our own

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analytical methods, we had coefficients of variation less than 5% for repeated measurements of the same sample.

During the feeding study, hens performed well with no significant differences in laying rates (94-98%). There were also no significant changes in hen weights taken weekly (1.48-1.50 kg/hen). An interesting trend was observed that all of the treatment diets (with added annatto) led to significantly more feed intake than the control (103.16 g/day/hen for control and as high as 108.28 g/day/hen for supplemented diets). More details on hen performance and egg quality are presented in Hansen et al. (2014).

Compositional Properties of Organs and Tissues as Affected by Feeding Treatment

Organ Weights. Organ weights averaged across treatments (due to no treatment effect in most), as presented in Table 3, were obtained in order to carry out various calculations. Some significant increases ($p<0.05$) were found, such as1 3.59% increase in the liver, 21.74% increase in the spleen, and 32.99% increase in the kidney by the TC 1000 diet. In these cases the treatment average of 4 replicates were used for calculations to account for these differences. The remaining organs were averaged across the 4 treatments treatments (n=16). Nockels et al. (1976) also reported increased liver size due to increased intake of alpha-tocopherol. This could also possibly account for the increase in spleen weight, although spleen may only have a minor role in vitamin E metabolism (Hosomi et el., 1998). Differences in the kidneys may have been due to the sampling error because of the difficulty in its complete tissue removal.

Weight of organs is very specific to breed, size of the chicken, and sampling procedure which makes comparison to literature values difficult. Ciftci et al. (2003) studied various organ weights of Babcock B-380 pullets and had similar organ weights

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for the pancreas and digestive tract, lower weights for heart and liver, and higher weights for the gizzard than that found in this study. Similar weights for the liver and spleen were reported by Brake and Thaxton (1979).

Moisture Content. The average moisture contents across treatments for all organs (except for manure and blood) are presented in Table 3. Significant differences were found in the moisture content of oviduct and fat pad (TC 1000 was higher), but these random differences are believed to be due to sample handling and not due to the treatments. If the oviduct was not emptied or lost some free fluid during sampling, this could change the moisture content. The difference in stage of the laying cycle in each hen may have impacted moisture content, because of the daily rhythmic water and mineral transfer in the oviduct during the egg laying process (Scanes et al., 2004). No other organs were found to have significantly different moisture contents among any of the treatments. Ruff et al. (1981) reported similar moisture contents for thigh, breast, and liver, but higher moisture content for the heart. This could be due to species differences (broiler verse laying hen), and that our hearts had the outer fat left on.

Lipid Content. No significant differences were found in lipid contents (dry-basis) of the organs across treatments, so Table 3 presents average values across treatments. The feed supplements were not expected to change the lipid content. Garlich et al. (1975) reported the range for lipid content in the liver to be 25.8-49.0% (on a dry basis), and the value determined in this study (31.50%) falls in this range. Even though the liver became heavier because of supplementation, its lipid content did not change.

Tres et al. (2012) reported that lipid content in dark meat of chicken was 10.8%, but higher thigh fat content was found (13.28%) in this study. The lipid content of the

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heart was unexpectedly high (50.50%), and a possible reason was that the fat tightly surrounding the heart was not removed. Ruff et al. (1981) reported a much lower amount of lipid (17.5%) in the heart than in this study.

Fatty Acid Composition. The fatty acid composition for various organ and tissue samples is shown in Table 4. No significant differences were found in any fatty acids in any tissue except for fat pad, oviduct, brain, and manure due to treatments. Forming and laid yolks had similar fatty acid profiles. The liver had higher percent composition of saturated fatty acids (16:0 and 18:0) and 20:4 than other organs and tissues. Breast and thigh samples did not show similar fatty acid composition, possibly due to different levels of muscle activity creating different energy needs in these muscles. The breast compared to the thigh contains much higher saturated fatty acids, and the thigh had 20:1 while the breast did not. Many of the fatty acids detected in the manure are typical of that found in the base diet (Table 1).

Significant differences were found across treatments in the fat pad (18:1), oviduct (20:5 and 22:6), brain (22:6), and manure (16:0, 18:0, 18:1, and 18:3). The fat pad fatty acid composition differences could be attributed to uneven sampling. The brain also had high levels of saturated fatty acids (especially 18:0). It had very different fatty acid profiles compared with other organs and tissues. The oviduct and brain were high in 20:5 (**EPA**) and 22:6 (**DHA**) and these significantly increased with vitamin E supplementation. The brain also seemed to increase in 20:4 (**ARA**) although not significant. The T3 2000 and TC 1000 led to significantly more 22:6 than in the control for the brain, indicating that annatto T3s played a role in this increase of polyunsaturated fatty acids.

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Surai et al (2000) found higher saturated fatty acids (16:0 and 18:0) in the liver in male chickens fed diets supplemented with vitamin E, with 16:0 increasing significantly with vitamin E supplementation. Similarly in this study, the liver had higher percent saturated fatty acids (16:0 and 18:0) but no significant changes by treatments. Also, the amount of 20:4 was similar between this study and ours. The liver has a central role in fatty acid synthesis and lipid processing in the body producing high amounts of 16:0 and 18:0 for the body (Berg et al, 2012). In the heart, muscles (breast and thigh), and fat pad the fatty acid profiles are similar to Surai et al. (2000) but the proportions vary. One factor that could impact these is gender of the chicken. Surai et al. (2000) studied male chickens and this study was on laying hens.

Our study shows similar major fatty acids (16:0, 18:1, and 18:2) in meat (thigh and breast) as reported in literature (Ponte et al., 2008; Tres et al., 2012; and Mourao et al., 2008). Tres et al. (2012) also reported similar major fatty acids in the liver.

Vitamin E Content and Mass Distribution

Overall, the main organs that accumulated the supplemented vitamin E (alpha-tocopherol, gamma-T3, and delta-T3) were the fat pad, liver and gall bladder, oviduct, forming yolks, laid yolks, kidney, brain, thigh, and breast (Table 5, concentration based on dry tissue; Table 6, concentration based on lipid). Much of the annatto supplement (gamma-T3 and delta-T3) was detected in the manure indicating it was excreted by the laying hens. Some organs and tissues that accumulated the supplement play a role vitamin E metabolism, especially the liver, kidney, and brain which have detectable amounts of the alpha-tocopherol transfer protein (Hosomi et el., 1998).

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Laying hens are efficient in transferring fat soluble nutrients to the yolks as shown by many previous studies (Yao et al., 2013; Walker et al., 2012; and Walde et al., 2013), and this was also shown in this study in both the forming yolks and laid yolks. The organs and tissues that accumulate and use vitamin E had different mass distribution depending on supplementation (Figure 1-3).

Alpha-tocopherol. Significantly more alpha-tocopherol was found in the highest supplementation diet (TC 1000) for fat pad, liver, oviduct, kidney, breast, and manure based on dry tissue weight (Table 5). The forming yolk, laid yolk, and brain concentrated stepwise with significantly more alpha-tocopherol in TC 200 than in the control or T3 2000 diets, and even higher yet in the TC 1000 treatment based on dry tissue (Table 5). When comparing the TC 1000 diet to TC 200 (5 times more alpha-tocopherol supplemented), interestingly, the liver, oviduct, kidney, and manure had responses that were greater than 5 times in concentration. Whereas in the fat pad, forming yolk, laid yolk, brain, and breast, the response was less than 5 times. Similar trends were seen when comparing the concentrations of alpha-tocopherol based on lipids (Table 6).

Some of the organs and tissues were resistant to change in alpha-tocopherol upon supplementation of feed. The heart was expected to have a change as a result of the increased amount of the alpha-tocopherol circulating in the body, especially when blood is transporting the nutrients. Although vitamin E was not quantified in the blood, it would increase with supplementation, as seen by Traber at el. (1992). However, no increase in vitamin E was seen in the heart in this study.

The liver of chickens has been well studied for the amount of vitamin E because it plays a vital role in the uptake and selectivity of vitamin E forms. Alpha-tocopherol

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content in the liver was increased disproportionally; a 5-fold increase in the feed concentration resulted in a more than proportional amount in the liver. A similar trend of vitamin E concentration in liver was reported by Tengerdy et al. (1977), as well as an increase in the concentration of vitamin E in the spleen, which was not observed here. Surai et al. (2000) carried out a feeding study with vitamin E on male broilers. Similar increases of alpha-tocopherol in the liver, kidney, meat, and brain were observed. In addition, increases were also seen in the heart, lungs, pancreas, and fat (Surai et al., 2000) that were not observed in this study.

The mass distribution of alpha-tocopherol (Figure 1) changed with supplementation, and most of the supplemented alpha-tocopherol (>70% on average) went to the forming yolks. This fat soluble nutrient's ability to accumulate in the yolk further supports that laying hen diets can effectively alter the composition of the resulting egg yolks. Adding alpha-tocopherol to the base diets (already containing 20 IU alpha-tocopherol from vitamin premix) caused decreases in the percent distributed to the breast meat (97.05% reduction) and brain (96.92% reduction) (Figure 1).

Gamma-tocotrienol. Significantly more gamma T3 was detected in TC 200 and TC 1000 for the liver, and only in TC 1000 for the brain compared to control. All treatments had significantly more gamma-T3 in breast meat compared to control based on dry weight. In the forming and laid yolks, T3 2000 had significantly more gamma-T3 than the control, and TC 1000 had significantly less gamma-T3 than the T3 2000 diet indicating that the presence of alpha-tocopherol at high concentrations decreased the transfer of gamma-T3. This is also seen in the mass distribution (Figure 2). This trend in the forming yolks was less pronounced when concentration of gamma-T3 was based on

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lipid (Table 6), with only the control being significantly lower. Overall, alpha-tocopherol impacted the transfer of gamma-T3 to tissues and organs differently; it helped transfer to liver, kidney, and brain, hindered transfer to laid and forming yolks, and had no effect on transfer to breast. Interestingly, the organs that alpha-tocopherol helped were the organs found to have detectable amounts of the alpha-tocopherol transfer protein (Hosomi et al., 1998).

Delta-tocotrienol. A significant increase of delta-T3 in fat pad, liver, oviduct, forming and laid yolks, brain, thigh meat, and breast meat was observed. Unlike gamma-T3, delta-T3 transfer was not impacted by the presence of alpha-tocopherol, with no significant differences between T3 2000 and TC 1000 (Table 5 and Table 6). Significant differences among treatments were not consistent based on dry tissue and lipid. T3 2000 was not always significantly higher than control but with added alpha-tocopherol (TC 200 and or TC 1000 depending on tissue), a significant increase was seen. This suggests that the presence of alpha-tocopherol may have assisted the uptake of delta-T3 in some tissues.

Concentration of delta and gamma-T3 was not uniform in all tissues. The fat pad, oviduct, and thigh accumulated significant amounts of delta-T3 but not of the gamma form. Organs and tissues seem to have preferences for certain forms of vitamin E. Also, accumulation of fat soluble nutrient did not always occur in the tissues predicted (i.e. higher fat tissues, such as skin, heart, and fat pad). In these tissues there is a need for these nutrients due to their anti-oxidation potential. This trend was also seen when comparing the breast and thigh meat, with the higher fat content in the thigh (13.28 %) than breast (8.54%) but lower vitamin E concentration. Both the gizzard and digestive

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tract were emptied of feed and any substance in them, but it should be noted that increases in these tissues may have been due to residual supplements in the feed. If any feed remained in a tissue sample (i.e. gizzard or digestive tract) this would largely skew the quantification.

Liver and kidney had significantly more gamma-T3 when alpha-tocopherol was added, and this is the opposite of what is reported in literature. Alpha-tocopherol is believed to attenuate the uptake of various forms of T3s (Ikeda et al., 2003), but in this study it seemed to facilitate the uptake.

Liver had significantly more of all of the forms of vitamin E. It is not surprising with its central role in the uptake and bioaccumulation of the vitamin E. However, just because a form of vitamin E was detected in the liver does not indicate it was distributed to other tissues in the body. The liver discriminates among the various forms, preferentially choosing alpha-tocopherol to be distributed to other tissues while most of the other forms usually are excreted (National Academy of Sciences, 2000 and Traber et al., 1999). This was verified with large amounts of T3s detected in the manure.

The oviduct also had increases in the concentration of the vitamin E nutrients, possibly stabilizing the fatty acids that are liable to oxidation. In the oviduct, significant increases in longer chain polyunsaturated fatty acids (20:4 and 22:6) were observed.

It is evident that alpha-tocopherol is distributed to the laying hen's body much better than the annatto T3s, as shown by the higher amounts quantified in various organs. Surai et al. (1998) reported that alpha-tocopherol was the main form of vitamin E (79-90%) in the brain, heart, lung, and adipose tissue in young chicks, supporting high biodiscrimination against other forms. All our treatment diets had 2000 ppm annatto in

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feed but over 90% of intake was excreted in the manure. The manure data was not included in the mass distribution calculation shown in Figures 1-3, so it would not heavily skew the distribution.

Increasing the concentration of vitamin E throughout the laying hen body by supplementing feed makes it possible to increase the amount of antioxidants in the meat. This could have economic, quality, and nutritional impacts of the chicken meat. Increased amount of vitamin E has been shown to significantly decrease the formation of oxidation products in chicken meat during storage (Brenes et al., 2008) and reduce oxidative stress in tissues (Gao et al., 2010). More work needs to be done to determine the oxidative stability of the breast and thigh meat in these laying hens or broilers to make further conclusive statements.

It is believed to be economically feasible to increase levels of alpha-tocopherol in the laying hen body by increasing the concentration in the feed. This is estimated to increase the cost of a dozen of eggs by approximately 10 to 15 cents.

Cholesterol Quantification

Blood Cholesterol. No significant differences were found in any blood lipid measurements across the treatments, with large natural variation in individual hens. Measurements of blood samples were 139.4-183.8 mg/dL total cholesterol, 13.9-18.3 mg/dL HDL cholesterol, and 3249.4-4447.5 mg/dL TAGs. Variances were very large across replicates in the same treatments. Many studies have shown decreased cholesterol concentration and improvements in the blood lipid profiles by T3 supplementation (Qureshi et al., 1996; Qureshi et al., 2001; Pearce et al., 1992; and Yu et al., 2006), but we did not.

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Qureshi et al. (1996) fed 6-week-old female chickens amaranth which showed reductions in the total serum cholesterol (10-30%) and LDL cholesterol (7-70%), minimal effect to HDL cholesterol, higher activity (10-18%) of 7-alpha-hydroxylase (responsible for converting cholesterol to bile salts,) and lower activity (~9%) of 3-hydroxy-menthyglutaryl coenzyme A reductase (rate limiting enzyme in cholesterol synthesis). It was suggested that such drastic improvements in cholesterol profiles may have been attributed to the presence of other substances in amaranth to enhance or mimic the effects of T3s. Qureshi et al. (2001) also showed that T3s from rice bran suppressed cholesterol synthesis and improved blood lipid chemistry in 4-month-old swine by lowering total serum cholesterol by 32-38% and LDL cholesterol by 35-43%.

Yu et al. (2006) showed that supplementing of 50-2000 ppm of delta and gamma-T3s (similar to this study) to 5-week old laying hens caused decreases in total serum cholesterol (32%), LDL level (66%), and TAGs, with minimal changes to HDL cholesterol. However, such trends were not seen in this study. Overall, many of the papers published in this area have used younger animal models.

In some of the previous studies (Qureshi et al., 1996), deprivation and re-feeding regimens were used just prior to euthanization. This was done to induce enzyme activities and to clear chylomicrons and VLDL from blood serum samples. In this study this protocol was not followed which could lead to differences observed among studies.

Organ Cholesterol. Larger variances in cholesterol concentration were observed. The cholesterol kit method used in this study was compared to the AOCS saponification and GC method (Ca 6b-53) as explained in detail in Hansen et al. (2014). It is noted that the kit used in reported 18.37-20.63% more cholesterol than the saponification and GC

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method on egg yolks, due to its quantification of all cholesterol forms, free and the esterified.

No significant differences were found in cholesterol across treatments in any of the organs except the heart using the cholesterol kit method. The highest amount of cholesterol was found in the egg yolks (14.19 mg/g yolks as-is averaged across treatments). The yolk has more than double of the cholesterol content in the heart and it was magnitudes greater than in other organs and tissues (breast, thigh, liver and gall bladder, kidney, and oviduct). Literature has shown that T3s could lower the amount of cholesterol made by the liver (Parker et al., 1993), but we did not observe such change. The brain was another organ that would have been analyzed for cholesterol with its natural high amounts, but low sample quantity did not allow for this.

Based on tissue as-is, T3 2000 treatment led to significantly less cholesterol in the heart than the control, even though the heart had no significant change in the concentration of T3s. Cholesterol is synthesized in the liver and packaged into lipoproteins and then distributed to the rest of the body through the vascular system. The liver however, did have significant increases in the amount of T3s but no significant changes in cholesterol. Surprisingly, the cholesterol reducing ability of the T3s was not seen in the liver but was observed in the heart. When the lipid and moisture percent for each treatment was used to convert the cholesterol concentration from an as-is basis to a dry-basis and lipid basis, the decreasing cholesterol trend based as-is is lost, making the TC 1000 treatment having the lowest cholesterol concentration. This suggests that alpha-tocopherol also has cholesterol lowering ability in the heart tissue.

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The function and health of the heart is vastly impacted by the level of cholesterol and lipid present, shown by a correlation between the level of cholesterol and incidence of cardiovascular disease (Martirosyan et al., 2007). Therefore, it is beneficial to reduce its cholesterol content by vitamin E supplementation.

Beyer et al. (1993) observed similar trends and amounts in blood and tissue cholesterol (in breast and thigh meat). T3s were supplemented to laying hen feed at 20 or 200 ppm, and they did not change the amount of cholesterol in the tissue or blood samples significantly. It is also interesting to compare cholesterol content as affected by the age of the laying hens. Yu et al. (2006) observed significant differences in cholesterol content in 5-week old hens, which was similar to Qureshi et al. (1996)'s 6-week-old female chickens when supplemented with T3s. Whereas, in Beyer et al. (1993) and in this study where 48-week and 30-week old laying hens were used, respectively, no effects were observed. Many factors impact function of a biological system, and age of the laying hens seems to be an important factor in observing the effects of T3s on cholesterol reduction. As a laying hen ages, the ways vitamin E is used or functions could change. With the various forms of vitamin E serving as strong antioxidants, especially T3s, there may be an increased need to reduce oxidative stress in the body or to protect the forming yolk.

Conclusions

Minimal changes occurred in the organ composition (moisture, lipid, fatty acid, and cholesterol) due to supplementation of laying hen feed with alpha-tocopherol and annatto T3s (gamma and delta T3). In some tissues (brain and oviduct), a significant increase in polyunsaturated fatty acids was seen with increased supplementation. Overall, the main

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organs that accumulated alpha-tocopherol, gamma-T3, and delta-T3 were fat pad, liver and gall bladder, oviduct, forming yolks, laid yolks, kidney, brain, thigh, and breast. Much more alpha-tocopherol is transferred into the hen's body from the feed than the annatto T3s. Alpha-tocopherol only significantly decreased the transfer of gamma-T3 to the laid and forming eggs, but this trend was not seen in delta-T3. Cholesterol content was not significantly impacted in most tissues (breast, thigh, liver and gall bladder, kidney, and oviduct) except the hear.

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References

Allain, C.; Poon, L.; Chan, C., Richmond, W.; Fu, P. Enzymatic determination of total serum cholesterol. *Clin. Chem.* **1974**, 20(4), 470-475.

American Oil Chemists' Society. AOCS Lipid Library. Available at http://lipidlibrary.aocs.org/ (last accessed Dec. 2014).

Berg, J.; Tymoczko J.; Stryer, L. In *Biochemistry*, 7th ed.; W.H. Freeman and Company: New Yolk, NY, **2012.**

Beyer, R.; Jensen, L. Tissue and egg cholesterol concentration of laying hens fed high-protein barley flour, alpha-tocotrienol, and cholesterol. *Poult. Sci.* **1993**, 72, 1339-1348.

Brake, J.; Thaxton, P. Physiological changes in caged layers during a forced molt 2. gross changes in organs. *Poult. Sci.* **1979**, 58, 707-716.

Brenes, A.; Viveros, A.; Goni, I.; Centeno, C.; Sayago-Ayerdy, S.; Arija, I.; Saura-Calixto, F. Effect of grape pomace concentrate and vitamin E on digestibility of polyphenols and antioxidant activity in chickens. *Poult. Sci*. **2008**, 87, 307-316.

Ciftci, I.; Yenice, E.; Eleroglu, H. Use of triticale alone and in combination with wheat or maize: effects of the diet type and enzyme supplementation on hen performance, egg quality, organ weights, intestinal viscosity and digestive system characteristics. *Anim. Feed Sci. Technol*. **2003**, 105, 149-161.

Folch, J.; Lees, M.; Sloane-Stanley, G. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **1957**, 226, 497-509.

Frega, N.; Mozzon, M.; Bocci, F. Identification and estimation of tocotrienols in the annatto lipid fraction by gas chromatography-mass spectrometry. *J. Am. Oil Chem. Soc.* **1998**, 75 (12), 1723-1727.

Garlich, J.D.; Olson, J.; Huff, W.; Hamilton, P. Liver lipid content of twenty varieties of laying hens from three confinement systems. *Poult. Sci.* **1975**, 54, 806-813.

Gao, J.; Lin, H.; Wang, X.; Song, Z.; Jiao, H. Vitamin E supplementation alleviates the oxidative stress induced by dexamethasone treatment and improves meat quality in broiler chickens. *Poult. Sci.* **2010**, 89, 318-327.

المنارة للاستشارات
www.manaraa.com

Hansen, H.; Wang, T.; Dolde, D.; Xin, H. Prusa, K. Supplementation of Laying-hen Feed with Annatto Tocotrienols for Egg Nutrient Enrichment. *J. Agric. Food.* **2015**. *Chem.* Accepted.

Hosomi, A.; Goto, K.; Kondo, H.; Iwatsubo, T.; Yokota, T.; Ogawa, M.; Arita, M.; Aoki, J.; Arai, H.; Inoue, K. Localization of alpha-tocopherol transfer protein in rat brain. *Neurosci.Lett*. **1998**, 256, 159-162.

Ikeda, S.; Tohyama, T.; Yoshimura, H.; Hamamura, K.; Abe, K.; Yanmashita, K. Dietary alpha-tocopherol decreases alpha tocotrienol but not gamma tocotrienol concentration in rats. *J. Nutr.* **2003**, 133, 428–434.

National Academy of Sciences. Vitamin E. In *Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids*, Washington D.C.*;* 186-283.

Nockels, C.; Menge, D.; Kienholz, W. Effect of excessive dietary vitamin E on the chick. *Poult Sci.* **1976**, 55, 649-652.

NRC, National Research Council. *Nutrient requirements of poultry*, 9th ed.; National Academy Press: Washington DC, **1994**.

Martirosyan, D.; Miroshnichenko, L.; Kulakova, S.; Pogojeva, A.; Zoloedov, V. Amaranth oil application for coronory heart disease and hypertenstion. *Lipids Health Dis*. **2007**, 6(1), 1-12.

Mourao, J.; Pinheiro, V.; Prates, J.; Bessa, R.; Ferreira, L.; Fontes, C.; Ponte, P. Effect of dieatary dehydrated pasture and citrus pulp on the performance and meat quality of broiler chickens. *Polt. Sci*. **2008**, 87, 733-743.

Parker, R.; Pearce, B.; Clark, R.; Gardon, D.; Wright, J. Tocotrienols regulate cholesterol production in mammalian cells by post-transcriptional suppression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *J. Biol. Chem.* **1993**, 268 (15), 11230-11238.

Pearce, B.; Parker, P.; Deason, M.; Qureshi, A.; Wright, J. Hypocholesteolemic activity of synthetic and natural tocotrienols. *J. Med. Chem.* **1992**, 35, 3595-3606.

Ponte, P.; Alves, S.; Bessa, R.; Ferreira, L.; Gama, L.; Bras, J.; Fontes, C.; Prates, J. Influence of pasture intake on the fatty acid composition and cholesterol, tocopherols, and tocotrienols content in meat from free-range broilers. *Polt. Sci*. **2008**, 87, 80-88.

المنارة للاستشارات

www.manaraa.com

Qureshi, A.; Burger, W.; Peterson, D.; Elson, C. The structure of an inhibitor of cholesterol biosynthesis isolated from barley. *J. Biol. Chem.* **1986**, 261 (23), 10544-10550.

Qureshi, A.; Lehmann, J.; Peterson, D. Amaranth and its oil inhibit cholesterol biosynthesis in 6-week-old female chickens. *J. Nutr.* **1996**, 126, 1972-1978.

Qureshi, A.; Mo, H.; Packer, L.; Peterson, D. Isolation and identification of novel tocotrienols from rice bran with hypocholesterolemic, antioxidant, and antitumor properties. *J. Agric. Food Chem.* **2000**, 48, 3130-3140.

Qureshi, A.; Peterson, D. The combined effects of novel tocotrienols and lovastatin on lipid metabolism in chickens. *Atherosclerosis*. **2001**, 156, 39-47.

Qureshi, A.; Peterson, D.; Hasler-Rapacz, J.; Rapacz, J. Novel tocotrienols of rice bran suppress cholesterogenesis in hereditary hypercholesterolemic swine. *J. Nutr.* **2001**, 131, 223-230.

Ruff, M.; Allen, P.; Chute, M. Composition of heart, liver, and skeletal muscle from broilers with coccidiosis. *Poult. Sci.* **1981**, 60, 1807-1811.

Scanes, G.; Brant, G.; Ensminger, M. Poultry Biology. In *Poultry Science*, 4th ed., New Jersey, USA. **2004**, 22-45

Sontag, T.; Parker, R. Influence of major structural features of tocopherols and tocotrienols on their ω-oxidation by tocopherol-ω-hydroxylase. *J. Lipid Res.* **2007**, 48, 1090-1098.

Spayd, R.; Bruschi, B.; Burdik, B. Multilayer film elements for clinical analysis: applications to representative chemical determinations. *Clin. Chem.* **1978**, 24, 1343-1350.

Surai, P.; Speake, B. Selective excretion of yolk-derived tocotrienols into the bile of the chick embryo. *Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol.* **1998**, 121, 393-396.

Surai, P.F.; Sparks, N. Tissue-specific fatty acid and alpha-tocopherol profiles in male chickens depending on dietary tuna oil and vitamin E providion. *Poult. Sci*. **2000**, 79, 1132-1142.

المنارة للاستشارات
www.manaraa.com

Surai, P.; Speake, B.; Sparks, N. Carotenoids in avian nutrition and embryonic development, absorption, availability and levels in plasma and egg yolk. *J. Poultry Sci.* **2001**, 38, 1-27.

Tengerdy, R.; Brown, J. Effect of Vitamin E and A on Humoral Immunity and Phagocytosis in E. *coli* infected chicken. *Poult. Sci.* **1977**, 56, 957-963.

Traber, M.; Arai, H. Molecular mechanisms of vitamin E transport. *Annu. Rev. Nutr.* **1999**, 19, 343-355.

Traber, M.; Burton, G.; Hughes, L/; Ingold, K.; Hidaka, H.; Malloy, M.; Kane, J.; Hyams, J.; Kayden, H. Discrimination between forms of vitamin E by humans with and without genetic abnormalities of lipoprotein metabolism. *J.Lipid Res.* **1992**, 33, 1171–82.

Tres, A.; Nuchi, C.; Magrinya, N.; Guardiola, F.; Bou, R.; Codony, R. Use of palm-oil by-products in chicken and rabbit feeds: effect on the fatty acid and tocol composition of meat, liver, and plasma. *Animal*. **2012**, 6(6), 1005-1017.

Walde, C.; Drotleff, A.; Ternes, W. Comparision of dietary tocotrienols from barley and palm oils in hen's egg yolk: transfer efficiency, influence of emulsification, and effect on egg cholesterol. *J. Sci. Food Agric*. **2013**, 94 (4), 810-818.

Walker, L.; Wang, T.; Xin, H.; Dolde, D. Supplementation of laying-hen feed with palm tocos and algae astaxanthin for egg yolk nutrient enrichment. *J. Agric. Food Chem.* **2012**, 60, 1989-1999.

Yang, Z.; Lee, M.; Sang, S.; Yang, C. Bioavailability and metabolism of tocotrienols. In *Tocotrienols Vitamin E Beyond Tocopherols*, 2nd ed. B. Tan, Watson, R. Preedy, V. CRC Press: Boca Raton, FL. **2013**, 37-51.

Yao, L.; Wang, T.; Persia, M.; Horst, R.; Higgins, M. Effects of vitamin D_3-enriched diet on egg yolk vitamin D_3 content and yolk quality. *J. of Food Sci.* **2013**, 78 (2), 78-183.

Yu, S.; Thomas, A.; Gapor, A,; Tan, B.; Qureshi, N.; Qureshi, A. Dose-response impact of various tocotrienols on serum lipid parameters in 5-week-old-female-chickens. *Lipids*. **2006**, 41, 453-461.

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Tables and Figures

Table 1. Composition of base diet fed to laying hens for 7 weeks

Ingredient	Amount (%)
Corn	61.00
Soybean meal	24.43
Calcium carbonate (coarse)	5.84
Calcium carbonate (fine)	3.90
Dicalcium phosphate	2.02
Animal rendered oil	1.57
Vitamin and trace mineral premix [a]	0.68
Sodium chloride	0.38
DL-methionine	0.18
Calculated Composition	**%**
Crude protein	16.05
Metabolizable energy (kcal/kg)	2,825
Crude fat	3.94
Linoleic acid	1.82
Calcium	4.20
Phosphorus (nonphytate)	0.48
Sodium chloride	0.18
Chloride	0.26
Lysine (digestible)	0.77
Methionine (digestible)	0.41
Methionine + cysteine (digestible)	0.63

[a] Premix includes (per kilogram of diet): vitamin A, 9000 IU; vitamin D3, 3000 IU; vitamin E, 20 IU; cobalamine, 13 µg; riboflavin, 6 mg; niacin, 45 mg; pantothenic acid, 12 mg; choline, 487 mg; menadione, 1.2 mg; folic acid, 1.5 mg; pyridoxine, 1.2 mg; thiamine, 1.5 mg; biotin, 45 µg; magnesium, 136 mg; manganese, 136 mg; zinc, 136 mg; iron, 140 mg; copper, 14 mg; and selenium, 0.27 mg.

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Table 2. Concentration of annatto tocotrienols (T3) and alpha-tocopherol (TC) in supplemented diets

Diet	Annatto [a] (ppm)	Alpha-Tocopherol [b] (TC, ppm)
Control	0	0
T3 2000	2000	0
TC 200	2000	200
TC 1000	2000	1000

[a] DeltaGold® 70 Annatto Tocotrienols: total tocotrienols, 74.5% (delta-tocotrienol 89.2%, gamma-tocotrienol 10.8%, and other tocotrienols/tocopherols <1%). [b]ADM Alpha-Tocopherol: alpha-tocopherol, 96.6%.

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Table 3. Content of moisture and lipid, and weight for various organ, tissues, and manure of hens fed diets supplemented with different levels of annatto tocotrienols (T3) and alpha-tocopherol (TC) (n=16) [a]

Organ	Moisture content (%)	Lipid (%, db)	Organ weight (g)
Skin	27.6 ± 3.3	71.2 ± 1.5	99.7 ± 8.7
Fat Pad	6.5 ± 1.4 [b]	78.2 ± 2.2	40.6 ± 6.1
Liver and gall bladder	73.6 ± 2.4	31.5 ± 4.5	57.2 ± 2.8 [b]
Heart	68.5 ± 4.9	50.5 ± 2.9	11.1 ± 0.9
Oviduct	78.2 ± 0.7 [b]	16.8 ± 1.3	192.1 ± 7.1
Forming yolks	53.2 ± 1.2	61.9 ± 0.9	43.1 ± 2.2
Laid yolks	48.5 ± 0.5	64.2 ± 0.9	14.6 ± 0.3
Lungs (pair)	76.1 ± 2.8	29.8 ± 3.4	29.6 ± 0.1
Spleen	72.0 ± 3.7	41.3 ± 7.0	1.6 ± 0.1 [b]
Kidney (pair)	76.6 ± 2.5	40.7 ± 2.9	41.8 ± 3.7 [b]
Pancreas	67.6 ± 2.8	43.8 ± 4.6	3.2 ± 0.3
Gizzard	67.7 ± 3.7	37.4 ± 8.4	19.9 ±1.5
Digestive tract	62.6 ± 3.3	74.8 ± 8.0	182.0 ± 10.2
Brain	74.6 ± 1.5	43.9 ± 2.8	8.0 ± 0.7
Thigh (both)	73.7 ± 0.9	13.3 ± 2.0	119.1 ± 7.3
Breast (both)	71.0 ± 0.4	8.5 ± 0.5	76.9 ± 7.3
Manure [c]	-	4.9 ± 0.7	-

[a] Values are means ± standard deviations across 4 treatments with 4 replicates for each. [b] Significant differences were found in these samples among the 4 treatments. [c] 75% and 120 grams/day were used as estimates of the manure moisture and weight, respectively.

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Table 4. Fatty acid composition (%) in various organ and tissue samples and manure for hens fed diets supplemented with different levels of annatto tocotrienols (T3) and alpha-tocopherol (TC) (n=4) [a]

Control (%)	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:1	20:4 (**ARA**)	20:5 (**EPA**)	22:6 (**DHA**)
Skin	-	19.1 ± 0.9	2.1 ± 0.2	6.9 ± 0.5	38.0 ± 1.1	30.1 ± 2.1	1.3 ± 0.1	-	-	-	-
Fat pad*	-	15.9 ± 2.3	1.7 ± 0.3	6.2 ± 0.7	32.0 ± 2.8 B	26.5 ± 2.6	1.1 ± 0.2	-	-	-	-
Liver	-	25.4 ± 0.9	1.9 ± 0.2	12.0 ± 1.0	35.5 ± 2.0	17.7 ± 2.7	-	-	3.3 ± 0.7	-	-
Heart	0.5 ± 0.1	20.3 ± 0.4	2.6 ± 0.3	7.7 ± 0.2	35.6 ± 3.1	27.5 ± 1.4	1.1 ± 0.2	-	0.4 ± 0.4	-	-
Oviduct*	-	24.0 ± 1.5	1.9 ± 0.5	8.3 ± 1.7	29.3 ± 0.6	22.3 ± 3.5	1.2 ± 0.1	-	0.9 ± 0.2	6.7 ± 0.9 A,B	1.9 ± 0.3 B
Forming yolk	-	25.6 ± 0.5	2.5 ± 0.2	9.7 ± 0.8	39.7 ± 0.1	16.1 ± 0.2	-	-	2.1 ± 0.2	-	0.5 ± 0.1
Laid yolk	-	24.3 ± 4.5	2.3 ± 0.2	9.7 ± 0.5	40.1 ± 1.7	16.5 ± 0.6	-	-	2.1 ± 0.2	-	0.5 ± 0.1
Lung	-	25.4 ± 0.5	2.0 ± 0.1	9.0 ± 0.2	36.4 ± 0.9	21.3 ± 1.2	0.9 ± 0.1	-	1.4 ± 0.2	-	-
Spleen	-	23.6 ± 1.0	1.7 ± 0.2	9.5 ± 1.1	36.0 ± 1.3	23.6 ± 0.7	0.9 ± 0.2	-	0.9 ± 0.2	-	-
Kidney	-	19.6 ± 0.9	1.6 ± 0.2	9.8 ± 0.5	35.7 ± 0.5	27.6 ± 1.2	1.2 ± 0.1	-	1.0 ± 0.2	1.2 ± 0.8	-
Pancreas	-	21.8 ± 2.3	1.6 ± 0.4	9.0 ± 1.0	37.2 ± 1.2	25.6 ± 3.7	0.9 ± 1.1				
Gizzard	0.4 ± 0.3	24.0 ± 5.4	1.9 ± 0.6	7.4 ± 4.0	36.0 ± 3.1	26.5 ± 2.1	0.8 ± 0.6	-	-	-	-
Digestive tract	-	20.6 ± 0.8	1.8 ± 0.1	7.5 ± 0.3	37.5 ± 0.6	28.5 ± 1.4	1.2 ± 0.1	-	-	-	
Brain*	1.3 ± 1.9	32.3 ± 1.8	23.0 ± 2.2	23.4 ± 3.2	13.7 ± 4.6	-	-	-	1.8 ± 2.5	-	- B
Thigh	-	19.3 ± 0.5	1.3 ± 0.9	9.9 ± 1.0	29.1 ± 3.1	30.4 ± 1.5	0.9 ± 0.6	2.4 ± 0.4	4.9 ± 6.9	-	-
Breast	1.4 ± 1.7	25.7 ± 10.5	10.5 ± 1.1	26.9 ± 1.8	25.0 ± 1.7	5.1 ± 1.5	2.1 ± 4.1	-	-	-	-
Manure*	-	12.4 ± 1.8 B	3.2 ± 1.83	22.9 ± 5.5 B,C	48.5 ± 6.7 A	4.0 ± 3.9	2.2 ± 1.8 A,B	-	-	-	-

T3 2000 (%)	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:1	20:4 (**ARA**)	20:5 (**EPA**)	22:6 (**DHA**)
Skin	-	18.7 ± 1.8	3.4 ± 2.3	6.7 ± 0.6	38.6 ± 2.9	27.5 ± 2.1	2.0 ± 2.5	-	-	-	-
Fat pad*	-	20.2 ± 2.0	2.3 ± 0.4	7.3 ± 0.8	37.0 ± 1.1 A,B	29.2 ± 1.1	0.9 ± 0.6	-	-	-	-
Liver	-	26.7 ± 1.5	1.9 ± 0.3	12.7 ± 1.3	38.4 ± 2.9	15.3 ± 1.6	-	-	2.1 ± 1.6	-	-
Heart	0.5 ± 0.1	20.6 ± 0.3	2.9 ± 0.2	7.5 ± 0.3	38.1 ± 0.5	26.0 ± 0.8	1.1 ± 0.1	-	0.7 ± 0.1	-	-
Oviduct*	-	23.7 ± 0.4	1.4 ± 1.1	8.3 ± 0.7	30.1 ± 1.4	22.0 ± 2.0	0.9 ± 0.6	-	0.7 ± 0.5	9.0 ± 2.4 A	1.9 ± 0.7 B
Forming yolk	-	24.7 ± 0.3	2.2 ± 0.1	10.3 ± 0.6	40.1 ± 0.6	15.9 ± 0.2	-	-	2.2 ± 0.1	-	0.6 ± 0.1
Laid yolk	-	24.3 ± 4.5	2.3 ± 0.2	9.7 ± 0.5	40.1 ± 1.7	16.5 ± 0.6	-	-	2.1 ± 0.2	-	0.5 ± 0.1
Lung	-	24.2 ± 1.5	1.9 ± 0.2	9.2 ± 0.7	38.2 ± 1.1	20.7 ± 0.4	0.6 ± 0.4	-	0.8 ± 0.6	-	-
Spleen	-	23.3 ± 0.9	1.8 ± 0.1	9.0 ± 0.5	37.1 ± 0.8	23.4 ± 1.7	0.9 ± 0.1	-	0.8 ± 0.5	-	-
Kidney	-	22.0 ± 3.1	1.8 ± 0.1	10.4 ± 2.1	37.2 ± 0.7	24.5 ± 3.1	0.8 ± 0.6	-	0.6 ± 0.4	1.0 ± 0.7	-
Pancreas	-	20.3 ± 0.7	1.8 ± 0.1	8.2 ± 0.2	38.5 ± 0.8	25.7 ± 1.2	1.1 ± 0.2	-	-	-	-
Gizzard	0.5 ± 0.1	21.2 ± 0.4	2.0 ± 0.1	8.0 ± 0.4	36.7 ± 0.4	26.8 ± 0.6	1.1 ± 0.1	-	-	-	-
Digestive tract	-	20.7 ± 0.3	2.0 ± 0.1	7.7 ± 0.3	38.6 ± 0.2	27.4 ± 0.8	1.1 ± 0.1	-		-	-
Brain*	-	22.0 ± 6.6	16.8 ± 4.6	14.6 ± 10.7	6.8 ± 5.0	-	-	-	11.6 ± 12.5	5.6 ± 2.4	10.2 ± 0.7 A
Thigh	-	19.2 ± 0.5	1.7 ± 0.3	9.7 ± 0.7	30.8 ± 1.3	30.4 ± 1.3	1.1 ± 0.1	2.7 ± 0.3	1.5 ± 0.4	-	-
Breast	1.6 ± 1.7	34.4 ± 9.3	9.3 ± 6.3	25.0 ± 7.2	20.1 ± 9.9	-	-	-	-	-	-
Manure*	-	20.2 ± 1.3 A	9.6 ± 1.9	37.7 ± 2.2 A,B	32.1 ± 5.9 B	0.4 ± 0.8	10.0 ± 2.0 A	-	-	-	-

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Table 4. cont'd

TC 200 (%)	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:1	20:4 (**ARA**)	20:5 (**EPA**)	22:6 (**DHA**)
Skin	-	19.4 ± 1.2	2.3 ± 0.3	6.7 ± 0.7	38.4 ± 1.8	29.5 ± 0.9	1.2 ± 0.1	-	-	-	-
Fat pad*	-	18.3 ± 0.8	1.9 ± 0.2	7.1 ± 0.8	36.9 ± 3.1 A,B	27.9 ± 3.1	1.3 ± 0.2	-	-	-	-
Liver	-	25.5 ± 3.6	1.9 ± 0.7	12.5 ± 0.6	37.4 ± 4.8	16.3 ± 5.3	-	-	2.7 ± 2.0	-	-
Heart	0.5 ± 0.1	20.3 ± 1.2	2.8 ± 0.3	7.8 ± 0.3	38.1 ± 0.8	26.3 ± 0.8	1.2 ± 0.1	-	0.7 ± 0.1	-	-
Oviduct*	-	23.6 ± 1.3	1.7 ± 0.3	8.5 ± 0.7	29.5 ± 2.1	21.0 ± 1.5	1.2 ± 0.2	-	0.7 ± 0.1	0.8 ± 0.6 B	8.3 ± 1.4 A
Forming yolk	-	24.9 ± 0.9	2.2 ± 0.2	9.8 ± 0.7	40.7 ± 0.7	15.9 ± 0.6	-	-	2.2 ± 0.2	-	0.6 ± 0.1
Laid yolk	-	24.3 ± 4.5	2.3 ± 0.2	9.7 ± 0.5	40.1 ± 1.7	16.5 ± 0.6	-	-	2.1 ± 0.2	-	0.5 ± 0.1
Lung	-	24.4 ± 0.5	1.9 ± 0.2	9.2 ± 0.3	37.3 ± 0.7	20.6 ± 0.8	0.7 ± 0.5	-	1.4 ± 0.4	-	-
Spleen	-	22.6 ± 1.7	1.7 ± 0.2	9.1 ± 0.6	37.6 ± 0.8	24.1 ± 1.6	1.0 ± 0.1	-	0.8 ± 0.4	-	-
Kidney	-	22.8 ± 4.2	2.0 ± 0.4	6.9 ± 4.6	37.7 ± 1.3	27.6 ± 2.1	0.6 ± 0.7		0.4 ± 0.4	0.6 ± 0.7	-
Pancreas	-	21.6 ± 1.5	1.7 ± 0.3	8.9 ± 0.4	36.8 ± 2.2	25.0 ± 2.4	1.0 ± 0.1	-	-	-	-
Gizzard	0.3 ± 0.2	22.3 ± 4.5	2.0 ± 0.3	6.3 ± 2.2	37.3 ± 0.6	27.9 ± 1.2	0.6 ± 0.4	-	-	-	-
Digestive tract	-	20.6 ± 0.7	1.9 ± 0.1	7.8 ± 0.2	38.5 ± 0.7	27.6 ± 0.4	1.1 ± 0.1	-	-	-	-
Brain*	2.1 ± 2.6	25.2 ± 3.6	19.2 ± 1.5	22.8 ± 1.5	10.8 ± 1.8	-	-	-	0.9 ± 0.8	5.6 ± 5.6	5.7 ± 4.4 A,B
Thigh	-	19.0 ± 0.6	1.6 ± 0.2	9.8 ± 0.5	30.9 ± 1.2	29.9 ± 1.8	1.1 ± 0.1	2.6 ± 0.5	3.1 ± 3.0	-	-
Breast	-	30.3 ± 11.9	11.9 ± 2.0	23.0 ± 9.3	23.5 ± 4.6	5.7 ± 5.7	2.2 ± 3.5	-	-	-	-
Manure*	-	21.6 ± 3.4 A	5.88 ± 7.07	44.1 ± 4.6 A	55.1 ± 11.3 A,B	1.9 ± 3.8	- B	-	-	-	-

TC 1000 (%)	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:1	20:4 (**ARA**)	20:5 (**EPA**)	22:6 (**DHA**)
Skin	-	20.0 ± 0.7	2.4 ± 0.2	6.8 ± 0.3	39.3 ± 1.8	28.3 ± 0.9	1.2 ± 0.1	-	-	-	-
Fat pad*	-	18.6 ± 0.7	1.9 ± 0.2	7.6 ± 0.2	39.7 ± 1.4 A	30.0 ± 1.0	1.4 ± 0.1	-	-	-	-
Liver	-	24.7 ± 0.6	1.9 ± 0.2	11.9 ± 0.5	39.0 ± 1.7	16.3 ± 1.2	-	-	2.6 ± 0.5	-	-
Heart	0.5 ± 0.1	20.2 ± 0.9	2.8 ± 0.2	7.5 ± 0.1	38.0 ± 1.3	26.6 ± 0.9	1.1 ± 0.1	-	0.7 ± 0.1	-	-
Oviduct*	-	24.0 ± 1.6	1.4 ± 1.0	7.8 ± 0.8	29.0 ± 0.8	23.7 ± 2.6	0.9 ± 0.6	-	0.7 ± 0.6	7.8 ± 4.7 A,B	3.2 ± 3.5 A, B
Forming yolk	-	24.8 ± 0.6	2.2 ± 0.1	10.0 ± 0.7	40.6 ± 0.7	15.5 ± 0.8	-	-	2.1 ± 0.1	-	0.5 ± 0.1
Laid yolk	-	24.3 ± 4.5	2.3 ± 0.2	9.7 ± 0.5	40.1 ± 1.7	16.5 ± 0.6	-	-	2.1 ± 0.2	-	0.5 ± 0.1
Lung	-	24.8 ± 0.7	2.2 ± 0.2	9.9 ± 0.3	40.4 ± 0.4	15.9 ± 0.3	-	-	2.2 ± 0.2	0.6 ± 0.1	-
Spleen	-	21.2 ± 0.4	1.6 ± 0.2	8.9 ± 0.5	37.1 ± 2.1	26.4 ± 2.0	1.1 ± 0.1	-	1.2 ± 0.4	-	-
Kidney	-	21.0 ± 0.7	1.7 ± 0.3	10.4 ± 1.0	37.8 ± 1.5	26.7 ± 0.9	0.7 ± 0.5	-	0.3 ± 0.6	0.5 ± 0.9	-
Pancreas	-	22.3 ± 1.7	1.3 ± 0.8	8.5 ± 0.7	37.2 ± 3.4	27.1 ± 1.9	0.7 ± 0.5	-	-	-	-
Gizzard	0.4 ± 0.3	21.6 ± 0.9	2.0 ± 0.2	7.8 ± 0.3	35.8 ± 1.4	28.5 ± 2.0	0.8 ± 0.3	-	-	-	-
Digestive tract	-	20.5 ± 0.4	1.8 ± 0.2	7.9 ± 0.2	37.7 ± 1.8	28.9 ± 1.6	1.2 ± 0.1	-	-	-	-
Brain*	-	30.4 ± 1.0	20.5 ± 1.0	12.6 ± 0.9	12.6 ± 0.9	-	-	-	0.2 ± 0.5	-	11.4 ± 0.2 A
Thigh	-	19.3 ± 0.5	1.7 ± 0.1	10.0 ± 0.6	31.4 ± 0.9	28.8 ± 1.8	1.1 ± 0.1	2.7 ± 0.5	3.4 ± 2.9	-	-
Breast	1.7 ± 2.0	27.8 ± 5.8	5.8 ± 5.8	24.4 ± 3.9	33.7 ± 8.0	0.7 ± 1.3	-	-	-	-	-
Manure*		18.2 ± 3.0 A,B	1.7 ± 2.2	16.4 ± 8.2 C	55.1 ± 11.7 A	5.3 ± 6.7	2.8 ± 5.5 A,B	-	-	-	-

[a] Values are means ± standard deviations. [b] Diets detailed in Table 2, T3 = ppm annatto, TC = ppm alpha-tocopherol with constant 2000 ppm annatto. [c] Different letters (comparing all treatments) with in the same organ or tissue indicate significant differences at the 99% confidence level. *p-value = 0.01, NS not significant at 1%. – indicates not detected. **ARA** = arachidonic acid, **EPA** = eicosapentaenoic acid, **DHA** = docosahexaenoic

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Table 5. Concentration (ppm based on dry organ) of alpha-tocopherol, gamma-tocotrienol, and delta-tocotrienol in various organs and tissues and manure from hens fed diets supplemented with different levels of annatto tocotrienols (T3) and alpha-tocopherol (TC) (n=4) [a]

Alpha-tocopherol (ppm based on dry organ)					
	Control[b]	T3 2000[b]	TC 200[b]	TC 1000[b]	Significance[c]
Skin	-	-	-	-	-
Fat pad	0.01 ± 0.02 B	0.02 ± 0.02 B	0.08 ± 0.07 B	0.27 ± 0.03 A	*
Liver and gall bladder	0.06 ± 0.07 B	0.18 ± 0.08 B	31.89 ± 36.51 B	187.71 ± 36.77 A	*
Heart	0.05 ± 0.09	-	0.03 ± 0.05	0.01 ± 0.02	NS
Oviduct	0.47 ± 0.86 B	0.02 ± 0.03 B	2.98 ± 5.11 B	34.18 ± 16.80 A	*
Forming yolk	2.59 ± 2.01 C	3.18 ± 1.83 C	302.48 ± 9.45 B	1154.41 ± 63.97 A	*
Laid yolks	58.30 ± 12.78 C	68.42 ± 7.32 C	694.98 ± 9.42 B	2570.80 ± 189.92 A	*
Lung	0.02 ± 0.03	0.01 ± 0.01	-	0.08 ± 0.07	NS
Spleen	0.09 ± 0.03	0.13 ± 0.03	0.11 ± 0.05	0.19 ± 0.08	NS
Kidney	0.11 ± 0.06 B	0.15 ± 0.04 B	2.73 ± 5.08 B	21.90 ± 9.72 A	*
Pancreas	0.08 ± 0.02	0.05 ± 0.01	0.08 ± 0.02	0.08 ± 0.06	NS
Gizzard	0.01 ± 0.01	0.01 ± 0.02	0.02 ± 0.03	0.02 ± 0.04	NS
Digestive tract	-	-	0.30 ± 0.60	3.09 ± 5.25	NS
Brain	2.40 ± 0.53 C	2.04 ± 0.70 C	5.78 ± 0.40 B	12.75 ± 2.21 A	*
Thigh	0.06 ± 0.01	0.05 ± 0.01	0.52 ± 0.87	8.82 ± 8.98	NS
Breast	0.87 ± 0.17 B	1.13 ± 0.11 B	2.05 ± 0.42 B	6.97 ± 1.41 A	*
Manure	3.68 ± 1.19 B	21.14 ± 19.24 B	98.82 ± 18.41 B	504.98 ± 96.37 A	*
Gamma-tocotrienol (ppm based on dry organ)					
	Control[b]	T3 2000[b]	TC 200[b]	TC 1000[b]	Significance[c]
Skin	0.03 ± 0.02	0.06 ± 0.06	0.02 ± 0.02	0.03 ± 0.04	NS
Fat pad	0.02 ± 0.02	0.02 ± 0.03	0.03 ± 0.04	0.15 ± 0.24	NS
Liver and gall bladder	- B	0.01 ± 0.03 B	0.58 ± 0.30 A	0.68 ± 0.14 A	*
Heart	-	0.01 ± 0.02	-	-	NS
Oviduct	0.01 ± 0.01	0.15 ± 0.15	0.24 ± 0.21	0.25 ± 0.05	NS
Forming yolk	0.07 ± 0.04 C	1.38 ± 0.24 A	1.08 ± 0.14 A,B	1.03 ± 0.16 B	*
Laid yolks	0.42 ± 0.02 C	12.12 ± 1.90 A	10.12 ± 0.98 A,B	9.24 ± 0.72 B	*
Lung	0.01 ± 0.02	-	-	-	NS
Spleen	-	-	-	-	NS
Kidney	0.01 ± 0.02 B	0.03 ± 0.02 B	0.18 ± 0.22 B	0.55 ± 0.12 A	*
Pancreas	-	-	-	-	NS
Gizzard	-	-	-	0.01 ± 0.01	NS
Digestive tract	0.02 ± 0.02	0.03 ± 0.02	0.16 ± 0.26	0.63 ± 0.85	NS
Brain	- B	0.12 ± 0.04 B	0.14 ± 0.03 B	0.15 ± 0.06 A	*
Thigh	-	0.19 ± 0.15	0.24 ± 0.09	0.24 ± 0.17	NS
Breast	0.02 ± 0.04 B	0.25 ± 0.05 A	0.22 ± 0.06 A	0.19 ± 0.02 A	*
Manure	0.63 ± 0.17 C	29.88 ± 7.90 A	18.41 ± 4.70 B	25.77 ± 5.27 A,B	*

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Table 5. cont'd

	Delta-tocotrienol (ppm based on dry organ)				
	Control[b]	T3 2000[b]	TC 200[b]	TC 1000[b]	Significance[c]
Skin	0.02 ± 0.04	0.07 ± 0.14	-	0.07 ± 0.09	NS
Fat pad	- B	0.76 ± 1.03 A,B	0.92 ± 1.03 A,B	3.08 ± 2.16 A	*
Liver and gall bladder	- B	1.28 ± 1.62 A,B	2.26 ± 1.21 A	2.20 ± 0.43 A	*
Heart	-	-	-	-	-
Oviduct	0.01 ± 0.02 B	0.78 ± 0.41 A,B	1.58 ± 1.44 A,B	0.81 ± 0.07 A	*
Forming yolk	- B	2.81 ± 0.39 A	2.59 ± 0.55 A	2.26 ± 0.34 A	*
Laid yolks	0.18 ± 0.36 B	22.70 ± 3.68 A	21.38 ± 3.62 A	18.98 ± 1.12 A	*
Lung	-	-	0.01 ± 0.02	0.01 ± 0.01	NS
Spleen	-	-	-	-	NS
Kidney	-	2.06 ± 1.75	1.70 ± 1.24	2.26 ± 0.45	NS
Pancreas	-	-	0.03 ± 0.05	0.04 ± 0.05	NS
Gizzard	0.01 ± 0.02	0.02 ± 0.02	0.03 ± 0.06	0.02 ± 0.02	NS
Digestive tract	0.01 ± 0.01	0.18 ± 0.10	1.67 ± 2.82	6.03 ± 8.73	NS
Brain	0.01 ± 0.03 B	0.45 ± 0.11 A,B	0.75 ± 0.26 A	0.64 ± 0.41 A	*
Thigh	0.01 ± 0.01 B	1.00 ± 0.45 A	1.16 ± 0.32 A	1.05 ± 0.10 A	*
Breast	0.01 ± 0.01 B	0.72 ± 0.14 A,B	1.11 ± 0.94 A	0.61 ± 0.12 A,B	*
Manure	0.32 ± 0.36 B	186.99 ± 55.87 A	120.88 ± 33.67 A	156.62 ± 33.26 A	*

[a] Values are means ± standard deviations. [b] Diets detailed in Table 2, T3 = ppm annatto, TC = ppm alpha-tocopherol with constant 2000 ppm annatto. [c] Different letters (comparing all treatments) in the same row indicate significant differences at the 95% confidence level. * p-value = 0.05, NS not significant at 5%. – indicates not detected.

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Table 6. Concentration (ppm based on lipid) of alpha-tocopherol, gamma-T3, and delta-T3 in various organs and tissues and manure from hens fed diets supplemented with different levels of annatto tocotrienols (T3) and alpha-tocopherol (TC) (n=4) [a]

Alpha-tocopherol (ppm based on lipid)					
Organ	**Control[b]**	**T3 2000[b]**	**TC 200[b]**	**TC 1000[b]**	**Significance[c]**
Skin	-	-	-	-	-
Fat pad	0.02 ± 0.03 B	0.03 ± 0.02 B	0.11 ± 0.10 B	0.40 ± 0.04 A	*
Liver and gall bladder	0.84 ± 0.97 B	1.91 ± 0.84 B	390.50 ± 477.08 B	2272.96 ± 445.27 A	*
Heart	0.28 ± 0.56	-	0.16 ± 0.32	0.09 ± 0.17	NS
Oviduct	12.33 ± 22.77 B	0.57 ± 0.68 B	83.97 ± 143.91 B	941.50 ± 462.84 A	*
Forming yolk	8.90 ± 6.89 C	10.88 ± 6.27 C	1056.90 ± 33.02 B	3986.93 ± 220.93 A	*
Laid yolk	79.29 ± 17.38 C	93.05 ± 9.96 C	945.14 ± 12.81 B	3496.29 ± 258.29 A	*
Lung	0.22 ± 0.45	0.08 ± 0.09	0.06 ± 0.12	1.19 ± 0.97	NS
Spleen	0.77 ± 0.28	1.24 ± 0.28	0.83 ± 0.36	1.71 ± 0.73	NS
Kidney	1.53 ± 0.83 B	1.70 ± 0.46 B	25.13 ± 46.68 B	222.79 ± 98.86 A	*
Pancreas	0.54 ± 0.16	0.34 ± 0.06	0.58 ± 0.16	0.55 ± 0.40	NS
Gizzard	0.08 ± 0.06	0.10 ± 0.20	0.08 ± 0.16	0.18 ± 0.37	NS
Digestive tract	-	0.01 ± 0.01	1.20 ± 2.39	11.28 ± 19.13	NS
Brain	21.34 ± 4.45 C	18.10 ± 6.26 C	52.16 ± 3.59 B	118.31 ± 20.47 A	*
Thigh	1.84 ± 0.38	1.42 ± 0.36	14.94 ± 25.03	223.38 ± 227.44	NS
Breast	36.39 ± 6.95 B	41.21 ± 4.18 B	83.08 ± 17.15 B	290.57 ± 58.81 A	*
Manure	303.81 ± 98.55 C	935.17 ± 52.73 B,C	9235.90 ± 1720.34 B	37199.18 ± 7098.76 C	*

Gamma-tocotrienol (ppm based on lipid)					
	Control[b]	**T3 2000[b]**	**TC 200[b]**	**TC 1000[b]**	**Significance[c]**
Skin	0.05 ± 0.04	0.12 ± 0.11	0.04 ± 0.05	0.06 ± 0.07	NS
Fat pad	0.02 ± 0.02	0.02 ± 0.04	0.04 ± 0.05	0.22 ± 0.35	NS
Liver and gall bladder	- B	0.16 ± 0.31B	7.13 ± 3.64 A	8.22 ± 1.66 A	*
Heart	-	0.07 ± 0.14	-	-	NS
Oviduct	0.15 ± 0.30	4.15 ± 3.98	6.85 ± 5.94	6.80 ± 1.35	NS
Forming yolk	0.22 ± 0.15 B	4.72 ± 0.83 A	3.79 ± 0.50 A	3.56 ± 0.56 A	*
Laid yolk	0.57 ± 0.03 C	16.48 ± 2.58 A	13.76 ± 1.33 A,B	12.57 ± 0.98 B	*
Lung	0.12 ± 0.23	-	-	-	NS
Spleen	-		-	-	-
Kidney	0.13 ± 0.26 B	0.29 ± 0.20 B	1.70 ± 2.03 B	5.60 ± 1.25 A	*
Pancreas	0.02 ± 0.04	-	-	-	NS
Gizzard	-	0.03 ± 0.06	-	0.05 ± 0.11	NS
Digestive tract	0.06 ± 0.07	0.09 ± 0.07	0.65 ± 1.05	2.30 ± 3.10	NS
Brain	- B	1.07 ± 0.38 A	1.30 ± 0.23 A	1.36 ± 0.55 A	*
Thigh	0.14 ± 0.27	5.60 ± 4.51	6.87 ± 2.54	6.12 ± 4.19	NS
Breast	0.86 ± 1.71 B	9.21 ±1.74 A	8.85 ± 2.58 A	8.08 ± 0.93 A	*
Manure	52.41 ± 13.87 B	2424.33 ± 641.24 A	1720.33 ± 439.19 A	1898.65 ± 388.31 A	*

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Table 6. cont'd

	Delta-tocotrienol (ppm based on lipid)				
	Control[b]	**T3 2000[b]**	**TC 200[b]**	**TC 1000[b]**	**Significance[c]**
Skin	0.04 ± 0.08	0.13 ± 0.26	-	0.14 ± 0.17	NS
Fat pad	- B	1.01 ± 1.37 A,B	1.24 ± 1.39 A,B	4.61 ± 3.24 A	*
Liver and gall bladder	- B	13.77 ± 17.39 A,B	27.66 ± 14.83 A	26.66 ± 5.17 A	*
Heart	-	-	-	-	NS
Oviduct	0.25 ± 0.49 B	21.02 ± 10.89 A,B	44.61 ± 40.43 A	22.26 ± 2.06 A,B	*
Forming yolk	0.02 ± 0.03 B	9.63 ± 1.32 A	9.06 ± 1.92 A	7.80 ± 1.19 A	*
Laid yolk	0.24 ± 0.49 B	30.87 ± 5.00 A	29.08 ± 4.92 A	25.81 ± 1.52 A	*
Lung	-	-	0.13 ± 0.26	0.08 ± 0.17	NS
Spleen	-	-	-	-	NS
Kidney	-	23.07 ± 19.54	15.58 ± 11.43	22.98 ± 4.57	NS
Pancreas	-	-	0.21 ± 0.42	0.32 ± 0.39	NS
Gizzard	0.07 ± 0.14	0.19 ± 0.14	0.17 ± 0.34	0.19 ± 0.14	NS
Digestive tract	0.04 ± 0.05	0.61 ± 0.35	6.70 ± 11.27	21.98 ± 31.82	NS
Brain	0.12 ± 0.24 B	4.01 ± 0.98 A,B	6.80 ± 2.37 A	5.95 ± 3.83 A	*
Thigh	0.18 ± 0.36 B	30.08 ± 13.54 A	33.28 ± 9.11 A	26.51 ± 2.44 A	*
Breast	0.24 ± 0.49 B	26.19 ± 5.02 A,B	45.25 ± 38.06 A	25.57 ± 5.16 A,B	*
Manure	26.43 ± 29.88 B	15171.61 ± 4532.67 A	11297.54 ± 3146.42 A	11537.68 ± 2449.77 A	*

[a] Values are means ± standard deviations. [b] Diets detailed in Table 2, T3 = ppm annatto, TC = ppm alpha-tocopherol with constant 2000 ppm annatto. [c] Different letters (comparing all treatments) in the same row indicate significant differences at the 95% confidence level. * p-value = 0.05, NS not significant at 5%. – indicates not detected.

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Table 7. Cholesterol content of select organs and tissues from hens fed diets supplemented with different levels of annatto tocotrienols (T3) and alpha-tocopherol (TC) (n=4) [a]

mg Cholesterol / g organ as-is	Control [b]	T3 2000[b]	TC 200[b]	TC 1000[b]	Significance [c]
Breast	0.33 ± 0.05	0.37 ± 0.12	0.34 ± 0.09	0.37 ± 0.12	NS
Thigh	0.94 ± 0.11	0.74 ± 0.09	0.75 ± 0.13	0.82 ± 0.12	NS
Liver and gall bladder	3.56 ± 0.29	4.24 ± 0.47	3.45 ± 0.22	3.45 ± 0.24	NS
Kidney	3.69 ± 0.57	3.88 ± 0.69	3.46 ± 0.22	3.33 ± 0.41	NS
Oviduct	2.47 ± 0.82	2.51 ± 0.48	2.14 ± 0.23	1.82 ± 0.23	NS
Heart	5.70 ± 0.71 A	3.64 ± 0.42 B	5.25 ± 0.42 A	4.32 ± 0.45 A,B	**
Laid egg yolks	14.87 ± 0.49	14.46 ± 0.25	13.49 ± 0.92	13.94 ± 1.42	NS
mg Cholesterol / g dry organ	Control [b]	T3 2000[b]	TC 200[b]	TC 1000[b]	Significance [c]
Breast	1.12 ± 0.16	1.27 ± 0.42	1.16 ± 0.31	1.24 ± 0.42	NS
Thigh	3.62 ± 0.44	2.86 ± 0.36	2.85 ± 0.49	3.08 ± 0.44	NS
Liver and gall bladder	13.70 ± 1.10	15.64 ± 1.73	13.10 ± 0.85	13.57 ± 0.96	NS
Kidney	16.60 ± 2.57	17.23 ± 3.08	14.75 ± 0.99	14.21 ± 1.82	NS
Oviduct	11.22 ± 0.37	11.31 ± 2.17	9.57 ± 1.01	8.75 ± 1.11	NS
Heart	10.89 ± 1.36 A,B	11.23 ± 1.29 A	9.81 ± 1.23 A,B	7.99 ± 0.49 B	**
Laid egg yolks	28.77 ± 1.51	28.04 ± 0.49	26.33 ± 1.79	27.25 ± 2.77	NS
mg Cholesterol / g lipid	Control [b]	T3 2000[b]	TC 200[b]	TC 1000[b]	Significance [c]
Breast	13.66 ± 1.94	13.49 ± 4.45	13.73 ± 3.64	14.68 ± 5.00	NS
Thigh	29.44 ± 3.59	22.35 ± 2.83	21.57 ± 3.72	20.80 ± 2.96	NS
Liver and gall bladder	47.32 ± 3.81	47.18 ± 5.22	42.64 ± 2.78	40.90 ± 2.92	NS
Kidney	42.74 ± 6.62	43.91 ± 7.86	33.18 ± 2.23	35.20 ± 4.51	NS
Oviduct	65.09 ± 2.17	67.34 ± 12.91	60.37 ± 6.40	50.21 ± 6.39	NS
Heart	22.67 ± 2.84 A	22.13 ± 2.55 A	19.62 ± 2.47 A,B	15.03 ± 0.92 B	**
Laid egg yolks	44.97 ± 2.36	43.61 ± 0.75	41.34 ± 2.80	42.97 ± 4.36	NS

[a] Values are means ± standard deviations. [b] Diets detailed in Table 2, T3 = ppm annatto, TC = ppm alpha-tocopherol with constant 2000 ppm annatto. [C] Different letters (comparing all treatments) in the same row indicate significant differences at the 99% confidence level. ** p-value = 0.01, NS not significant at 1%. – indicates not detected.

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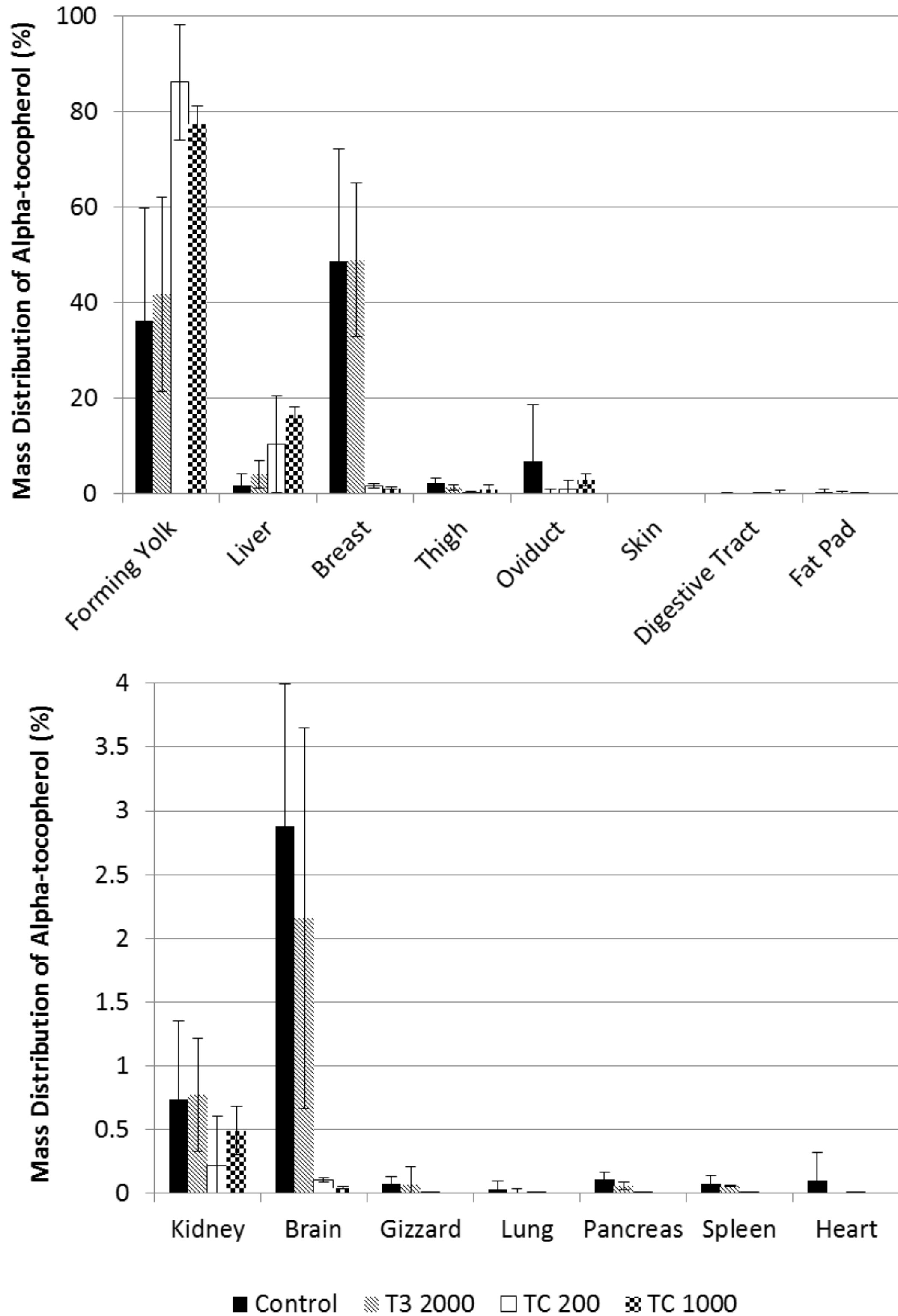

Figure 1. Mass distribution of alpha-tocopherol in various organs and tissues of laying hens.
All diets with supplementation have 2000ppm annatto and manure was excluded. Error bars = SD.

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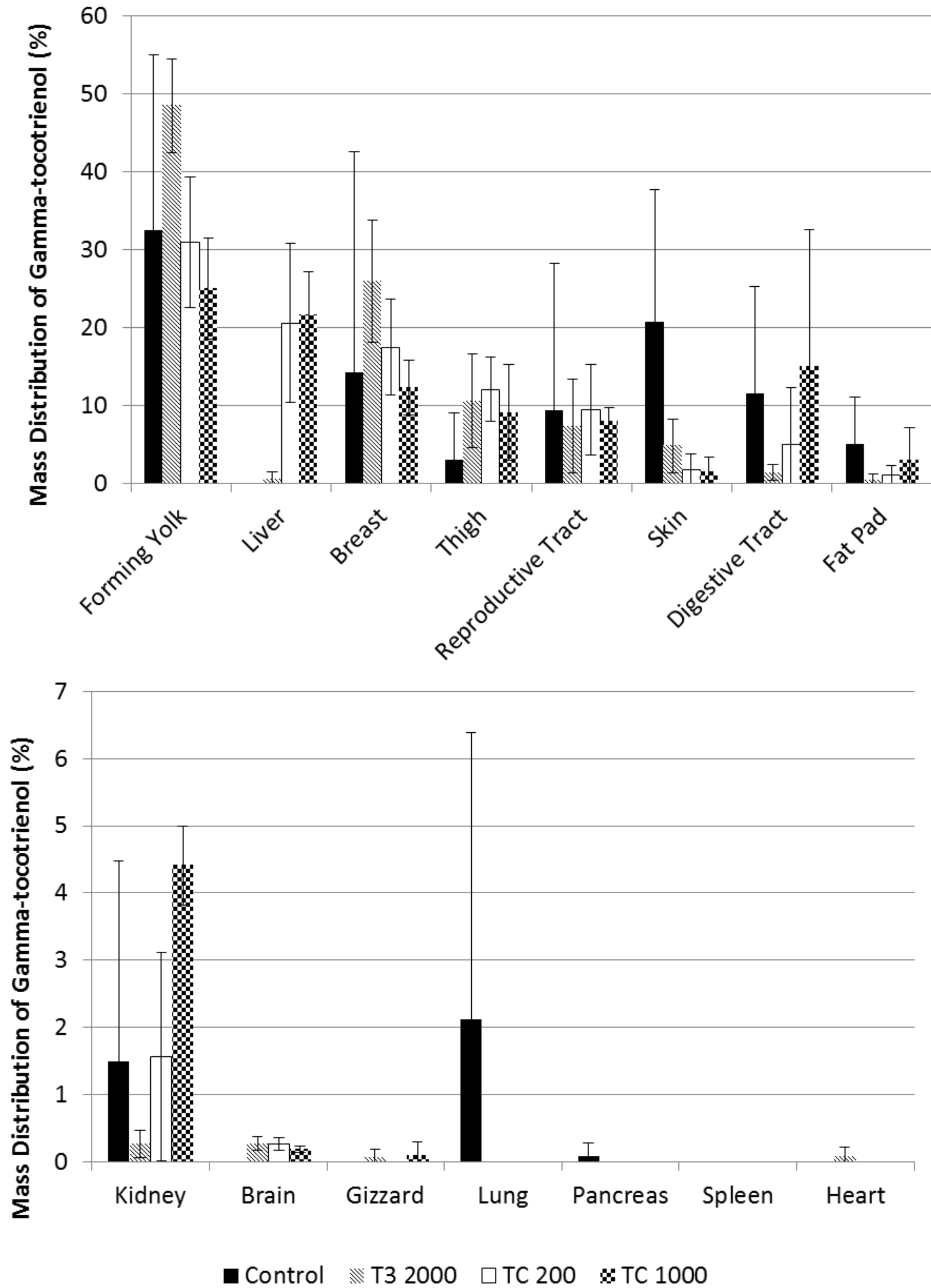

Figure 2. Mass distribution of gamma-tocotrienol in various organs and tissues of laying hens.

All diets with supplementation have 2000ppm annatto and manure was excluded. Error bars = SD.

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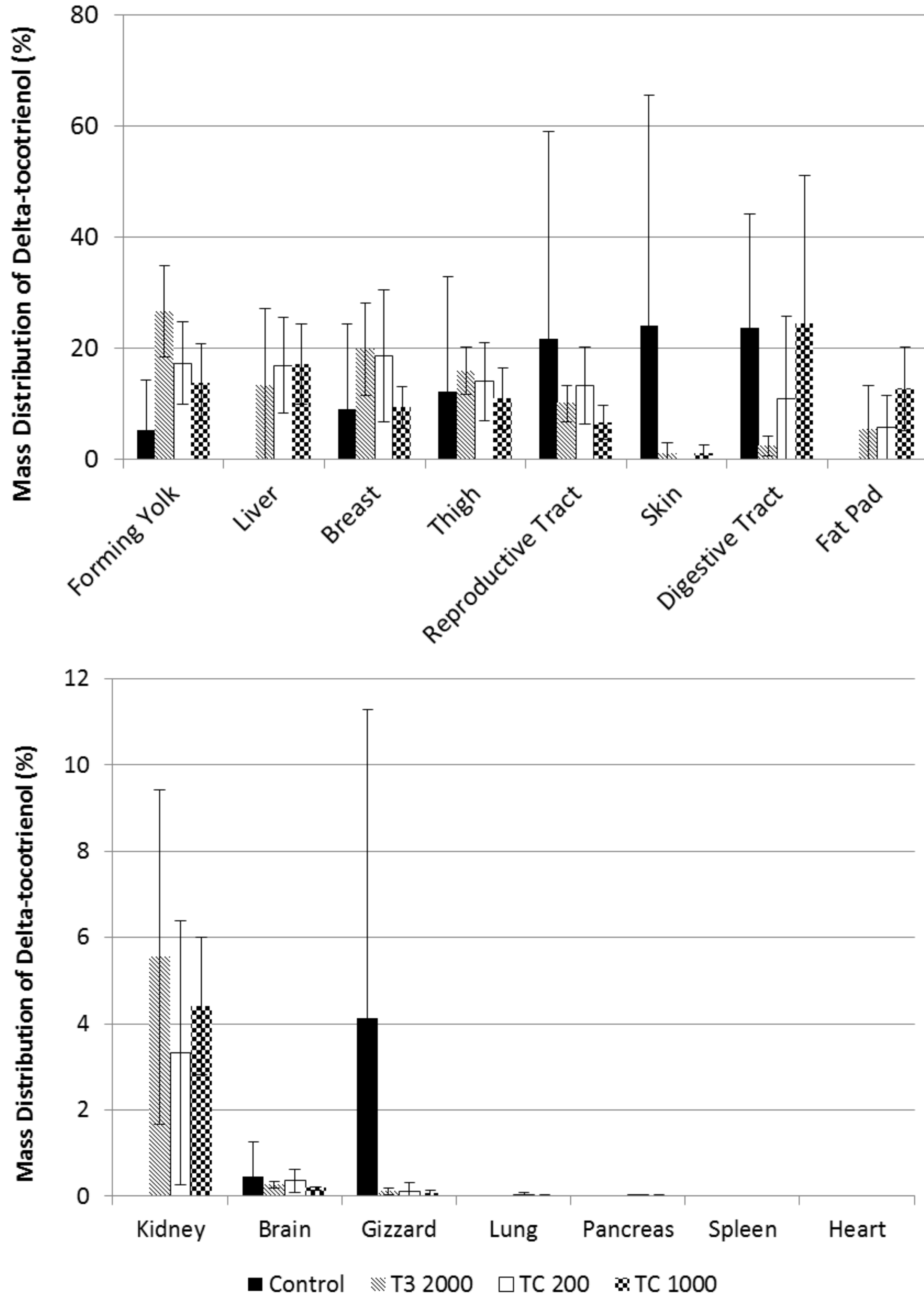

Figure 3. Mass distribution of delta-tocotrienol in various organs and tissues of laying hens.
All diets with supplementation have 2000ppm annatto and manure was excluded. Error bars = SD.

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CHAPTER 5

SUMMARY AND CONCLUSIONS

In summary, annatto and alpha-tocopherol did not significantly impact most quality parameters analyzed of the resulting egg yolks (hen performance: laying rate and hen weight, physical or quality parameters; whole egg weight, egg yolk weight, eggshell strength, yolk color, HU, and eggshell thickness; sensory attributes: moistness, smoothness, chalkiness/mouth-drying, bitterness, and off-flavor; and chemical measurements: egg yolk total moisture, egg yolk lipid, egg yolk phospholipids, egg yolk fatty acids, and egg yolk cholesterol). Significant differences were found in the daily feed intake, egg yolk viscosity, sensory yolk color and savory yolk flavor, and in the amount and transfer efficiency of the vitamin E supplements to the egg yolks. Based on both the transfer efficiencies at maximum value and data over time for alpha-tocopherol, gamma-T3, and delta-T3, it is evident that alpha-tocopherol is much better transferred to hen egg yolks and does play a role in how the other forms of vitamin E are absorbed.

Minimal changes occurred in the organ composition (moisture, lipid, fatty acid, and cholesterol) due to supplementation of laying-hen feed with alpha-tocopherol and annatto T3s (gamma and delta T3). Overall, the main organs that accumulated alpha-tocopherol, gamma-T3, and delta-T3 were the fat pad, liver and gall bladder, oviduct, forming yolks, laid yolks, kidney, brain, thigh, and breast. Same as seen in the previous study (Hansen et al., 2014), much more of alpha-tocopherol is transferred into the hen's body from the feed than the annatto T3s. Alpha-tocopherol only significantly decreased the transfer of gamma-T3 to the laid and forming eggs, but this trend was not seen in delta-T3. Cholesterol content was not significantly impacted in most tissues (breast, thigh, liver and

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gall bladder, kidney, and oviduct) except the heart. When determining how a nutrient is distributed in a biological system, such as a laying-hen, it is important to consider what the function(s) that the nutrient has for each unique organ or tissue.

This study illustrated that by changing nutrient composition of laying-hen feed it is possible to produce a more nutritious egg that and alter to the distribution of alpha-tocopherol, gamma-T3, and delta-T3in various organs and tissues. Increasing these nutrients in a biological system may have benefits to the health of the laying-hen and stability of the meat products, although minimal impacts to cholesterol content in the eggs and various organs were seen. With much of the annatto T3s being excreted a more efficient delivery system is needed to overcome the bio discrimination for alpha-tocopherol. Supplementing chicken feed with vitamin E may have future applications in the broiler industry with increased feed intake that could lead to faster weight gain and increased oxidative stability with more vitamin E present in the laying-hen.

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Appendix

Table 1. Yolk Moisture and Oil Contents for Diets with Different Levels of Annatto (T3) and Alpha-Tocopherol (TC) Supplementation (n=4) [a]

Diets	Yolk Moisture (%)	Yolk Oil (%, db)
Control	48.30 ± 0.21	63.96 ± 1.25
T3 100	48.34 ± 1.01	64.34 ± 1.23
T3 500	48.50 ± 0.27	63.88 ± 1.48
T3 2000	48.42 ± 0.62	64.30 ± 0.43
TC 200	48.76 ± 0.23	63.68 ± 0.87
TC 600	48.50 ± 0.15	64.00 ± 0.44
TC 1000	48.84 ± 0.34	63.43 ± 0.53
Significance [c]	NS	NS

[a] Values are means ± standard deviations. [b] Diets in Table 2 above, T3=Annatto, TC=alpha-tocopherol. Different letters in the same column indicate significant differences at the 95% confidence level. [C] p-value= 0.05, NS not significant at 5%.

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Table 2. Phospholipid Composition of Egg Yolk Oil Determined by ^{31}P NMR for diets with Different Levels of Annatto (T3) and Alpha-Tocopherol (TC) Supplementation (n=4) [a]

Diets	PC (Wt. %)	PI (Wt. %)	LPC (Wt. %)	SM (Wt. %)	PE (Wt. %)	LPE (Wt. %)	Total PLs (Wt. %)
Control	75.40 ± 0.90	0.82 ± 0.56	1.36 ± 0.31	2.17 ± 0.33	19.45 ± 0.51	0.80 ± 0.10	24.94 ± 5.58
T3 100	75.41 ± 0.71	1.42 ± 0.44	1.35 ± 0.24	2.12 ± 0.24	19.00 ± 0.91	0.70 ± 0.22	23.71 ± 4.80
T3 500	75.54 ± 0.66	1.04 ± 0.21	1.30 ± 0.065	2.31 ± 0.35	19.05 ± 0.53	0.75 ± 0.050	23.20 ± 6.33
T3 2000	74.93 ± 1.20	1.00 ± 0.076	1.22 ± 0.29	1.90 ± 0.39	20.25 ± 1.03	0.70 ± 0.23	24.03 ± 4.94
TC 200	75.46 ± 1.08	1.17 ± 0.59	1.35 ± 0.12	2.03 ± 0.38	19.19 ± 0.50	0.79 ± 0.19	21.11 ± 6.02
TC 600	74.79 ± 1.77	1.48 ± 0.58	1.43 ± 0.10	2.47 ± 0.29	19.17 ± 1.5	0.66 ± 0.15	22.31 ± 3.78
TC 1000	74.74 ± 1.48	1.41 ± 0.61	1.44 ± 0.18	1.90 ± 0.37	20.18 ± 1.12	0.59 ± 0.093	22.19 ± 3.39
Significance	NS	NS	NS	NS	NS	NS	NS

[a] Values are means ± standard deviations. [b] Diets in Table 2 above, T3=Annatto, TC=alpha-tocopherol. Different letters in the same column indicate significant differences at the 95% confidence level. [C] * p-value= 0.05, NS not significant at 5%.

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Table 3. Fatty Acid Composition for Egg Yolks from Diets with Different Levels of Annatto (T3) and Alpha-Tocopherol (TC) Supplementation (n=4) [a]

Diets [b]	16:0 (%)	16:1 (%)	18:0 (%)	18:1 (%)	18:2 (%)	20:4 (%)	22:6 (%)
Control	26.26 ± 0.72	2.57 ± 0.09	9.47 ± 0.22	40.92 ± 4.15	16.46 ± 0.20	2.09 ± 0.08	0.53 ± 0.01
T3 100	25.03 ± 0.81	2.47 ± 0.11	9.29 ± 0.78	39.59 ± 1.25	17.05 ± 0.95	1.88 ± 0.38	0.48 ± 0.10
T3 500	24.67 ± 0.28	2.15 ± 0.23	9.78 ± 0.64	40.15 ± 0.79	16.90 ± 0.43	1.99 ± 0.24	0.48 ± 0.19
T3 2000	25.43 ± 0.40	2.29 ± 0.12	10.05 ± 0.22	39.53 ± 0.95	16.14 ± 0.48	2.10 ± 0.08	0.57 ± 0.04
TC 200	25.06 ± 1.36	2.35 ± 0.37	9.51 ± 0.11	40.01 ± 1.11	16.48 ± 0.66	2.10 ± 0.11	0.57 ± 0.03
TC 600	24.37 ± 0.92	2.04 ± 0.10	9.97 ± 0.74	40.23 ± 1.18	16.60 ± 0.59	2.19 ± 0.11	0.60 ± 0.04
TC 1000	24.77 ± 0.66	2.19 ± 0.19	9.92 ± 0.30	40.44 ± 0.41	15.91 ± 032	2.17 ± 0.19	0.58 ± 0.06
Significance [c]	NS	NS	NS	NS	NS	NS	NS

[a] Values are means ± standard deviations. [b] Diets in Table 2 above, T3=Annatto, TC=alpha-tocopherol. Different letters in the same column indicate significant differences at the 99% confidence level. [C] *p-value= 0.01, NS not significant at 1%.

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