Enriching Eggs of Laying Hens with Multiple Bioactive Nutrients for Improving Human Health

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The USA faces an epidemic of obesity and other non-communicable disorders. Major causes of death including cardiovascular disease (CVD) and diabetes are correlated with poor nutrition and dietary habits, which can be modulated through improving the nutritional quality of largely consumed foods such as eggs. Animal products, particularly eggs, have a lot of potential for improvement in terms of nutritional composition. Previous studies by our group have demonstrated that laying hens are capable of enriching into their eggs health promoting compounds including a potent antioxidant astaxanthin (AST), the long chain omega-3 fatty acid docosahexaenoic acid (DHA), and the pre-activated form of vitamin D3 calcifediol. When these compounds are transported into yolks, their molecular forms are changed to be incorporated into phospholipids in the case of DHA, bound to proteins, or hydroxylated such as calcitriol. In order to evaluate how the metabolism of the laying hens changes the bioavailability and potency of these three compounds, murine models were used to simulate dietary consumption of these eggs by humans. Overall, the objectives of this thesis research were: to determine if it's possible to produce eggs which contain AST, DHA, and calcifediol at concentrations which can meet a person's daily requirement, understand how these compounds influence the metabolism and endogenous pathways of laying hens to further manipulate them, produce a practical combination of these compounds for producers, and evaluate the health promoting effects of these eggs for human consumption.

The first series of experiments were to produce eggs enriched with the aforementioned health promoting agents which involved three laying hen trials: 1) Enrich the eggs and tissues of hens with AST from the microalgae *Haematococcus pluvialis* (Heliae Development, Arizona), 2) Enrich eggs and tissues with microalgal DHA from *Aurantiochytrium* (Heliae Development, Arizona), and 3) Enrich eggs and tissues with calcifediol from the vitamin D supplement Rovimix (DSM Industries, Netherlands) alongside both DHA and AST from the previous two studies. All three studies had similar experimental designs with 10 hens per treatment groups and incremental or combined dietary doses of the testing ingredients for 6 weeks after which hens were euthanized and blood and tissues were collected. Major findings included an accumulation of AST in the eggs of tissues which improved overall oxidative status of hens and eggs, an enrichment of DHA in eggs and tissues but with a sharp decrease in enrichment efficiency past 2% of the microalgae and a suppression of overall lipid metabolism. Calcifediol was capable of being enriched at levels up to 9 ug/100 g of yolk when included in the diets of hens at 200 ug/kg of diet. When added in combination, DHA, calcifediol, and AST did not impair each other's enrichment.

The second phase of experiments involved feeding the enriched egg yolks from the first three layer studies to mice to evaluate if these yolks could improve their health. Two feeding trials were conducted with the first involving either DHA or AST enriched egg yolks, and the second involving DHA, AST, and calcifediol enriched egg yolks. Mice were fed yeast-sucrose mash diets which were supplemented with freeze-dried egg yolk products enriched with DHA, AST, and(or) calcifediol. Studies were either 6 or 8 weeks long. At the end, mice were euthanized and tissues collected. Parameters of lipid metabolism and general health were measured in various tissues including the liver, adipose, gastrocnemius muscle, and cardiac

muscle. Major findings included a very sharp decrease in heart triglyceride content by yolk enriched with AST. The yolk enriched with DHA down-regulated lipid anabolism and storage in the tissues at both the biochemical and molecular levels.

BIOGRAPHICAL SKETCH

Andrew Magnuson attended high school at the Bronx High School of Science in NYC, NY. Upon graduating, he began an undergraduate study in the field of Animal Science at Cornell. During his Junior year he started conducting poultry related research in the lab of Dr. Xingen Lei and graduate with a distinction in research. Afterwards, he went to the University of Arkansas for a masters in Poultry Science with Dr. Craig Coon. Upon completion he returned to Dr. Lei's lab at Cornell to pursue a PhD related to further poultry and layer research. Afterwards he plans to join Dr. Picklo's lab at the USDA research facility in Grand Forks North Dakota to study the lipidomic profile of milk from cows fed different feed rations.

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LIST OF ABBREVIATIONS

5-LOX	Arachidonate 5-Lipoxygenase
ACC	Acetyl CoA Carboxylase
ACOT4	Acyl-CoA Thioesterase 4
ADFI	Average Daily Feed Intake
AKP	Alkaline Phosphatase
ALA	Alpha-Linolenic Acid
ALT	Alanine Transaminase
AST	Astaxanthin
BW	Body Weight
CHPT1	Choline Phosphotransferase 1
COX1	Cyclooxygenase 1
CPT1A	Carnitine Palmitotransferase 1A
CVD	Cardiovascular Disease
CYP2R1	Cytochrome P450 2 Subfamily R Member 1
CYP24A1	Cytochrome P450 24 Subfamily A Member 1
DEGS1	Delta-4 Desaturase
DGAT1	Diacylglycerol O-Acyltransferase 1
DGAT2	Diacylglycerol O-Acyltransferase 2
DHA	Docosahexaenoic Acid
ELOVL2	Fatty Acid Elongase 2
ELOVL5	Fatty Acid Elongase 5
EPA	Eicosapentaenoic Acid
EPT1	Ethanolaminephosphotransferase 1
FADS2	Delta-6 Desaturase
FASN	Fatty Acid Synthase
GPX	Glutathione Peroxidase

GR	Glutathione Reductase
GSH	Glutathione
GSSG	Glutathione Disulfide
GST	Glutathione Transferase
LPL	Lipoprotein Lipase
ME	Metabolizable Energy
MDA	Malondialdehyde
MUFA	Monounsaturated Fatty Acid
NEFA	Non-Esterified Fatty Acid
n3	Omega-3 Fatty Acid
n6	Omega-6 Fatty Acid
ORAC	Oxygen Radical Absorbance Capacity
PL	Phospholipid
PLPPP1	Phospholipid Phosphatase 1
PPARA	Peroxisome Proliferator Activator Receptor Alpha
PUFA	Polyunsaturated Fatty Acid
SCD1	Stearoyl-CoA Desaturase 1
SFAS	Saturated Fatty Acid
SOD	Superoxide Dismutase
SREBP1c	Sterol Regulatory Element Binding Protein 1c
TBARS	Thiobarbituric Acid Reactive Substances
TC	Total Cholesterol
TG	Triglycerides
TRAP	Tartrate Resistance Acid Phosphatase
VDR	Vitamin D Receptor

Chapter One
Introduction

1.1 Laying Hen Industry in the USA

Beginning in the early 1890s, the most highly consumed poultry product in the U.S. has been eggs, higher than that of chicken and turkey meat [1]. While households in those times may have been able to manage their own hens and supply themselves with eggs, in the modern era a majority of egg production happens within large scale industrial farms. Production of eggs, relative to other animal products, requires much less energy inputs and is more sustainable from an environmental standpoint requiring as little as 4.8 kg of CO2 emission per kg of eggs (USDA, 2020). Eggs require much less processing and intensive storage relative to meat as they only need to be washed and can last several months at room temperature before spoilage [2]. Laying hen production has been advanced over the last century due to various discoveries which optimize their performance including maximization of hours of light, benefits of adding dietary high levels of vitamin D, improved housing conditions, intensive selection for better genetics, precise nutrition, and automated egg collection. Within the U.S. alone, approximately 113,253 million eggs were produced from 400 million hens 2020, with an average consumption of 267.4 eggs per capita. While not every egg is eaten directly by consumers, a vast majority are incorporated into other industries and processed foods. Eggs themselves are hailed as a “super food” due to the nutrient dense composition of the eggs’ edible components: the yolk and albumen. Egg yolk serves as a source of fatty acids and cholesterol, and albumen as protein, both necessary for proper human nutrition and health. Modern focus of improving the laying hen industry has shifted from

producing more eggs to rather increasing their nutritional value and reducing the carbon footprint for producing them [3].

Animal product consumption is highest in the United States with over 57 billion pounds of poultry meat and 9.34 billion eggs produced annually, with less than 10% of it being exported (USDA) [3]. Non-communicable diseases account for roughly 70% of all deaths globally and over 88% of deaths in the United States which can be prevented in part by proper nutrition [4]. The United States is attributed to the western "sweet meat" diet that consists of an abundance of animal products and an inadequate intake of fruits and vegetables [5]. While animal products such as meat, dairy, and eggs are an excellent source of nutrients including protein, calcium, iron, vitamin B-12, and are calorie dense, they are associated with these non-communicable diseases which leads to a desire to improve their nutrient composition.

1.2 Fortifying Eggs to Tackle Non-Communicable Diseases

High caloric intake by itself is not unhealthy, however, this stigma stems from the incidence of obesity within the United States and the improper balance of omega-6 (n6) to omega-3 (n3) fatty acids, lack of phytochemicals from fruits and vegetables, and micronutrient deficiencies associated with the western diet [6]. The major crops grown within the U.S., corn and soybean, contain high concentrations of these n6 fatty acids which are readily absorbed and stored within animals when eaten, which then pass on to humans. Metabolically n6 fatty acids are essential for growth, immune function, and inflammatory response when converted to lipo-hormones known as eicosanoids [7]. Docosahexaenoic acid is a long chain n3 which can also be utilized to synthesis eicosanoids and is involved in other metabolic processes such as controlling

overall fat storage and deposition, however n3 fatty acids are scarce in the western diet unless actively sought after by consumers. The balance of these long chain n6 and n3 fatty acids dictates what type of metabolic effects eicosanoids will have; when synthesized from n6 fatty acids they have pro-inflammatory and vasoconstrictive effects, conversely from n3 they are anti-inflammatory and vasodilators [8]. Fruit and vegetables are rich sources of fiber and phytochemicals which can improve digestion and aid in reducing inflammation, something which is lacking in terrestrial animal products [9]. Carotenoids, a sub-category of phytochemicals, are responsible for pigment of fruits and vegetables which arguably have anti-carcinogenic and anti-hypotensive capacity through improving antioxidant function in animals [10]. Astaxanthin is a particularly potent carotenoid synthesized in marine plants well known for its health benefits derived from antioxidant function [11]. Similar to DHA and AST, a micro-nutrient that is difficult to obtain is Vitamin D which is not naturally present in most foods without fortification. Known as the “sunshine vitamin” vitamin D is essential for proper metabolic functions spanning from the cardiovascular system to bone and brain health, however most of the population is insufficient or deficient in this nutrient [12]. Calcifediol is a pre-activated form of Vitamin D3 which has a higher activity making it desirable for food enrichment.

As the average American consumes approximately one half to two thirds of an egg per day a realistic goal would be to enrich said eggs with a combination of these health promoting compounds. By incorporating AST, DHA, and calcifediol at concentrations which would meet the daily requirement of a person in 1-2 eggs it is possible to increase the value of eggs for commercial producers and farmers and to increase the health benefit of eating eggs for consumers.

1.3 Hen Lipid Metabolism

Laying hens have the unique metabolic demand of producing one egg per day for many months. This is a unique and demanding challenge for hens as they must mobilize protein from their pectoralis muscle for albumen, fat from their fat pads for egg yolk, and calcium from their medullary bone in the tibia for shell [13]. Simultaneously, all these nutrients may be directly deposited from what is circulating in the blood which was absorbed from the GI tract. While the GI tract of layers and birds is slightly different than mammalian species, with the crop and gizzard adding an extra degree of food storage and mechanical processing prior to absorption, lipid breakdown and transport is conserved. Once lipids (and lipophilic compounds such as calcifediol and AST) are emulsified in bile salts in the jejunum they are subsequently absorbed through the enterocytes and brought to the liver via chylomicrons for further processing. Vitamin D requires its own transporter protein, vitamin D binding protein, but it general this will accumulate where other lipophilic compounds go such as the liver and developing yolks in hens. Once these compounds reach the liver they will then be subjected to processing by either being broken down into constitutive free fatty acids, modified by being bound to specific proteins such as vitamin D, remain in the liver, or exported with high and or low density lipoproteins such as AST. Unlike most fatty acids, DHA is primarily stored in the phospholipid form which is quite recalcitrant against being broken down into free fatty acids and thus will mostly stay in this form unless oxidized [14].

While both DHA and AST have potent anti-lipogenic effects they are quite readily transported and stored in various tissues. The major challenge for AST deposition is crossing the GI barrier, but once inside the body it accumulates quite readily within muscle, adipose, egg yolk, and hepatic tissue [15]. Ovarian tissue is the only barrier between the blood and developing

follicles which become egg yolks once fully formed. While much is known regarding the processes behind how ovarian tissue forms egg yolks with enhanced machinery for developing follicles and modified organelles such as the enlarged smooth ER, it has yet to be studied how dietary inclusion of AST, DHA, and or calcifediol may modulate yolk formation. While there is hormonal signaling regarding overall energy status and health for hens to continue to produce eggs, there doesn't seem to be any mechanism for controlling specifically what enters egg yolks aside from what is limiting (inadequate total lipid for follicle development) allowing the health promoting compounds of interest, AST, DHA, and calcifediol to freely enter yolks once in taken.

1.4 Research Aims

Evaluate the potential to enrich novel bioactive compounds and health promoting agents into the eggs of layers. Considerations include adverse effects of dietary inclusion, tolerable ranges the animal can intake, and maximizing enrichment efficiencies. The second series of studies involving murine models aim to evaluate the health promoting effects of intaking said enriched eggs and how they modulate various health parameters within the body.

Chapter Two
Literature Review

2.1 History of Egg Omega 3 Fatty Acid & Docosahexaenoic Acid Enrichments

Enhancing the nutritional value of eggs by improving their omega 3 fatty acid content has been accomplished many times before through various nutritional methods including ALA containing flaxseed and other terrestrial plants, and DHA mainly via marine fish and algal sources [16]. It is well known that EPA and DHA produce a much more profound health benefit for humans thus making them more popular to enrich into eggs [17]. Increasing the long chain omega 3 content of eggs has been correlated with improved immune function, reduced inflammation, and an enhanced protection against CVD [18]. Increasing the DHA content of eggs is especially important for pregnant women and young children as this has been shown to improve brain and eye development [19]. With regards to most of the US population, long chain omega 3 fatty acids have been shown to help prevent many non-communicable chronic diseases including cancer, obesity, and diabetes, however more research is needed to substantiate this [20]. It is generally accepted across various government and health organizations that the recommended daily intake for omega 3s ranges from 1.4 to 2.5 g/day, with long chain EPA and DHA ranging between 250 to 500 mg/d [21]. Fish oil and strains of microalgae selected for high EPA and DHA production are the densest sources of said fatty acids which have been utilized for enrichment of eggs. One strain of DHA rich algae, *Aurantiochytrium*, also known as "DHA gold" has been demonstrated to enrich eggs with levels up to 100 mg DHA/egg, an amount which could provide the daily requirement in 4 to 5 eggs [22] [23]. Strikingly, fish oil provides a higher enrichment efficiency of 55% with more DHA being incorporated into the egg than the

algal *Arantiochytrium* at 45% [24]. While it seems that there is a limit to how much DHA can be stored directly by fish oil and algal dietary supplementations perhaps this could be overcome with additional dietary supplements related to DHA metabolism [25].

2.2 Efficacy of Enriching Phytochemicals including Astaxanthin

While eggs naturally contain phytochemicals and particularly carotenoids which give them their yellow color such as lutein, beta carotene, zeaxanthin, and crypotoxanthin which stem from the diet of the laying hens, there is potential to further enrich these compounds to increase the health benefit of consuming these animal products [26]. Like omega 3 fatty acids, phytochemicals are known for their health promoting effects including reducing the risk of CVD albeit through a different mechanism of reducing oxidative stress [27]. Several phytochemicals have been of interest for enrichment into animal products including catechin, lycopene, curcumin, and resveratrol, however, the algal AST is especially potent and has demonstrated a more profound antioxidant effect than the former [28] [29]. Astaxanthin itself is a dark red pigment and the major carotenoid from algae. As carotenoids can serve as a precursor for vitamin E synthesis the enrichment of these compounds including d, l-α-tocopheryl acetate has been studied in layers before with enrichments of up to 132 ug/g egg having been reached [30]. A designer strain of algae grown to produce AST, *Haematococcus pluvialis* has been utilized to enrich said compound with concentrations of up to 2 mg/egg being achieved [31]. As AST accumulated within eggs and in the tissue of laying hens there was an increased overall antioxidative status and improved response to oxidative stress. While AST by itself may improve the nutritional value of consuming eggs it has yet to be studied how this compound may work in conjunction with compounds susceptible to oxidation such as DHA.

2.3 Potential to Increase Yolk Calcifediol Concentrations

Animal products historically have served as an excellent supplementary source of vitamin D3 including cholecalciferol, ergocalciferol, and their respective hydroxylated metabolites [32]. As humans have the capacity to synthesis vitamin D in the skin by utilizing ultraviolet radiation to modify 7-dehydrocholesterol this makes vitamin D a conditionally limiting nutrient which depends not only on nutrition but also exposure to sunlight [33]. Young children and elderly are especially at risk of vitamin D insufficiency or deficiency in the northern hemisphere regions, and particularly during the winter [34]. Similar to both DHA and AST, increased vitamin D intake can help reduce the risk for non-communicable disorders including diabetes, CVD and hypertension, and cancer [35]. The generally agreed intake for vitamin D is between 7.5 to 15 *u*g/day depending on one's age, average sunlight exposure, and health status [36]. While vitamin D is commonly found within the fat of various animal products it is difficult to obtain from any other food source outside of taking supplements [37]. Egg yolks naturally are an excellent source of vitamin D, however, the maximum potential to store this compound has yet to be discovered. Hens fed a standard industry diet will produce eggs which contain between 0.2-3.4 ug/100 g yolk and 0-0.71 ug/100 g yolk of vitamin D3 and calcifediol, respectively [38]. While humans are capable of performing the enzymatic reactions to convert vitamin D3 into calcifediol and ultimately calcitriol which occur in the liver and then kidney, it has been demonstrated that dietary intake of calcifediol directly is much more potent as it has roughly 5x the activity compared to the unhydroxylated D3 [39]. By directly adding 122 ug/kg of calcifediol to the diets of laying hens it is possible to enrich up to 4.3 ug/100 g yolk of calcifediol. Despite this it remains to be studied if this could be further increased due to the toxic dose of vitamin D3OH ranging between 150 to 400 ug/kg of diet for layers [40].

2.4 Biochemical Approaches to Enrich DHA, AST, and Calcifediol and Related Pathways

Carotenoids are the major class of phytochemicals responsible for pigment and color within plants, necessary for light absorption and hormone like functions. Characterized by being lipid soluble, having a low bioavailability, and metabolically inactive in animals these compounds are not considered nutrients nor essential for proper growth and thus have no requirement. Despite this, certain carotenoids serve as precursors for vitamin A or have high antioxidant capacity due to their molecular structure composing of many unsaturated double bounds such as AST. Astaxanthin is well known for its blood red pigmentation, function as a "super vitamin E" due to its extraordinarily high antioxidant capacity, and is considered essential for marine life such as salmon and shrimp which utilize AST for its color and ability to reduce inflammation [11,12]. Naturally AST is produced in marine plants and algae, of which the strain *Haematococcus pluvialis* produces the known highest concentration. Astaxanthin has gained recognition as a health promoting compound known to improve cardiovascular health and reduce inflammation but there is no recommended daily amount. This compound is commercially available dietary supplements, isolated from said algae, or present with marine foods. Our group has demonstrated that poultry including both broilers and laying hens are capable of absorbing and enriching this compound into their meat and eggs respectively. As a lipophilic compound AST was transported in the blood of these animals and stored in the fat within several tissues including liver, muscle, and adipose where it exerted antioxidant effects. One potential benefit of this improved antioxidant status is the protection of long chain fatty acids which are particularly sensitive to oxidation including the health promoting omega-3 fatty acids eicosapentaenoic acid (EPA) and DHA.

The American Heart Association recommends that the average adult receives a daily intake of 200 mg/day of EPA + DHA and 300 mg/day for pregnant woman. Unlike shorter chain fatty acids the long chain EPA and DHA are utilized for more tissue growth and creation rather than for energy and ATP synthesis [41, 42]. Particularly DHA is utilized in phospholipid synthesis in the membrane of cells, this is very pronounced in the brain, eyes, and heart muscle for tissue function. The n6 equivalent ARA is also utilized for phospholipid synthesis and the mechanism behind how DHA exerts it's health promoting effects may be due to competition with ARA for sites of incorporation in these phospholipids, as well as eicosanoid synthesis [43]. Terrestrial plants are unable to naturally produce DHA but can make the shorter chain n3 alpha-linoleic acid (ALA) which can be converted to EPA and DHA in animals via endogenous elongases and desaturases but with limited capacity. Marine plants, such as microalgae, can synthesis DHA de novo which is normally consumed and incorporated into other forms of marine life. In particular one commercial strain of microalgae, *Aurantiochytrium*, is capable of synthesis approximately this fatty acid as 16% of its total fatty acid composition, and capable of storing up to 60% of its total volume as lipids. As phospholipids are the major storage form for the long chain fatty acid DHA and additional dietary phospholipid components may further promote deposition of this fatty acid into the egg yolks of hens. Structurally phospholipids consist of a diacylglyceride bound to a phosphate which has a head group including serine, ethanolamine, inositol, and choline. One trait these compounds share is that they can be interconverted through the Kennedy & Phosphatidylserine synthesis pathways, remodeled on phospholipid membranes by the Lands cycle, or ultimately converted into trimethylamine N-oxide by gut microbiota. Once a diacylglyceride is formed and committed to being incorporated into a phospholipid it can then be bound to either cytidine diphosphate choline or cytidine

diphosphate ethanolamine by choline phosphotransferase 1 (*CHPT1*) or ethanolamine phosphotransferase 1 (*EPT1*) respectively [44]. Previous work involving rodents has demonstrated that increasing dietary choline and ethanolamine enhance hepatic phospholipid concentrations however this has yet to be studied in poultry [45] [46] [47]. High levels of dietary choline have also been implicated to increase the synthesis of trimethylamine N-oxide, a metabolite correlated with CVD and hypertension [48]. While this may not have significance in production poultry, people consuming eggs containing greater phosphatidylcholine or lecithin may potentially be at risk for high levels of trimethylamine N-oxide and subsequent CVD. Our group has also demonstrated the ability of both broilers and laying hens to absorb and store this fatty acid in their meat and eggs, the latter capable of meeting the AHS daily requirement in a single egg. While meeting the AHS requirement of DHA can be difficult without actively seeking this nutrient. Another micronutrient which is scarcely found in foods is Vitamin D.

Vitamin D3 and its prehormone hydroxylated form, calcifediol, are naturally only found within animal fat including meat and egg yolks. A desire to fortify this nutrient has led to its incorporation into orange juice, grains, and milk through mixture of an isolate made microbially, a practice which had started back in the 1940s [49]. Vitamin D3 can be made by endogenously via UVB radiation dependent synthesis in cells under the epidermis of the skin and converted to calcifediol and calcitriol in the liver and kidney, respectively[50]. A problem with this endogenous synthesis is that there are regions of the world which do not have persistent sunlight to promote adequate synthesis of this compound, particularly the north hemisphere including the United States. Deficiencies of Vitamin D3 are associated with bone depletion, CVD, cancer, diabetes, poor cognition, and multiple sclerosis making this a very essential nutrient. The WHO recommends an approximate intake of 600 IU of vitamin D3 per day, the equivalent of 15 *u*g

daily [51]. An average egg contains roughly 1 *u*g of vitamin D3 which is in part due to feeding the laying hens a high concentration of this nutrient in their diets to enhance calcium absorption for egg shell synthesis, but nonetheless the proves beneficial by being incorporated into their products [38]. Based upon this concentration it would take 15 eggs to meet the daily requirement which is not realistic. Calcifediol when absorbed directly has approximately 3-5 times the activity of the non-hydroxylated vitamin D3 making it a more desirable form to enrich. Laying hens are capable of enriching their eggs with both vitamin D3 and calcifediol with up to 6.8 and 4.3 ug respectively when these compounds are incorporated into their diets. Chicken meat has not been extensively studied but it has been reported that normal chicken thighs contain 0.25 *u*g/100 g of meat. Ideally eggs and meat could be enriched with higher levels of calcifediol to make this nutrient more accessible for the average consumer including those who are lactose intolerant and do not wish to actively intake it via pills.

Animal products can potentially serve as a vehicle to deliver scarce marine and desired micro-nutrients to consumers. Poultry eggs are the most affordable and globally consumed animal product, including the United States, making them ideal for this role. Astaxanthin, DHA, and Calcifediol are all lipophilic compounds which are readily absorbed and stored within the eggs and meat of laying hens and broilers but the combination of enriching of all three of these has not been studied.

Chapter Three

Supplemental Microalgal Astaxanthin Produced Coordinated Changes in Intrinsic Antioxidant Systems of Layer Hens Exposed to Heat Stress[1]

3.1 Summary

Microalgal biomass has been shown to serve as an excellent substitute for conventional ingredients corn and soybean in diets for both broiler chicks and laying hens, and is capable of enriching their meat and eggs with omega-3 fatty acids. Certain strains of microalgae contain high concentrations of phytochemicals including phenolics, flavonoids, and other bioactive compounds that benefit both chicken and human health. One carotenoid, in particular, AST, is abundant in many microalgal species and has strong anti-oxidant potency. It is an excellent electron donor and has the ability to protect cells from free radicals and subsequent oxidative stress under both in vitro and in vivo conditions. Supplementation of AST at 7 to 100 mg/kg of diets can be readily absorbed by chickens, including laying hens, and subsequently incorporated into their tissues. Another benefit of AST is to improve meat quality, by increasing shelf life through decreasing oxidative radicals and lipid peroxidation. However, it remained to be determined if this elevated antioxidant potential by supplemental microalgae could be translated into a protection against oxidation and lipid peroxidation induced by heat stress in hens.

3.2 Introduction

Over 8 billion chickens are slaughtered for meat and 99 billion eggs are produced within the United States alone every year (USDA) [3]. Microalgal biomass has been shown to serve as

[1]Adapted from authors publication: Magnuson AD, Sun T, Yin R, Liu G, Tolba S, Shinde S, Lei XG: **Supplemental microalgal astaxanthin produced coordinated changes in intrinsic**

antioxidant systems of layer hens exposed to heat stress. *Algal Research-Biomass Biofuels and Bioproducts* 2018, **33:**84-90.

an excellent substitute for conventional ingredients corn and soybean in diets for both broiler chicks and laying hens, and is capable of enriching their meat and eggs with omega-3 fatty acids [52] [53] [54]. Certain strains of microalgae contain high concentrations of phytochemicals including phenolics, flavonoids, and other bioactive compounds that benefit both chicken and human health [10]. One carotenoid, in particular, AST, is abundant in many microalgal species and has strong anti-oxidant potency [9]. It is an excellent electron donor and has the ability to protect cells from free radicals and subsequent oxidative stress under both in vitro and in vivo conditions [55] [56]. Supplementation of AST at 7 to 100 mg/kg of diets can be readily absorbed by chickens, including laying hens, and subsequently incorporated into their tissues [57] [58] [59] [60] [61]. Another benefit of AST is to improve meat quality, by increasing shelf life through decreasing oxidative radicals and lipid peroxidation [62].

However, it was unclear if AST could be digested by hens and improve their performance under heat stress conditions. The majority of poultry production in the U.S. occurs within southern States where temperature is high in the summer, resulting in heat stress in laying hens [63]. Oxidative stress is the key factor as to how heat stress impacts the health and performance of the birds [64] [65]. Dietary supplementation of anti-oxidants ameliorated the negative impacts of heat stress [66] [67] [68]. Likewise, dietary supplementation of microalgae rich in phytochemicals improved the antioxidant status of animals. However, it remained to be determined if this elevated antioxidant potential by supplemental microalgae could be translated into a protection against oxidation and lipid peroxidation induced by heat stress in hens [69] [70].

Therefore, this study was conducted to feed laying hens under heat stress with supplemental microalgal AST at 10, 20, 40, and 80 mg/kg of a corn-soybean meal diet for 6 wk.

Our objectives were to determine: 1) if the supplemental AST was bioavailable to laying hens to be deposited into their eggs and tissues and how this metabolism was affected by heat stress; 2) if the enriched AST improved the antioxidant status of the eggs and tissues; 3) if the supplemental AST affected the egg production performance, egg quality, and health status of the hens.

3.3 Materials and Methods

All protocols of this experiment were approved by the Cornell University Institutional Animal Care and Use Committee. Supplemental microalgae *Haematococcus pluvialis* were provided by Heliae Development, LLC (Gilbert, Arizona, USA). Proximate analyses and AST concentration of these ingredients are presented in **Table 3.1** A total of 50 shaver leghorn laying hens (19-week old, donated by Kreher Farmers, Clarence, NY, USA) were used. They were housed in individual cages in an environmentally controlled room maintained at 25°C, 55% relative humidity with 8:16 dark:light cycles and with free access to water and fed for 6 weeks. Starting at week 4 temperatures were gradually elevated over several days to a set point of 28°C to induce heat stress which was maintained until the end of the experiment.

Table 3.1 Composition (g/kg) of laying hen diets

Treatment	Control	Treatment 1	Treatment 2	Treatment 3	Treatment 4
Calculated AST (mg/kg)	0	10	20	40	80
Corn, grain	653	651	653	643	651
Soybean meal 48%	215	211	214	201	213
Defatted Microalgae	0.00	5.00	0.00	20.0	0.00
Full Fatted Microalgae	0.00	0.00	0.80	0.00	3.20
Corn oil	20.0	20.0	20.0	20.0	20.0
Dicalcium Phosphate	9.50	9.50	9.50	9.50	9.50
Limestone	78.9	78.9	78.9	78.9	78.9
Choline	2.60	2.60	2.60	2.60	2.60
DL-Methionine	1.60	1.60	1.60	1.60	1.60
Isoleucine	1.40	1.40	1.40	1.40	1.40
Tyrosine	4.20	4.20	4.20	4.20	4.20
Valine	1.20	1.20	1.20	1.20	1.20
Tryptophan	0.20	0.20	0.20	0.20	0.20
Sodium Chloride	4.00	4.00	4.00	4.00	4.00
Vit/Min mixture*	0.90	0.90	0.90	0.90	0.90
Cellite	7.80	8.80	7.90	11.7	8.20
Nutritive Value					
ME, MJ/kg	12.13	12.13	12.13	12.13	12.13
Crude protein, g/kg	150	150	150	150	150
AST,mg/kg	0.00	7.96	23.9	54.0	109
Methionine, g/kg	3.88	3.89	3.88	3.93	3.89
Methionine + Cysteine, g/kg	6.32	6.32	6.33	6.29	6.33
Lysine, g/kg	7.16	7.18	7.17	7.26	7.19
Phosphorus, g/kg	2.52	2.51	2.52	2.49	2.52
Calcium, g/kg	32.5	32.5	32.5	32.5	32.5

* Vitamin and mineral mixture provided the following nutrients per kilogram of diet: vitamin A, 11,000 IU; vitamin D, 5,000 IU; vitamin E, 75 IU; menadione bisulfite, 3 mg; riboflavin,8 mg; D-Ca pantothenate, 15 mg; niacin, 60 mg; vitamin B-12, 0.016 mg; biotin, 4 mg; folic acid, 2 mg; thiamine-HCl, 3mg; pyridoxine-HCl, 4 mg; $CuSO_4 \cdot 5H_2O$, 16 mg; KI, 1.25 mg; $MnSO_4 \cdot H_2O$, 120 mg; Na_2SeO_3, 0.3 mg; ZnO, 100 mg; $Na_2MoO_4 \cdot 2H_2O$, 0.001261 mg

Hens were divided into five treatment groups (n = 10/treatment) based on average egg production and body weight **(Table 3.3)**.The control group was fed a corn-soy basal diet without microalgae. Treatment groups 1 and 3 were fed the basal diet supplemented with 0.5% and 2.0% of defatted *Haematococcus pluvialis* to provide 10 and 40 mg of AST/kg of diets, respectively. Treatment groups 2 and 4 were fed the basal diet supplemented with 0.08% and 0.32% of full fatted *Haematococcus pluvialis* to provide 20 and 80 mg of AST/kg of diets, respectively. All experimental diets were formulated based on NRC requirements (1994) [71].

Body weights were measured weekly, feed intake biweekly, and egg production daily. Eggs from each treatment were collected at the beginning and at the ends of weeks 3 and 6 to be evaluated for egg component weights and biochemical parameters. Blood was collected from the wing veins at the three same time-points to prepare plasma samples that were stored at -20°C until analyses.

Plasma alkaline phosphatase (AKP) activity was determined using the method established by Bowers and McComb [72]. Plasma tartrate-resistant acid phosphatase (TRAP) activity was determined using the methods of Lau et al. [73]. Plasma inorganic phosphorus was analyzed using the method of Gomori [74]. Kits for total cholesterol (TC), triglyceride (TG) and non-esterified fatty acid (NEFA) were purchased from Wako Chemicals (Richmond, VA, USA). Kits for determining uric acid, urea nitrogen, and alanine aminotransferase (ALT) were obtained from Thermo Scientific, Inc. (Waltham, MA, USA). The kit for determining glucose concentration was purchased from Sigma Aldrich (St. Louis, Missouri, USA).

Astaxanthin in the diets, plasma, liver, and egg yolk were extracted using the method of Lopez et al [75] with modification. Concentrations of the extracted AST from the samples were

measured using the methods of Sowell et al [76], Breithaupt et al [77], and Rohrle et al [78] with modification. Briefly, extracted AST was eluted isocratically with methanol and acetonitrile (50:50) with 0.1% triethylamine (TEA) at a flow rate of 1 mL/min, carried on a Agilent Eclipse plus C18 reverse phase column (5 μm, 4.6 x 250 mm) using a HPLC system (Shimadzu, Japan) (LC-10AD vp pumps, an SIL-10Ai auto injector, and an SPD-10 AV vp UV-vis detector). Column temperature was set up at 30^{o}C. Mobile phase was sonicated at room temperature for 15 min before using. Chromatographic peaks were identified by comparison of the retention time of standard AST. To validate the results, sample extracts were spiked with standard AST to determine its appearance on the chromatogram in relation to the sample peak being identified. Astaxanthin and β-carotene standards were purchased from Sigma Aldrich (St. Louis, Missouri, USA).

Oxygen radical absorbance capacity of plasma, liver, and egg yolk were measured using an adapted method of Ou et al [79]. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as the standard. All results were expressed as μM of trolox equivalence.

Malondialdehyde (MDA) levels were determined by the method of Esterbauer et al [80], using 2-thiobarbituric acid assay and1,1,3,3-tetraethoxypropane as a standard. Glutathione peroxidase (GPX), glutathione transferase (GST), glutathione reductase (GR), and superoxide dismutase (SOD) activities were determined using previous methods of [81] [82] [83] [84]. Tissue and plasma GSH and GSSG concentrations were determined using the methods of Anderson [85].

Lipids were extracted from egg yolk, plasma, and liver according to Folch et al. [86]. Fatty acids were methylated with methanolic sulfuric acid (1%) as described by Christie [87]. Tritridecanoin was used as an internal standard, and each fatty acid was identified by its

retention time of the fatty acid methyl ester standard (Sigma-Aldrich Co., St. Loius MO). Methyl esters of fatty acids were analyzed using a gas chromatography (Agilent 6890N, Agilent Technologies, Santa Clara, CA) fitted with a flame-ionization detector. A fused-silica capillary column coated with CP-SIL 88 for fatty acid methyl esters (100 m × 0.25 mm i.d., 0.2 mm film thickness) was used (Varian Inc., Lake Forest, CA). Oven temperature was programmed to be held for 4 minutes at 140^{o}C, increased by 4^{o}C per minute to 220^{o}C, and then held for 5 minutes. Carrier gas was N_2 with a constant flow rate of 45 mL/minute. The injector temperature was 230^{o}C and the detector temperature was 280^{o}C.

Software R (version 3.3.1, R Foundation for Statistical Computing, Vienna, Austria) was used for the data analyses. Each hen or cage was the experimental unit. Main effects of the treatments were analyzed by one-way ANOVA and the mean comparisons were conducted with Duncan's multiple-ranged method. Data were presented as means ± SEM, and the significance level for differences was $P < 0.05$.

3.4 Results

Dietary supplementation of AST resulted in dose-dependent increases of total carotenoids and AST in plasma ($P < 0.05$), except for total carotenoid concentrations in the 20 and 40 mg/kg groups at week 6 and AST concentrations in the 40 and 80 mg/kg groups at week 6 (**Figure 3.1**, **Table 3.2**). Total carotenoid concentration in the liver of the hens fed 80 mg AST/kg diet was higher ($P < 0.05$) than that of the other groups. Dietary supplementation of AST resulted in a linear ($P < 0.05$) enrichment of the phytochemical in the liver. Concentrations of total carotenoids and AST in the egg yolk were positively correlated ($P < 0.05$) with dietary AST

inclusion, except for total carotenoids in the 40 mg AST/kg group at week 6 (**Figure 3.2, Table 3.2**).

Table 3.2 Effects of different concentrations of dietary microalgal astaxanthin on total carotenoids and astaxanthin concentrations of plasma, liver, and egg yolk of layer hens

Treatment AST (mg/kg)	Control 0	Treatment 1 10	Treatment 2 20	Treatment 3 40	Treatment 4 80	SEM
Total Carotenoids						
Plasma, ug/ml						
Week 3	0.090^{c}	1.05^{bc}	1.87^{b}	6.87^{a}	6.99^{a}	1.07
Week 6	0.154^{d}	2.12^{c}	1.90^{c}	4.38^{b}	6.10^{a}	0.59
Liver, mg/kg						
Week 6	5.91^{b}	51.8^{a}	48.0^{a}	55.4^{a}	74.8^{a}	15.9
Egg Yolk, mg/kg						
Week 3	17.1^{c}	15.5^{c}	28.3^{bc}	48.2^{b}	114^{a}	18.7
Week 6	13.8^{c}	11.7^{c}	35.9^{b}	22.4^{bc}	86.1^{a}	17.2
AST Concentration						
Plasma, ug/ml						
Week 3	0.00^{d}	0.242^{d}	0.630^{c}	1.10^{b}	2.11^{a}	0.21
Week 6	0.00^{c}	0.940^{b}	1.50^{b}	1.34^{b}	4.06^{a}	0.64
Liver, mg/kg						
Week 6	0.00^{c}	3.39^{b}	3.50^{b}	4.92^{a}	5.78^{a}	0.75
Egg Yolk, mg/kg						
Week 3	0.00^{c}	13.2^{b}	15.8^{b}	15.7^{b}	36.2^{a}	4.94
Week 6	0.00^{c}	18.4^{b}	17.7^{b}	30.1^{a}	31.1^{a}	4.40

*Data expressed as means (n=5 plasma, n=3 liver).

Data analyzed via one-way ANOVA.

$^{a, b, c, d}$ Means in the same row without a common letter differ ($P < 0.05$).

Figure 3.1 Regression analysis of dietary astaxanthin supplementation and its incorporation into plasma at weeks 3 (A) and 6 (B) and the liver at week 6 (C).

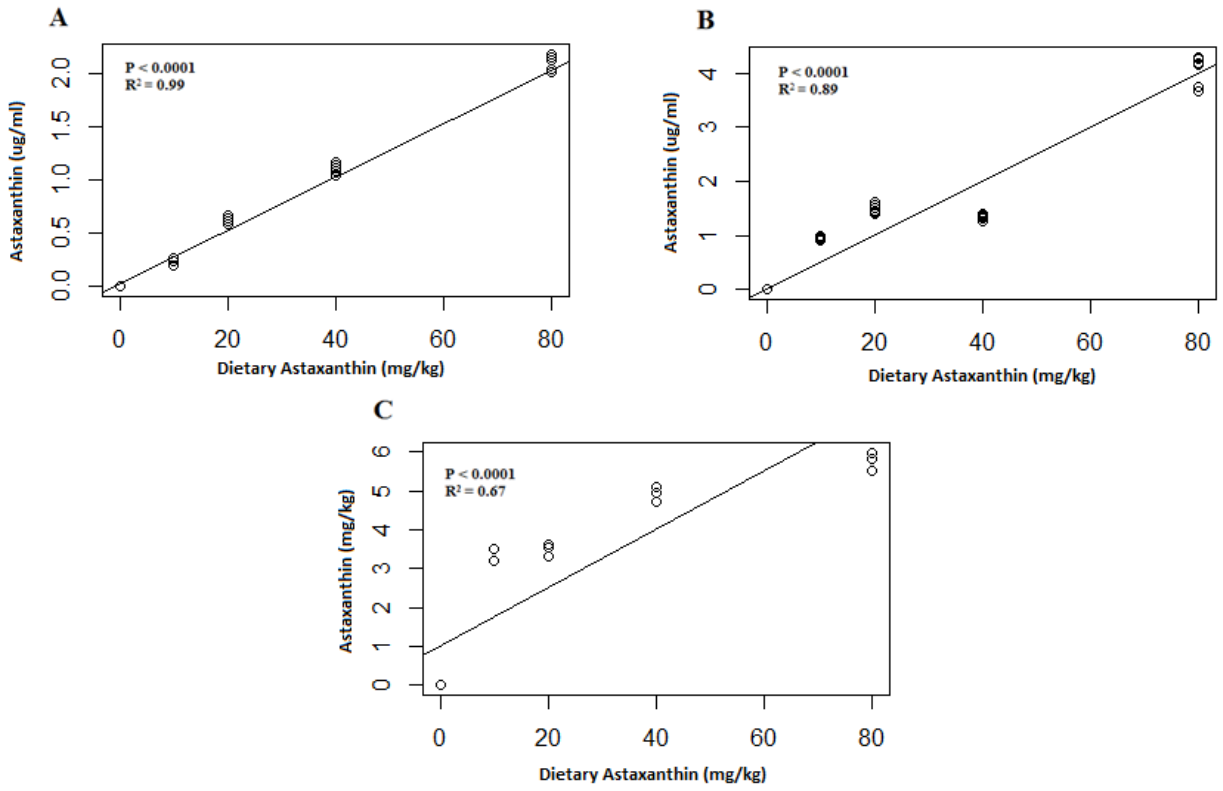
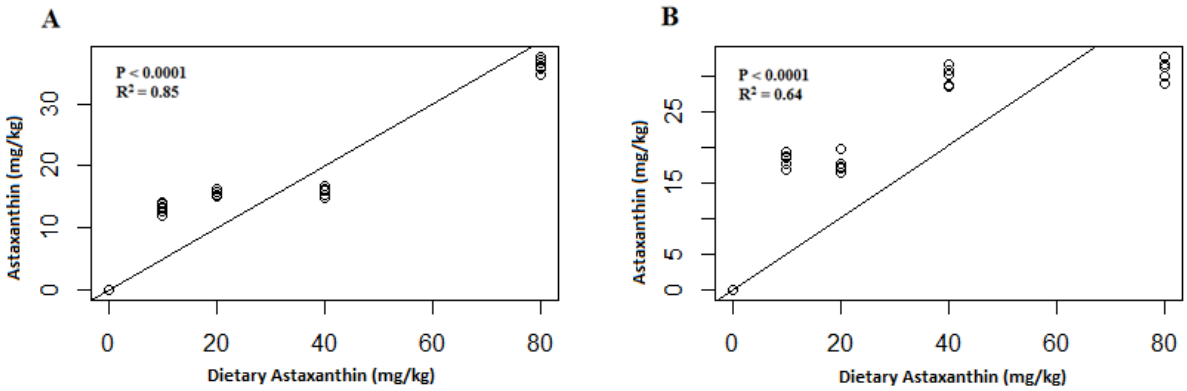


Figure 3.2 Regression analysis of dietary astaxanthin supplementation and its incorporation into the egg yolk at weeks 3 (A) and 6 (B).



Dietary supplementation of AST did not affect average daily feed intake or egg production, although there was a non-significant negative correlation with the final body weights of hens (**Table 3.3**). Similarly, dietary supplemental AST did not affect plasma activities of AKP and TRAP or plasma concentrations of inorganic phosphorus, glucose, total TG, NEFA, and total cholesterol. Plasma ALT activity was increased with the dietary AST inclusion ($P < 0.05$) at week 6, while such correlation was not statistically significant at week 3.

Table 3.3 Effects of different concentrations of dietary microalgal astaxanthin on performance and health parameters of layer hens

Treatment	Control	Treatment 1	Treatment 2	Treatment 3	Treatment	SEM
AST (mg/kg)	0	10	20	40	80	
Average Body Weight						
Initial, g	1460	1460	1470	1470	1470	78.7
Final, g	1470	1460	1380	1390	1360	103
ADFI[1], g/hen/day	107.6	102.3	94.40	103.3	99.6	13.4
Egg Production, %	98.6	95.3	94.8	98.8	95.0	10.6
Alanine aminotransferase[2], U/L						
Week 3	42.4	53.4	53.8	74.1	81.6	18.0
Week 6	59.9^{c}	82.4^{c}	99.1^{bc}	123^{ab}	126^{a}	23.8

*Data expressed as means over 6 weeks (n=10).

Data analyzed via one-way ANOVA.

[1]ADFI, average feed intake.

[2]ALT, activity defined as the rate of decreasing in absorbance at 340nm due to the oxidation of NADH.

Compared with the control, supplemental AST at 20 and 80 mg/kg decreased ($P < 0.05$) total liver glutathione concentrations (**Table 3.4**). Total GPX and GR activities in the liver were also decreased ($P < 0.05$) by supplemental AST at all levels except for the 80 mg/kg group on GPX activity. Total ORAC in both egg yolk and liver were positively correlated ($P < 0.05$) with dietary AST supplementation. However, supplemental AST did not affect liver GST and SOD activities or MDA concentrations in the liver and egg yolk.

Table 3.4 Effects of different concentrations of dietary microalgal astaxanthin on biomarkers of oxidative stress and antioxidant status of layer hens

Treatment	Control	Treatment 1	Treatment 2	Treatment 3	Treatment 4	SEM
AST (mg/kg)	0	10	20	40	80	
Oxygen radical absorbance capacity[1], U/mg tissue						
Egg Yolk						
Week 3	0.832^{c}	0.865^{b}	0.878^{b}	0.950^{a}	0.942^{a}	0.025
Week 6	0.878^{c}	0.916^{b}	0.923^{b}	0.971^{a}	0.976^{a}	0.029
Liver						
Week 6	0.458^{b}	0.589^{a}	0.575^{a}	0.601^{a}	0.629^{a}	0.039
Enzyme Assays, Liver Week 6						
GSH[2], uM/g tissue	0.91^{ab}	1.09^{a}	0.78^{ab}	0.99^{a}	0.56^{b}	0.19
GSSG[3], uM/g tissue	0.07	0.06	0.09	0.08	0.10	0.04
GPX[4]	30.2^{a}	12.1^{b}	15.6^{b}	15.6^{b}	20.4^{ab}	8.39
GST[5]	12.5	13.7	13.3	13.8	13.5	1.50
GR[6]	225^{a}	173^{b}	190^{b}	173^{b}	172^{b}	20.8
SOD[7]	1.64	1.19	0.85	1.39	0.44	0.93
Malondialdehyde[8], umol/g protein						
Egg Yolk						
Week 3	0.037	0.030	0.028	0.028	0.020	0.006
Week 6	0.031	0.029	0.037	0.044	0.024	0.008
Liver						
Week 6	0.179	0.318	0.159	0.219	0.073	0.062

*Data expressed as means (n=3 liver, n=6 eggs).

Data analyzed via one-way ANOVA.

$^{a, b, c}$ Means in the same row without a common letter differ ($P < 0.05$).

[1]ORAC, Umol of Trolox equivalent.

[2]GSH, reduced glutathione; [3]GSSG, oxidized glutathione.

[4]GPX, glutathione peroxidase. Activity defined as the amount of NADP+ formed when GPX is exposed to H_20_2 with GSH. Normalized by total protein content.

[5]GST, glutathione transferase. Activity defined as the amount of GS-DNB conjugate formed when GST is exposed to GSH and CDNB. Normalized by total protein content.

[6]GR, glutathione reductase. Activity defined as the amount of NADP+ formed when GR is exposed to GSSG. Normalized by protein content.

[7]SOD, superoxide dismutase (U/g protein). Activity defined as the rate of Xanthine reduction. Normalized by protein content.

[8]MDA, Activity defined as the amount of tetraethoxypropane (TEP) equivalent normalized with total protein content.

Supplemental AST had no consistent impact on egg total weights, albumen weights, shell weight, shell thickness or TC, TG, and NEFA concentrations in the egg yolk at week 3. Egg yolk weight was lower ($P < 0.05$) in the 20 and 80 mg AST/kg groups than that of the other groups at week 3, but there was no such negative effects at week 6 (**Table 3.5**). Shell thickness at week 6 was increased ($P < 0.05$) in the 40 mg AST/kg group compared with the other groups. Egg yolk color was changed ($P < 0.05$) by dietary supplementation of AST at both weeks 3 and 6.

Table 3.5 Effects of different concentrations of dietary microalgal astaxanthin on egg quality

Treatment	Control	Treatment 1	Treatment 2	Treatment 3	Treatment	SEM
AST (mg/kg)	0	10	20	40	80	
Yolk weight, g						
Week 3	13.0^{ab}	12.3^{b}	13.2^{ab}	14.2^{a}	12.4^{b}	1.17
Week 6	13.5	13.6	12.8	12.9	12.5	1.01
Shell Thickness, mm						
Week 3	0.39	0.39	0.38	0.38	0.38	0.03
Week 6	0.37^{b}	0.37^{b}	0.39^{a}	0.36^{b}	0.37^{b}	0.03
Fatty Acid Concentration, mg/egg						
Week 3						
SFA[1]	1230^{a}	1161^{a}	1151^{a}	1216^{a}	843.0^{b}	144.2
MUFA[2]	1236^{a}	1209^{a}	1197^{a}	1242^{a}	836.9^{b}	163.7
PUFA[3]	649.3^{ab}	691^{ab}	664.4^{ab}	762.7^{a}	494.5^{b}	130.9
n-3	0.00	0.00	0.00	0.00	0.00	N/A
Week 6						
SFA[1]	1024	1103	1008	932.0	898.0	153.2
MUFA[2]	1008^{ab}	1134^{a}	974.9^{ab}	938.7^{ab}	843.2^{b}	178.0
PUFA[3]	558.2	588.6	571.0	557.4	554.2	94.42
n-3	0.00	0.00	5.954	0.00	6.348	8.500
Yolk Color						
Week 3	6.00^{c}	11.1^{b}	15.0^{a}**	14.1^{a}	15.0^{a}**	1.57
Week 6	5.33^{d}	7.47^{c}	14.6^{a}	12.2^{b}	15.0^{a}**	1.13

*Data expressed as means (n=5).

**Yolk color outside the roche fan range.

Data analyzed via one-way ANOVA.

^{a,b,c,} Means in the same row without a common letter differ ($P < 0.05$).

$^{1, 2, 3}$SFA, saturated fatty acid; MUFA, mono-unsaturated fatty acids; PUFA, polyunsaturated fatty acids.

The 80 mg AST/kg group had lower ($P < 0.05$) concentrations of fatty acids including palmitic, palmitoleic, stearic, oleic, total saturated, total monounsaturated, and total polyunsaturated fatty acids at week 3, and lower stearic and total monounsaturated fatty acids at week 6 in the egg yolk relative to the control. This group also had lower ($P < 0.05$) concentrations of n-3 fatty acids in the liver than the 60 mg AST/kg group (**Table 3.6**). However, liver SFA, MUFA, PUFA, TC, TG, and NEFA concentrations were not affected by dietary AST inclusions **(Table 3.6).**

Table 3.6 Effects of different concentrations of dietary microalgal astaxanthin on liver triglyceride, cholesterol, and lipid profile of layer hens

Treatment AST (ppm)	Control 0	Treatment 1 10	Treatment 2 20	Treatment 3 40	Treatment 80	SEM
Fatty Acid, mg /g of tissue						
SFA[1]	11.1	12.1	11.0	13.5	10.1	2.87
MUFA[2]	9.43	9.98	9.29	11.6	9.51	3.37
PUFA[3]	6.00	6.94	6.92	7.61	5.79	1.42
n-3[4]	0.312[ab]	0.404[ab]	0.403[ab]	0.475[a]	0.215[b]	0.133
Total cholesterol, mg/g protein						
Week 6	0.012	0.010	0.009	0.012	0.010	0.001
Total triglycerides, mg/g protein						
Week 6	0.039	0.038	0.055	0.042	0.069	0.007
NEFA[1], μmol/g protein						
Week 6	10.6	18.2	6.21	9.71	8.19	4.82

*Data expressed as means (n=3).

Data analyzed via one-way ANOVA.

[a,b,] Means in the same row without a common letter differ ($P < 0.05$)

[1]SFA, saturated fatty acid; [2]MUFA, mono-unsaturated fatty acid; [3]PUFA, polyunsaturated fatty acid; [4]n-3, omega-3 fatty acid; [5]NEFA, non-esterified fatty acid.

3.5 Discussion

Our present study has demonstrated that supplemental microalgal AST was absorbed by the hens and deposited into their egg yolks, plasma, and liver in dose-dependent fashions [57] [58]. These dose-dependent enrichments of AST and total carotenoids were observed at week 3 and remained the same at week 6 aside from AST in the egg yolks which plateaued at 40 mg/kg at week 6. This pattern of AST enrichment was different from the previously described relationship between dietary and egg yolk AST by Walker et al. [61] in which an apogee was reached at day 7, followed by a subsequent decrease over time. One possible reason for this discrepancy was the relatively higher doses of supplemental AST (66 to 396 mg/kg) that might

have caused a negative feedback mechanism within the hens to limit phytochemical absorption [57]. Both hen plasma and liver total carotenoid and AST concentrations were increased proportionately, with some exceptions, with the amount present in the diet, reaching levels up to 6.99 μg/mL in plasma and 5.78 mg/kg in the liver, which was indicative of the hens' ability to absorb, transport, and store the algal derived phytochemicals. Astaxanthin and other lipophilic phytochemicals are known to be absorbed in the same fashion as vitamins E where they are incorporated into chylomicrons in the small intestine and subsequently transported via the lymph system which ultimately joins the systemic circulation [88] [89]. Once in the blood the phytochemicals are brought to the different tissues and particularly the liver where they can be stored or shuttled out once more with other fatty acids bound to lipoproteins. Plasma total carotenoid content was linearly increased with the amount of AST in the diet. However the concentration of plasma AST was doubled within the same treatment groups from week 3 to 6, suggesting that there was an increased enrichment of AST over time. Astaxanthin was seemingly physiologically inert and might not be metabolized by animals unlike most other carotenoids, which may explain why its retention is different [90]. Hen liver total carotenoid and AST concentrations shared similar responses to the diet treatments and exhibited marginal enrichments beyond the 20 mg/kg group. This implies that there was a limitation in the amount of phytochemicals that could be stored within this tissue.

Egg yolk ORAC was enhanced proportionately to the enrichment of AST, along with a non-significant decrease in the concentration of MDA, an indicator of lipid peroxidation. It is believed that free radicals accumulate in the lipid-rich yolk over time, and shorten the product shelf life. Because AST may scavenge and remove free radicals, its enrichment should improve egg stability and reduce lipid oxidation [9] [91]. While dietary AST did not affect the hen

performance under heat stress conditions, there was an improved whole body anti-oxidant status within the laying hens, as shown by the changes of glutathione concentration and related enzymes within their blood and liver tissue. Total glutathione was decreased in the treatment groups fed the full fatted *Haemotococcus Pluvialis,* which is indicative of less cellular oxidative stress due to the homeostatic nature of glutathione being synthesized in response to the presence of free radicals [92]. Strikingly the enzyme activity of both glutathione peroxidase and superoxide dismutase were also reduced in the AST treatment groups. This suggests that the presence of AST was neutralizing free radicals, the direct or indirect substrates to be removed by these enzymes [93]. The decrease of SOD activity was consistent with the findings of another study which investigated the anti-oxidant properties of AST in melanocytes against UV damage [94]. Conversely there have been conflicting responses regarding the effect of AST on tissue GSH and GPX. Nevertheless, our study was the first effort to evaluate its effect under heat stress conditions [95] [96] [97] [98]. One group found that AST reduced the activity of GPX and GR of diabetic rats but increased their respective activities in healthy rats implying that the effect of AST on antioxidant enzymes depended on the physiological condition of the animal [99]. It remained unclear why AST exhibited GSH and redox enzyme attenuating effects. One possible explanation is that enriched AST, as one of the most powerful lipophilic antioxidant and probably mainly residing on the cellular membrane [100], could interrupt the free radical formation and lipid peroxidation chain reaction induced by extracellular oxidants, and then spares the need for the intrinsic or intracellular response via a feedback homeostatic mechanism[92].

Prior to heat stress (week 3), there was a negative correlation in the total amount of egg yolk weight and component fatty acids including SFA, MUFA, and PUFA with the amount of

dietary AST inclusion. After heat stress only MUFA was significantly decreased by the microalagal AST. A possible explanation for this change in fatty acid profile is that PUFAs are especially susceptible to oxidation which would have allowed AST to protect them during stress conditions causing the relative concentration of PUFAs within the yolk to increase compared with the control group. The lipophilic nature of AST enables it to be co-transported with other lipids, which allows it to directly interact and potentially protect fatty acids within the egg yolk from oxidation [91].

Similar to Walker et al., increased levels of dietary *Haemotococcus Pluvialis* positively correlated with an increased redness in yolk color as well as no negative impacts on laying hen performance, body weight, or egg component weights [13]. While phytochemicals are considered to be anti-oxidant, anti-carcinogenic, and anti-inflammatory, and have the ability to lower risks of chronic diseases including CVD, obesity, and diabetes [10] [101] [102], these compounds have low bio-availability to humans, with only a small percentage absorbed by the small intestine [103]. Enriching eggs with AST and using this bio-fortified source for human ingestion may improve the biological value of AST and may serve as a convenient protection of PUFAs present in the egg yolks. As n-3 PUFAs, particularly the long chain EPA and DHA, are enriched in the egg yolk by different dietary treatments, combining the protection by AST against oxidation of fatty acids may lead to a synergistic benefit to produce highly desirable eggs for human nutrition and health.

3.6 Conclusion

In short, AST from the microalgae *Haematococcus pluvialis* is biologically available to laying hens and was capable of being transported across the GI tract and deposited into various

tissues and egg yolk. Once uptaken into yolk, AST was able to exert anti-oxidant effects by reducing total lipid oxidation as demonstrated by reduced MDA formation, and enhance free radical removal shown by elevated ORAC. Despite changes in egg yolk color the nutritionally value of these eggs is dramatically higher due to AST being a potent antioxidant which may shine when protecting other compounds susceptible to oxidation such as the long chain omega 3 DHA.

Chapter Four

Maximizing Docosahexaenoic Acid Deposition into Egg Yolks & Manipulations of the Kennedy Pathway & Phosphatidylserine Synthesis

4.1 Summary

The present study was to determine if feeding laying hens a DHA-rich microalgal biomass would produce a dose-dependent enrichment of this fatty acid into their egg yolks and tissues, and if there were ways to improve this deposition through enhancing phospholipid synthesis with novel dietary additives. Two studies were conducted. In Experiment 1, a total of 40 White Leghorn Shavers (42-wk old) were divided into 4 groups (n = 10/group), caged in an environmentally-controlled room, and fed a corn-soybean meal basal diet supplemented with the microalgal biomass (*Aurantiochytrium)* (Heliae, Gibert, AZ) at 0, 1, 2, and 4% of the diet (0, 1.7, 3.4, and 6.8 g DHA/kg of diet) for 6 wk. In Experiment 2, a total of 50-White-Leghorn-Shavers (42-wk old) were individually-caged and divided into 5-groups (n=10/group). The 5-groups of hens were fed the following diets for 3-wk: Diet-1= a corn soybean-meal basal-diet, Diet-2=Diet-1 + 4%-microalgae (*Aurantiochytrium*, Heliae, Gibert, AZ, 1.81g-DHA/kg) + choline-chloride (26.3-g/kg diet, 60%-purity, DSM-Co., Basel, Switzerland), Diet-3=Diet-2 + 1.41% of L-serine (100%-purity, Ajinomoto-Co., Inc., Kawasaki, Japan), Diet-4=Diet-2 + 100-mg of ethanolamine/kg (99%-purity, Sigma-Aldirch-Co., St Loius, MO), and Diet-5=Diet-3 + 100-mg of ethanolamine/kg. Body weight, feed intake, and egg production and quality were recorded weekly. Blood and eggs were collected (n = 10) and liver (n = 6) were collected at wk 3 and (or) wk 6 for analyses. Data were analyzed by one-way ANOVA. The supplementation of microalgae resulted in dose-dependent enrichments of DHA ($P < 0.05$) in the plasma ($R^2 = 0.66$-0.85), liver

(R^2 = 0.80), and egg yolk (R^2 = 0.85-0.74) of hens at wk 3 and (or) wk 6. The maximal concentrations of DHA reached 0.83 mg/mL, 2.4 mg/kg, and 225 mg/kg, respectively, in these samples. The microalgae supplementation decreased ($P < 0.05$) linoleic and arachidonic acid concentrations and improved ratios of n-6 to n-3 fatty acids (from 20 to 1.7) in the liver and egg yolk at wk 3 and (or) wk 6. The microalgae supplementation showed no effect on feed intake, body weight, or egg production of hens, but the 4% microalgae decreased ($P < 0.05$) egg yolk and total egg weights, plasma concentrations of lipids, glucose, and uric acid, and elevated ($P < 0.05$) concentrations of lipids, compared with the control. Regarding the second study, compared with Diet-1, Diet-2 enhanced ($P < 0.05$) DHA concentrations in egg-yolk and liver by 213-mg/egg and 2.98-mg/g tissue, respectively, but decreased ($P < 0.05$) total phospholipid-concentrations of yolk and liver by 50%, and hepatic-mRNA levels of elongases-2/5 and desaturases-4/6 by-25-50%. Compared with Diet-2, Diet-5 enhanced ($P < 0.05$) DHA (by-20%) and phospholipid (by-40%) concentrations in the egg-yolk, and upregulated ($P < 0.05$) lipid-metabolism genes involved in the citicoline (CDP, up-to-3-fold) and CDP-ethanolamine (up-to-2.5-fold) pathways in the liver and ovary-tissue. In conclusion, supplemental dietary DHA from the *Aurantiochytrium* biomass was highly bioavailable for producing high DHA-enriched eggs and improving their n-6 to n-3 fatty acid ratio, and feeding hens a high DHA and choline diet enriched DHA in the egg-yolk and down-regulated lipogenesis-gene-expression in the tissues. Supplementing the diet with extra-serine and ethanolamine further-enhanced the DHA enrichment in the egg yolk and restored the high DHA-mediated changes in the gene-expression.

4.2 Introduction

Eggs are unique in that they are economical to produce, low carbon footprint as animal protein, a rich source of both protein and healthy lipids, are used in a wide range of cultures, and are incorporated into many food products. The fatty acid profile of eggs can be modulated through feeding laying hens diets which contain certain types of lipids and fatty acids. The staple feedstuffs chickens are fed within the United States are typically corn and soybean. While these ingredients are rich in many nutrients and contain an excellent amino acid profile for chicken growth and egg production they both contain a very high n6/n3 fatty acid ratio which ultimately creates meat and eggs with this unbalanced ratio [6]. Many non-communicable diseases such as CVD, cancer, and diabetes have been attributed to this unbalanced ratio of fatty acids which leads to a desire to produce omega-3 enriched animal products [4].

Long chain omega-3 fatty acids, EPA and DHA, are known to be more metabolically active and have much more profound health benefits than the shorter chain alpha-linolenic acid ALA [5] [7]. Part of the reason as to why the long chain n3 fatty acids have stronger health benefits is that they are utilized in creating eicosanoids, lipid derived hormones, which are known to have pro-inflammatory or anti-inflammatory effects depending on whether they are made from either n6 and n3 fatty acids respectively [8]. Fish oils are established as both a rich dietary source of DHA and EPA for humans and for fortifying animal products with these nutrients [4]. While fish oil and meat are both excellent sources of these long chain omega-3 fatty acids fish themselves do not synthesize DHA or EPA but instead acquire them nutritionally from marine plants including microalgae [104] [105] [31] [106] [107] [108] [109] [53] [110] [54].

While laying hens demonstrate a high efficacy to absorb, transport, and store fat soluble compounds in their egg yolks, such as phytochemicals including AST and omega-3 fatty acids, the mechanism behind how these compounds are stored is unknown. Regarding fatty acids, a family

of enzymes are responsible for elongating and desaturating fatty acids which are utilized in animal liver tissue have been shown to be highly regulated and expressed in laying hens [9]. Free fatty acids (FFA) and triglycerides may be absorbed from the small intestine via chylomicron formation which is facilitated through bile salts released by the liver. Once in the systemic circulation these lipids will be absorbed and processed in the liver to be utilized for energy, converted into triglycerides for energy storage, and or exported as FFA in a lipoprotein complex. Longer chain fatty acids including EPA, DHA, and arachidonic acid are primarily utilized for phospholipid synthesis, a structural form of the lipids used in cellular membrane creation which is the primary form these fatty acids are found within egg yolk and meat [43] [111] [112]. The liver may be the major organ for lipid conversion and processing but in laying hens there is an additional step prior lipid incorporation into an egg yolk which is regulated by ovarian tissue [113]. The effect of enriching eggs with DHA and EPA and how this changes ovary regulation of forming egg yolks has not been explored.

Thus in the present study we investigated the potential to maximize DHA supplementation into the egg yolks of laying hens through two approaches: 1) determining the dose of DHA-rich microalgae for optimizing DHA enrichment efficiency, and 2) evaluating the potential of additional phospholipid component enhancers for further upregulating DHA deposition. We also evaluated the expression of key genes related to fatty acid metabolism, conversion, and phospholipid synthesis in lipid regulating tissue liver, adipose, and ovaries. Our objectives were to 1) determine the optimal dietary dose of *Aurantionchytrium* for producing DHA enriched eggs 2) evaluate how laying hen tissue changes biochemically in response to DHA 3) gain further understanding of the mechanism behind how laying hen liver, adipose, and ovary tissue respond to DHA

4.3 Materials and Methods

Protocols were approved by the Institutional Animal Care and Use Committee of Cornell University (Ithaca, NY). Supplemental microalgae, *Aurantiochytrium*, were provided by Heliae Development, LLC (Gilbert, Arizona, USA).

Experiment 4.1 – Dose-dependent effect of algal DHA supplementation: Shaver-White laying hens (42-wk old, donated by Kreher Farmers, Clarence, NY, USA) were divided into 4 dietary treatments based on egg production and body weight (n=10). Hens were maintained on dietary treatments for 6 wk. Supplemental *Aurantiochytrium* was incorporated into a common corn-soy basal at 0, 1, 2, and 4% of the total diet to make an equivalent 0, 1708, 3415, and 6830 mg DHA/kg diet.

Experiment 4.2 – Potential of dietary phospholipid components in enhancing DHA deposition: A total of 50-White-Leghorn-Shavers (42-wk old) were individually-caged and divided into 5-groups (n=10/group). The 5-groups of hens were fed the following diets for 3-wk: Diet-1= a corn soybean-meal basal-diet, Diet-2=Diet-1 + 4%-microalgae (*Aurantiochytrium*, Heliae, Gibert, AZ, 1.81g-DHA/kg) + choline-chloride (26.3-g/kg diet, 60%-purity, DSM-Co., Basel, Switzerland), Diet-3=Diet-2 + 1.41% of L-serine (100%-purity, Ajinomoto-Co., Inc., Kawasaki, Japan), Diet-4=Diet-2 + 100-mg of ethanolamine/kg (99%-purity, Sigma-Aldirch-Co., St Loius, MO), and Diet-5=Diet-3 + 100-mg of ethanolamine/kg.

Hens were individually caged in 0.44 m high x 0.30 m wide x 0.45 m deep units inside an environmentally controlled room maintained at 25°C, 55% relative humidity. Hens had free access to feed and water and exposed to 8:16 dark:light cycles. Vitamins, minerals, and crystalline amino acids were added to meet laying hen nutrient requirements (NRC, 1994) and diets were formulated

to be isocaloric and isonitrogenous [71]. Proximate and mineral analyses were completed by Dairy One, Inc. (Ithaca, NY). Diet formulations for Experiment 4.1 are presented in **Table 4.1** and for Experiment 4.2 in **Table 4.2**, diet fatty acid profiles in **Table 4.3**, and nutrient compositions in **Table 4.4**. Body weights were measured weekly, feed intake biweekly, and egg production daily. Eggs from each treatment were collected at wk 3 and 6 to be evaluated for egg component weights and biochemical composition. Blood was drawn from wing veins at wk 0, 3, and 6 to prepare plasma samples which were subsequently stored at -20°C until analyses. At week 6, 5 representative birds per diet (selected based on average egg production and body weight) were euthanized by carbon dioxide asphyxiation. Subsamples of liver, breast muscle, adipose, and ovary were harvested and immediately frozen in liquid nitrogen and stored at -80^{o}C for gene expression analyses. Additionally, frozen liver, breast muscle, adipose, and ovary tissue samples stored at -20^{o}C to be used for compositional analyses.

Table 4.1 Ingredient composition of layer diets in Experiment 4.1

Diet algae, %	0	1	2	4
Ingredient, %				
Corn	63.2	62.9	62.7	62.1
Soybean Meal	23.9	23.7	23.5	23.2
DHA Algae	0.00	1.00	2.00	4.00
DHA Calculated (ppm)	0	1710	3420	6840
DHA Actual (ppm)	0	1450	2890	5780
Corn Oil	3.00	2.50	2.00	1.00
Dicalcium Phosphate	0.94	0.94	0.94	0.94
Limestone	7.89	7.89	7.89	7.89
NaCl	0.35	0.35	0.35	0.35
Methionine	0.09	01.07	0.05	0.01
Isoleucine	0.06	0.06	0.06	0.06
Valine	0.03	0.03	0.03	0.03
Cellite	0.24	0.20	0.16	0.08
Vitamin/Mineral Mix[1]	0.35	0.35	0.35	0.35

[1]The vitamin/mineral mix contained vitamin A 4550 IU/kg, vitamin D 450 IU/kg, vitamin E 7.5 IU/kg, vitamin K 0.752 mg/kg, biotin 0.152 mg/kg, choline 1575 mg/kg, folacin 0.376, niacin 15.147 mg/kg, pantothenic acid 3 mg/kg, riboflavin 3.76 mg/kg, thiamine 1.07 mg/kg, vitamin B63.78 mg/kg, vitamin B12 0.006 mg/kg, copper 12 mg/kg, iodine 0.05 mg/kg, 30.23 mg/kg, manganese 53 mg/kg zinc, selenium 0.09 mg/kg, and iron 67.82 mg/kg.

Table 4.2 Ingredient composition of layer diets in Experiment 4.2

Treatment	Treatment 1	Treatment 2	Treatment 3	Treatment 4
	4% DHA, 300% Choline	4% DHA, 300% Choline, 200% Serine	4% DHA, 300% Choline, 100 ppm Ethanolamine	4% DHA, 300% Choline, 200% Serine, 100 ppm Ethanolamine
Ingredient, %				
Corn	62.1	62.1	62.1	62.1
Soybean Meal	23.2	23.2	23.2	23.2
DHA Algae	4.00	4.00	4.00	4.00
DHA Calculated (ppm)	6840	6840	6840	6840
DHA Actual (ppm)	5780	5780	5780	5780
Choline	0.26	0.26	0.26	0.26
Ethanolamine (ppm)	0.00	0.00	100	100
Serine	0.00	0.80	0.00	0.80
Corn Oil	1.00	1.00	1.00	1.00
Dicalcium Phosphate	0.94	0.94	0.94	0.94
Limestone	7.89	7.89	7.89	7.89
NaCl	0.35	0.35	0.35	0.35
Methionine	0.01	0.01	0.01	0.01
Isoleucine	0.06	0.06	0.06	0.06
Valine	0.03	0.03	0.03	0.03
Cellite	0.08	0.08	0.08	0.08
Vitamin/Mineral Mix[1]	0.35	0.35	0.35	0.35

[1]The vitamin/mineral mix contained vitamin A 4550 IU/kg, vitamin D 450 IU/kg, vitamin E 7.5 IU/kg, vitamin K 0.752 mg/kg, biotin 0.152 mg/kg, choline 1575 mg/kg, folacin 0.376, niacin 15.147 mg/kg, pantothenic acid 3 mg/kg, riboflavin 3.76 mg/kg, thiamine 1.07 mg/kg, vitamin B63.78 mg/kg, vitamin B12 0.006 mg/kg, copper 12 mg/kg, iodine 0.05 mg/kg, 30.23 mg/kg, manganese 53 mg/kg zinc, selenium 0.09 mg/kg, and iron 67.82 mg/kg.

Table 4.3 Fatty acid profiles of Experiment 4.1 laying hen diets and *Aurantiochytrium*

Treatment	Control	Treatment 1	Treatment 2	Treatment 3	*Aurantiochytrium*
DHA[1] Algae (%)	0	1	2	4	
DHA (ppm)[**]	0	1708	3415	6830	
Fatty Acid, g/kg					
C14	0.00	0.00	0.00	0.00	0.74
C16	2.20	2.50	3.40	4.10	11.3
C18	0.33	0.30	0.33	0.30	0.00
C18:1	3.40	3.10	3.20	2.20	0.00
C18:2	8.00	7.00	7.10	5.20	0.00
C18:3	0.40	0.38	0.37	0.33	0.00
C22:5	0.00	0.00	0.00	0.00	4.00
C22:6 (DHA)	0.00	1.50	3.20	6.50	160
SFA[2]	2.50	2.80	3.70	4.40	12.0
MUFA[3]	3.40	3.10	3.20	2.20	0.00
PUFA[4]	8.40	8.90	11.0	12.0	164
n-3	0.44	1.90	3.60	6.80	164
n-6	8.00	7.00	7.10	5.20	0.00
n-6/n-3	18.0	3.70	2.00	0.76	N/A

*Data expressed as means (n=5).

**DHA concentration calculated based upon *Aurantiochytrium* fatty acid profile.

Data analyzed via one-way ANOVA.

[a,b,c,] Means in the same row without a common letter differ ($P < 0.05$).

[1]DHA, docosahexaenoic acid.

[2, 3, 4]SFA, saturated fatty acid; MUFA, mono-unsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Table 4.4 Nutrient composition of laying hen diets for Experiment 4.1[1]

Microalgae, %	0.0	1.0	2.0	4.0	*Aurantiochytrium*
Moisture	10.2	10.2	10.2	10.1	1.30
Crude fat	5.40	5.63	5.86	6.32	49.6
Crude Protein	16.0	16.0	16.0	16.0	5520
ME[2] (kcal/kg)	2970	2970	2970	2970	9.55
Ca, %	3.26	3.26	3.26	3.26	0.10
P, %	0.52	0.53	0.54	0.55	0.22
Mg, %	0.29	0.29	0.29	0.29	0.14
K, %	0.69	0.69	0.69	0.69	0.49
Na, %	0.15	0.15	0.15	0.15	2.50
Fe, mg/kg	67.5	67.5	67.5	67.5	20.0
Zn, mg/kg	52.5	52.5	52.5	52.5	3.00
Cu, mg/kg	12.0	12.0	12.0	12.0	<1.0
Mn, mg/kg	30.0	30.0	30.0	30.0	20.0

[1] Proximate and mineral analyses were carried out by Dairy One Coop Inc. (Ithaca, NY)

[2]ME, Metabolizable Energy.

Egg components were measured using the same protocol as described by Kim et al. [109] Blood was drawn from wing veins at wk 0, 3, and 6 and was held on ice during collection, centrifuged at 2,000 g for 20 min at 4^{o}C, and stored at -20^{o}C until analyses. Plasma glucose was determined spectrophotometrically with a glucose assay kit (GAG020, Sigma-Aldrich, Sigma Chemical Co., St. Louis, MO). Plasma uric acid was analyzed with Infinity Uric Acid Liquid Stable Reagent (Thermo-Fisher Scientific, Inc., MA). Plasma non-esterified fatty acids, triglyceride, total phospholipid, and total cholesterol were analyzed using commercial enzymatic kits following manufacturer's protocols (Wako Pure Chemical Industries, Ltd., Richmond, VA). All samples were analyzed in duplicate. Lipids were extracted from egg yolk, plasma, and tissue utilizing a modified methodology created by Folch et al. as described by Manor et al. [105] which involved homogenizing samples in TRIZMA-EDTA followed by two extractions in Folch solution, 2:1 chloroform-methanol (v/v), and 4:1 chloroform-methanol. Chloroform layers were separated via centrifugation at 3000 RPM for 15 minutes at 10 °C. Prior to methylation the fatty acids concentrated in the chloroform were evaporated using industrial N_2 gas in a water bath at 60 °C. Fatty acids were methylated with methanolic sulfuric acid (1%) and tritridecanoin was used as an internal standard. Fatty acids were identified by their respective retention time of the fatty acid methyl ester standard (Sigma-Aldrich Co., St. Loius MO). Methyl esters of fatty acids were analyzed within 24 hours of methylation using a gas chromatography (Agilent 6890N, Agilent Technologies, Santa Clara, CA) fitted with a flame-ionization detector. A fused-silica capillary column coated with CP-SIL 88 for fatty acid methyl esters (1.00 m x 0.25 mm i.d., 0.2 mm film thickness) was used (Varian Inc., Lake Forest, Ca). Oven temperature was programmed to be held for 4 min at 140 °C, increased by 4 °C per minute to 220 °C, and then held for 5 min. Carrier gas was N_2 with a constant flow rate of 45 mL/min. The injector temperature was 230°C

and the detector temperature was 280°C. For analysis of individual lipid species, prior to methylation a separate extraction procedure was utilized following the methodology was described by Corl et al. [114] which involved extraction of lipids followed by passing them through a solid phase extraction column to isolate triglyceride, cholesterol, and phospholipid components. Protein extraction and western blot analysis was performed utilizing the same methods described by Gatrell et al. [107].Total mRNA extraction and quantification of select genes were performed using the same methods described by Zhao et al. [115]. Primers are listed in **Table 4.5**.

Table 4.5 List of primers used for QPCR analysis

Gene	Forward Primer (5' to 3') Reverse Primer (5' to 3')
5-LOX	GAGAAGGCTGGGTTTTGTGG
	CAAAGCCCAGATACGTGAGC
ACC	GTTCCAGGAGGACCAAACAA
	TCTCCTAAAGCCCACATTGC
ACOT4	GCCATCATCTGGTGAGAGGT
	GATTTCGGTTTTGCTGCCTA
CHPT1	GTTGTCCTTGGATCCTGCAT
	GGCCTACCAAATGCACCTTA
COX1	CAACGGCATCTTTGGGGAAA
	CTCCAGGGATGCTGTCTTGA
CPT1A	CCATCCATTTCCATGTCTCC
	CTGCTTTCATTCGCTGTTCA
DEGS1	TCAGCCACTCCATGACTCTG
	GTGCAGAAAAACCAGCCTTC
DGAT2	GAAGATCCCAGTGGGTGAGA
	TGTGCTGAAGTTGCAAAAGG
ELOVL2	GGATTACGCTGCTCTCCTTG
	TCTGGCTGCTTTTCTTCCTC
ELOVL5	CCAAAGTACATGCGGAACAA
	CCACCAGAGGACACGTATGA
EPT1	CCCTGGGGATATGACATCAG
	CGGTGAGAGCACAAGCAATA
FADS2	GCCGTAGGTGTCCTCATTGT
	CGCATTCAGCAGATGAGTCT
FASN	GCAGGGAAAATTCTGTGGAA

	CAGCGGTCAACAACAACATC
LPL	TGAGGGAATCGAGAGCAAGT
	TACATTCCTGTCACCGTCCA
PLPP1	TCCGTTCCATGACAGTACCA
	CTCCGAAAATGAAGGTTCCA
PPARA	GATCGCCCAGGTTTGTTAAA
	TGCACGTGTTCCGTTACAAT
SREBP1c	GCAAGGTGAAGCAGGAGAAC
	AGCCATGATGCTTCTTCCAG

Abbreviation: *ACC*, Acetyl-CoA carboxylase; *ACOT4*, Acyl-CoA thioesterase 4; *CHPT1*, Choline Phosphotransferase 1; *COX1*, Cyclooxygenase 1; *CPT1A*, Carnitine Palmitotransferase 1; *DEGS1*, Delta-4 Desaturase; *ELOVL2*, Elongase 2; *ELOVL5*, Elongase 5; ; *EPT1*, Ethanolaminephosphotransferase 1*FADS2*, Delta-6 Desaturase; *FASN*, Fatty acid synthase; *LPL*, Lipoprotein Lipase; *PLPP1*, Phospholipid Phosphatase 1; *PPARA*, Peroxisome Proliferator Activator Receptor Alpha; *SREBP1c*, Sterol Regulatory Element Binding Protein 1c.

Software R (version 3.3.1, R Foundation for Statistical Computing, Vienna, Austria) was used for the data analyses. Each hen or cage was the experimental unit. Main effects of the treatments were analyzed by one-way ANOVA and the mean comparisons were conducted with Duncan's multiple-ranged method. Data were presented as means ± SEM, and the significance level for differences was $P < 0.05$. The P value was adjusted using a *Bonferroni* correction procedure for multiple regression analyses, with the significance level at $P \leq 0.01$.

4.4 Results

Experiment 4.1 - Optimizing the amount of algal DHA supplementation:

Dietary supplementation of *Aurantiochytrium* did not negatively impact body weight, average daily feed intake, or egg production (**Table 4.6**). Inclusion of *Aurantiochytrium* at 4% of the diet significantly decreased ($P < 0.05$) total egg and yolk weights at week 6.

Table 4.6 Effects of the *Aurantiochytrium* on body weight, feed intake, egg production, and egg components of laying hens

Treatment	Control	Treatment 1	Treatment 2	Treatment 3	SEM	P Value
DHA Algae (%)	0	1	2	4		
DHA (ppm)	0	1708	3415	6830		
Initial BW[1], g	1700	1680	1690	1684	142	0.82
Final BW, g	1630	1640	1550	1580	175	0.32
ADFI[2], g/d	102	108	103	108	15.4	0.50
Egg Production, %	0.867	0.934	0.900	0.881	0.097	0.99
Week 0						
Total egg, g	59.3	59.2	57.1	58.6	2.86	0.45
Yolk, g	16.5	17.0	15.6	16.3	1.29	0.42
Albumen, g	33.2	33.6	32.4	33.5	2.23	0.96
Shell, g	5.44	5.60	5.63	5.63	0.372	0.38
Shell Thickness, mm	40.7	40.0	41.3	40.6	1.81	0.45
Week 3						
Total egg, g	58.2	61.4	54.8	56.9	5.09	0.31
Yolk, g	16.6	17.4	13.8	15.8	2.63	0.27
Albumen, g	34.1	35.6	33.3	33.1	2.59	0.34
Shell, g	5.08	5.41	5.30	5.55	0.587	0.28
Shell Thickness, mm	36.5	37.8	38.5	38.5	2.66	0.088
Week 6						
Total egg, g	65.2[a]	64.1[ab]	64.5[ab]	60.2[b]	3.16	0.032
Yolk, g	16.8[a]	16.7[a]	16.9[a]	15.2[b]	1.03	0.042

Albumen, g	36.1	34.8	35.2	34.1	2.61	0.30
Shell, g	5.74	5.53	5.56	5.47	0.509	0.46
Shell Thickness, mm	38.8	38.6	37.9	39.5	2.62	0.78

*Data expressed as means (n=5).

[a,b] Means in the same row without a common letter differ ($P < 0.05$).

[1]BW, Body Weight.

[2]ADFI, Average Daily Feed Intake.

Egg yolk fatty acids profiles at week 3 were different ($P < 0.05$) with an increase in palmitic acid (C16) in the 4% algae group and a positive correlation with algae in the diet (**Table 4.7**). Conversely, arachidonic acid (C20:4n-6) decreased with algae inclusion. Docosahexaenoic acid (C22:6n-3) increased in a dose dependent fashion ($P < 0.001$) and reached levels of 116, 144, and 225 mg/g of egg in the 1%, 2%, and 4% algae treatments respectively. The ratio of total omega 6 fatty acids to omega 3 decreased from 6.67 in the 1% algae treatment to 2.56 in the 4% group. At the week 6 point there were other changes in yolk fatty profile including a decrease ($P < 0.001$) in stearic acid (C18) which was lower ($P < 0.05$) in the 2% and 4% algae groups relative to the control. Linoleic acid (C18:2n-6) was lower ($P < 0.001$) in algae fed groups with the control being higher than the 1% and 2% treatments and the 4% group being significantly lower than the other algae groups. Arachidonic acid (C20:4n-6) was significantly lowered ($P < 0.001$) in a dose dependent fashion having a negative correlation with algae inclusion. The amount of DHA was positively correlated ($P < 0.001$) with algal inclusion with enrichments reaching up to 123, 183, and 196 mg/g of egg for the 1%, 2%, and 4% groups respectively. The omega 6 to omega 3 ratio was changed from 5.41 in the 1% algae group to 2.55 in the 4% group. Yolk total phospholipids, triglycerides, and non-esterified fatty acids were decreased ($P < 0.05$) by DHA supplementations. The combination of choline, serine, and ethanolamine increased ($P <$

0.05) yolk palmitic acid (C16), and DHA concentrations reaching up to 256 mg/egg with an enrichment efficiency of 37.9% (**Table 4.14**). Both yolk and hepatic fatty acids in the form of phospholipid, TG, and NEFA were all decreased in the same fashion by algal DHA supplementation, except for DHA, which was increased and had a plateau in phospholipid incorporation between 2 and 4% DHA enrichment but continued to accumulate in the TG form (**Table 4.8**).

Table 4.7 Effects of different concentrations of dietary *Aurantiochytrium* on egg yolk fatty acid concentration and lipid profiles

Treatment	Control	Treatment 1	Treatment 2	Treatment 3	SEM	P Value
DHA*1* Algae (%)	0	1	2	4		
DHA (ppm)	0	1708	3415	6830		
Fatty Acid, mg/egg						
Week 0						
C20:4	104	94.0	97.3	95.0	9.32	0.33
C22:6 (DHA)	N.D.	N.D.	N.D.	N.D.		
SFA[2]	1300	1170	1220	1240	79.8	0.46
MUFA[3]	1560	1370	1430	1500	133	0.73
PUFA[4]	678	586	610	711	90.3	0.61
n-3	N.D.	N.D.	N.D.	N.D.		
n-6	678	586	610	711	90.3	0.61
n-6/n-3	0.00	0.00	0.00	0.00		
Week 3						
C20:4	128[a]	93.3[b]	66.7[c]	62.0[c]	13.5	<0.001
C22:6 (DHA)	0.00[c]	116[b]	144[b]	225[a]	33.1	<0.001
Enrichment Efficiency (%)	N/A	68.0[a]	42.0[b]	33.0[c]	12.0	<0.001
SFA	1310	1400	1310	1450	108	0.26
MUFA	1510	1550	1460	1530	135	0.95

PUFA	730	895	705	804	127	0.92
n-3	0.00[c]	116[b]	144[b]	225[a]	33.1	<0.001
n-6	730	779	560	579	114	0.046
n-6/n-3	0.00[d]	6.67[c]	3.85[b]	2.56[a]	0.233	<0.001
Week 6						
C20:4	119[a]	88.6[b]	65.6[c]	46.6[d]	8.17	<0.001
C22:6 (DHA)	0.00[c]	123[b]	183[a]	196[a]	43.5	<0.001
Enrichment Efficiency (%)	N/A	72.0[a]	54.0[b]	29.0[c]	15.0	<0.001
SFA	1420	1330	1320	1300	151	0.25
MUFA	1520	1380	1300	1310	169	0.047
PUFA	810	788	812	706	87.0	0.11
n-3	0.00[c]	123[b]	183[a]	196[a]	43.5	<0.001
n-6	810[a]	665[b]	629[b]	510[c]	78.0	<0.001
n-6/n-3	0.00[d]	5.41[a]	3.44[b]	2.55[c]	0.214	<0.001
Lipid						
Total Phospholipid, g/dL						
Week 0	2.20	2.15	2.23	2.26	0.15	0.45
Week 3	2.24[a]	2.01[a]	1.38[b]	1.29[b]	0.14	<0.001
Week 6	2.25[a]	1.98[b]	1.34[c]	1.21[c]	0.18	<0.001
Total Cholesterol, mg/dL						
Week 0	374	370	365	380	32.5	0.66
Week 3	350	372	366	375	45.2	0.54
Week 6	360	375	399	378	40.3	0.71
Triglycerides, g/dL						
Week 0	4.85	4.80	4.75	4.96	0.52	0.46
Week 3	4.78[a]	3.62[b]	2.65[c]	2.52[c]	0.49	<0.001
Week 6	4.62[a]	3.55[b]	2.72[c]	2.32[d]	0.41	<0.001
Non-esterified fatty acid, μmol/mL						

Week 0	8.70	8.52	8.90	9.10	0.56	0.78
Week 3	8.53[a]	7.60[b]	7.12[c]	6.85[d]	0.25	0.03
Week 6	8.64[a]	7.41[b]	6.80[c]	6.65[c]	0.21	0.02

*Data expressed as means (n=5).

[a,b,c,d] Means in the same row without a common letter differ ($P < 0.05$).

[1]DHA, Docosahexaenoic Acid.

[2]SFA, saturated fatty acid; [3]MUFA, mono-unsaturated fatty acids; [4]PUFA, polyunsaturated fatty acids.

Table 4.8 Effects of different concentrations of dietary *Aurantiochytrium* on hepatic fatty acid distributions in different lipid species

Treatment	Control	Treatment 1	Treatment 2	Treatment 3	SEM	P Value
DHA[1] Algae (%)	0	1	2	4		
DHA (ppm)	0	1708	3415	6830		
Liver, mg/g tissue						
Week 3						
C20:4						
PL[2]	114[a]	83.0[b]	59.4[c]	55.2[c]	5.47	0.04
TG[3]	2.56[a]	1.87[b]	1.33[c]	1.24[c]	0.17	0.03
NEFA[4]	11.5[a]	8.39[b]	6.00[c]	5.58[c]	0.64	0.01
C22:6 (DHA)						
PL	0.00[d]	103[c]	125[b]	187[a]	15.4	0.01
TG	0.00[d]	2.32[c]	5.76[b]	18.0[a]	14.3	0.01
NEFA	0.00[d]	10.4[c]	12.9[b]	20.3[a]	2.4	0.01
Week 6						
C20:4						
PL	106[a]	78.9[b]	58.4[c]	41.5[d]	4.65	0.01
TG	2.38[a]	1.77[b]	1.31[c]	0.93[d]	0.18	0.01

NEFA	10.7[a]	7.97[b]	5.90[c]	4.19[d]	0.65	0.01
C22:6 (DHA)						
PL	0.00[c]	109[b]	159[a]	163[a]	14.5	0.01
TG	0.00[d]	2.46[c]	7.32[b]	15.7[a]	1.60	0.01
NEFA	0.00[c]	11.1[b]	16.5[a]	17.6[a]	1.41	0.01

*Data expressed as means (n=5).

[a,b,c,d] Means in the same row without a common letter differ ($P < 0.05$).

[1]DHA, Docosahexaenoic Acid.

[2]PL, Phospholipid.

[3]TG, Triglyceride.

[4]NEFA, Non-esterified Fatty Acid.

The DHA-rich *Aurantiochytrium* exerted effects on endogenous tissue and blood plasma fatty acid concentrations of laying hens at week 3 and or 6 (**Table 4.9**). Hepatic concentrations of palmitic acid (C16), stearic acid (C18), oleic acid (C18:1n-9), linoleic acid (C18:2n-6), and arachidonic acid (C20:4n-6) were all significantly lowered ($P < 0.05$) while DHA was elevated ($P < 0.001$). Concentrations of total SFA, MUFA, PUFA, total n6 fatty acids, and n6/n3 fatty acid ratios were all significantly lowered ($P < 0.05$) in the liver with total n3 fatty acids increasing ($P < 0.01$). Plasma linoleic acid (C18:2n-6) and total n6 fatty acids decreased ($P < 0.05$) in the 4% algae group with the n6/n3 ratio being lowered ($P < 0.05$) in all algae groups. Strikingly, at week 6 the plasma concentration of arachidonic acid (C20:4n-6) decreased ($P < 0.05$) with the concentration of dietary algae with the 4% group being non-detectable. Conversely plasma DHA was elevated ($P < 0.001$) in the 2 and 4% treatment groups. Total PUFA, n6 fatty acids, and n6/n3 were significantly lowered ($P < 0.05$) while total n3 was

increased (P < 0.001) by dietary algae. Additional serine decreased (P < 0.05) palmitic acid (C16) liver concentration but increased arachidonic acid (C20:4n-6) and total n6n/3 ratio.

Table 4.9 Effects of different concentrations of dietary *Aurantiochytrium* on hen liver and plasma fatty acid concentrations

Treatment	Control	Treatment 1	Treatment 2	Treatment 3	SEM	P Value
DHA[1] Algae (%)	0	1	2	4		
DHA (ppm)	0	1708	3415	6830		
Liver, mg/g						
C20:4	2.15[a]	1.74[b]	1.35[c]	0.93[d]	0.281	<0.001
C22:6 (DHA)	0.474[c]	1.64[b]	2.06[ab]	2.38[a]	0.436	<0.001
SFA[2]	14.9[a]	15.6[a]	12.5[b]	11.0[c]	1.63	<0.001
MUFA[3]	14.2[a]	14.5[a]	10.7[b]	8.83[b]	2.25	<0.001
PUFA[4]	8.64[a]	8.56[a]	7.35[ab]	6.16[b]	1.22	0.0024
n-3	0.474[c]	1.64[b]	2.06[ab]	2.38[a]	0.436	<0.001
n-6	8.16[a]	6.92[a]	5.29[b]	3.78[c]	1.05	<0.001
n-6/n-3	17.1[a]	4.29[b]	2.60[b]	1.68[b]	3.52	<0.001
Plasma, mg/ml						
Week 3**						
C20:4	0.493	0.290	0.411	0.271	0.179	0.145
C22:6 (DHA)	0.175	0.367	0.428	0.260	0.216	0.472
SFA	4.86	5.33	5.20	3.45	1.60	0.191
MUFA	5.11	5.62	5.53	3.83	1.84	0.3
PUFA	2.88	3.16	3.02	1.96	0.865	0.111
n-3	0.175	0.367	0.428	0.260	0.216	0.472
n-6	2.71[ab]	2.80[a]	2.59[ab]	1.70[b]	0.750	0.046
n-6/n-3	16.2[a]	7.96[b]	9.07[b]	9.09[b]	5.03	0.041
Week 6***						

C20:4	0.410[a]	0.104[b]	0.353[ab]	N.D.[b]	0.207	0.021
C22:6 (DHA)	0.0709[b]	0.169[b]	0.88[a]	0.825[a]	0.363	<0.001
SFA	5.34	3.99	9.24	4.67	4.11	0.699
MUFA	6.01	4.10	9.36	4.89	3.92	0.812
PUFA	3.09[ab]	1.98[b]	4.46[a]	2.35[ab]	1.61	0.033
n-3	0.0709[b]	0.169[b]	0.702[a]	0.825[a]	0.363	<0.001
n-6	3.01[ab]	1.81[ab]	3.75[a]	1.53[b]	1.52	0.042
n-6/n-3	22.3[a]	4.41[b]	3.29[b]	1.89[b]	4.45	<0.001

*Data expressed as means (n=5).

**Hens were not fasted prior to blood collection.

***Hens were fasted prior to blood collection.

[a,b,c,d] Means in the same row without a common letter differ ($P < 0.05$).

[1]DHA, Docosahexaenoic Acid.

[2, 3, 4]SFA, saturated fatty acid; MUFA, mono-unsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Parameters indicative of health status were altered by *Aurantiochytrium* supplementation; alanine aminotransferase, an enzyme indicative of liver damage, was significantly higher ($P < 0.05$) in the 4% algae group for week 3 and the 2% algae group for week 6 compared to other treatment groups (**Table 4.10**). Plasma inorganic phosphorus was significantly lower ($P < 0.05$) in the 4% algae group for week 6. Total plasma cholesterol was significantly decreased ($P < 0.05$) in the 1% and 2% algae groups at week 3. Total plasma triglycerides were significantly decreased ($P < 0.05$) in the 4% algae group for both weeks 3 and 6. Plasma non-esterified fatty acids were significantly lower ($P < 0.05$) in all algae groups for week 3 compared to the control and in the 2% and 4% algae groups for week 6. Plasma glucose was significantly lower ($P < 0.05$) in the 4% algae treatment for week 3. Plasma uric acid was significantly lower ($P < 0.05$) in the 1% and 4% treatment groups for week 6.

Table 4.10 Effect of different concentrations of *Aurantiochytrium* on plasma lipid and health biomarkers in laying hens

Treatment	Control	Treatment 1	Treatment 2	Treatment 3	SEM	P Value
DHA[1] Algae (%)	0	1	2	4		
DHA (ppm)	0	1708	3415	6830		
Enzyme						
ALT[2], U/L						
Week 0	104	122	115	119	28.2	0.723
Week 3	93.8^{ab}	78.5^{b}	99.4^{ab}	105^{a}	19.2	0.013
Week 6	124^{ab}	111^{ab}	144^{a}	101^{b}	23.6	0.117
AKP[3], U/mL						
Week 0	55.7	50.6	56.9	50.3	17.2	0.922
Week 3	78.8	109	82.2	84.3	39.3	0.685
Week 6	158	165	120	92.3	73.6	0.399
Inorganic phosphorus, mg/dL						
Week 0	35.7	37.0	38.4	36.6	7.90	0.957
Week 3	40.8	42.5	50.3	38.6	11.6	0.466
Week 6	34.8^{a}	32.7^{a}	27.3^{ab}	20.6^{b}	6.92	0.020
Lipid, g/dL						
Total Phospholipid						
Week 0	13.5	13.9	12.1	13.0	2.43	0.41
Week 3	14.6^{c}	18.5^{b}	23.1^{a}	23.0^{a}	1.12	0.03
Week 6	14.2^{c}	19.3^{b}	22.5^{a}	22.7^{a}	1.89	0.02
Total cholesterol, mg/dL						
Week 0	69.6	68.3	64.8	64.6	15.0	0.930
Week 3	89.9^{a}	76.2^{b}	77.4^{b}	80.7^{ab}	9.44	0.085
Week 6	45.9	39.4	45.2	41.0	5.66	0.210
Triglycerides, g/dL						
Week 0	1.26	1.25	1.09	1.07	0.399	0.863

Week 3	1.95[a]	1.56[ab]	1.57[ab]	1.32[b]	0.457	0.224
Week 6	1.74[a]	1.39[ab]	2.02[a]	0.743[b]	0.449	0.010
Non-esterified fatty acid, μmol/mL						
Week 0	0.326	0.344	0.330	0.306	0.075	0.895
Week 3	0.343[a]	0.268[b]	0.270[b]	0.252[b]	0.043	0.022
Week 6	0.343[a]	0.428[a]	0.306[ab]	0.161[b]	0.103	0.022
Glucose, g/L						
Week 0	4.19	4.25	4.26	4.40	0.524	0.907
Week 3	2.87[a]	2.76[ab]	2.68[ab]	2.52[b]	0.310	0.226
Week 6	2.20	2.26	2.20	2.26	0.126	0.696
Uric acid, mmol/L						
Week 0	0.869	0.757	0.864	0.699	0.228	0.707
Week 3	0.883	0.757	0.853	0.615	0.310	0.509
Week 6	0.961[ab]	0.693[b]	1.46[a]	0.463[b]	0.421	0.016

*Data expressed as means (n=5).

Data analyzed via one-way ANOVA.

[a,b] Means in the same row without a common letter differ ($P < 0.05$).

[1]DHA, Docosahexaenoic Acid.

[2]ALT, Alanine aminotransferase, U/L: activity defined as the rate of decreasing in absorbance at 340nm due to the oxidation of NADH.

[3]AKP, Alkaline phosphatase, U/mL: activity defined as the amount of p-nitrophenol product formed per minute when alkaline phosphatase exposed to phosphatase substrate.

Dietary inclusion of *Aurantiochytrium* downregulated the hepatic expression of genes related to fatty acid and lipid anabolism *SREBP1c*, *ACC*, and *DGAT2*, with an exception being *FASN*, a gene responsible for de novo fatty acid synthesis (**Table 4.11**). Expression of hepatic genes responsible for long chain fatty acid and phospholipid catabolism, *ACOT4* and *PLPP1*, were both upregulated with *Aurantiochytrium*. Hepatic expression of genes regulating general fatty acid catabolism, *CPT1A* and *PPARA*, were downregulated by the DHA algae. Expression of liver 5-LOX and COX1, genes which control lipid-hormone synthesis were upregulated ($P < 0.05$) in the 1% algae group but this returned to baseline levels with additional supplementation. Genes which regulate fatty acid elongation and desaturation, *ELOVL2*, *ELOVL5*, *DEGS1*, and *FADS2* were downregulated ($P < 0.05$) with algae supplementation. Expression of the gene which controls lipoprotein absorption, *LPL*, was enhanced ($P < 0.001$) by over 10 fold with the highest level of *Aurantiochytrium*. Hepatic mRNA expression of genes controlling phosphatidylethanolamine and phosphatidylcholine, *EPT1* and *CHPT1* respectively, were upregulated ($P < 0.05$) with DHA algae supplementation (**Figure 4.1**).

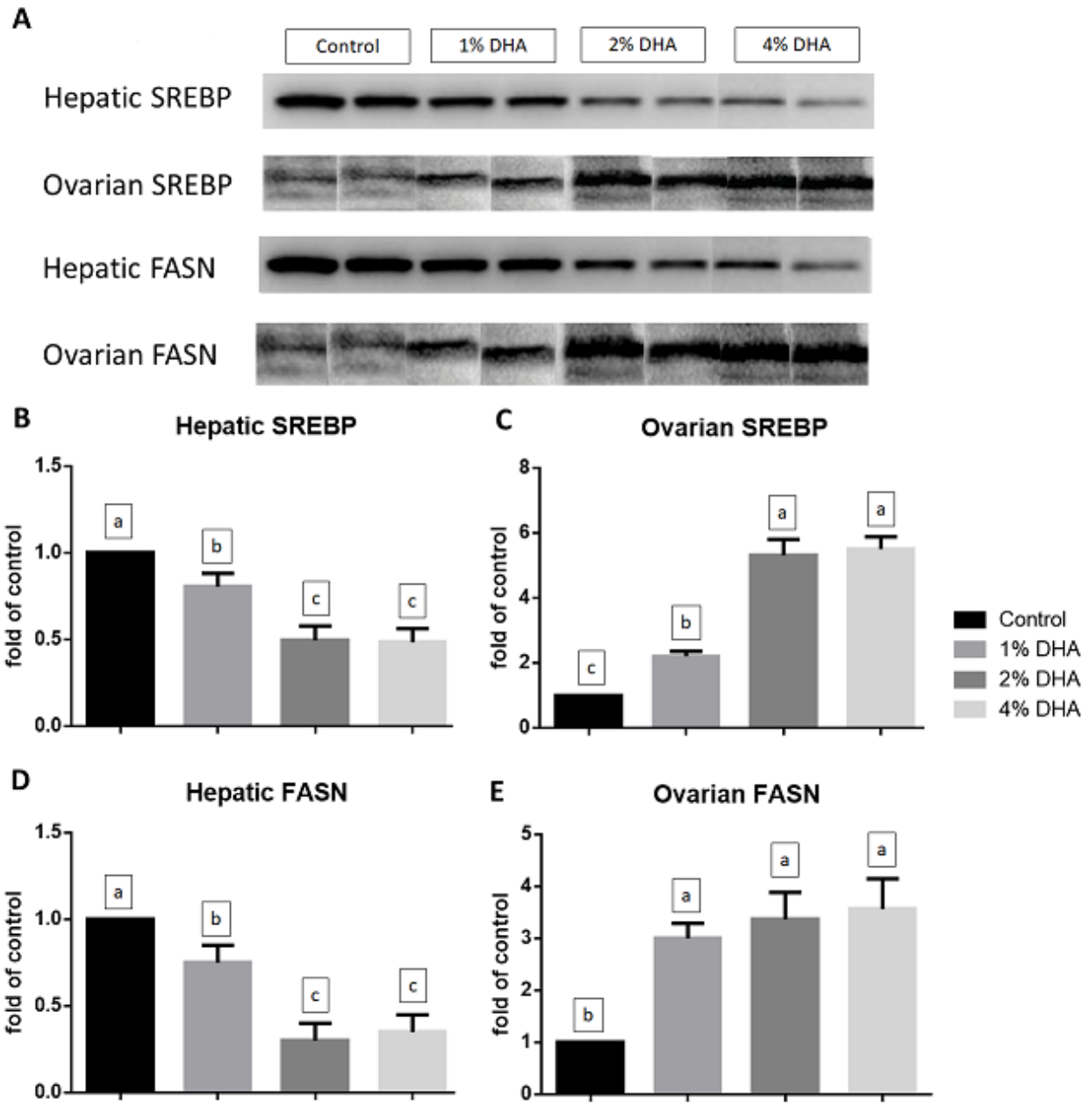


Figure 4.1 Effects of algal DHA on relative concentrations of lipid regulatory proteins fatty acid synthase and sterol regulatory binding element protein. Western blot analysis (a) was performed to measure levels of hepatic SREBP1c (b), ovarian SREBP1c (c), hepatic FASN (d), and ovarian FASN (e).

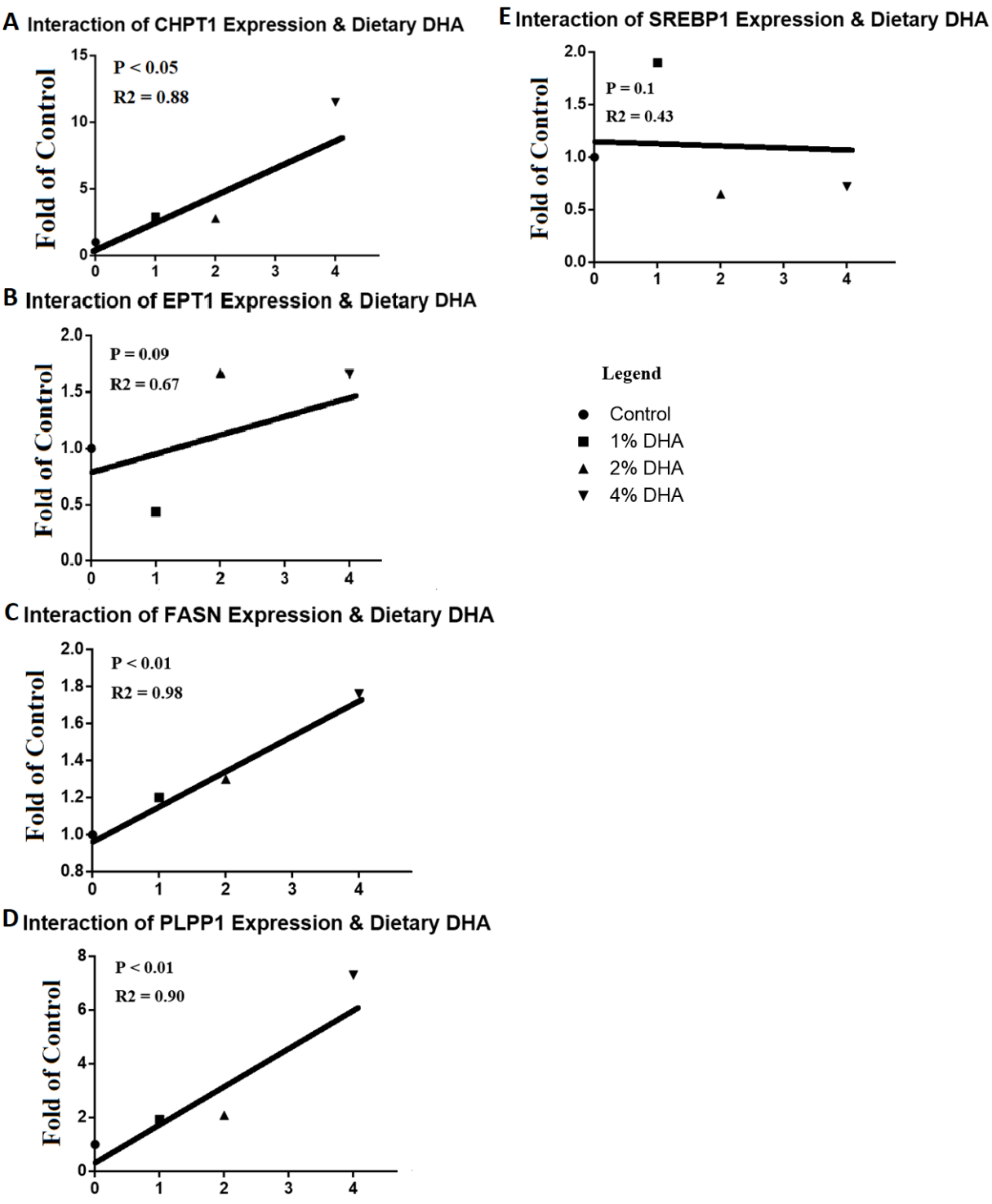


Figure 4.2 Effects of algal DHA on hepatic gene expression of key regulators of lipid metabolism and conversion. Quantitive PCR was performed to measure levels of hepatic CHPT1 (a), EPT1 (b), FASN (c), PLPP1 (d), and SREBP1c (e).

Table 4.11 Hepatic gene expression of layers fed *Aurantiochytrium*

Treatment	Control	Treatment 1	Treatment 2	Treatment 3	SEM	P Value
DHA Algae (%)	0	1	2	4		
DHA (ppm)	0	1708	3415	6830		
			Anabolic			
ACC	1.00±0.062[a]	0.61±0.031[b]	0.67±0.047[b]	0.71±0.037[b]	0.060	0.043
COX1	1.00±0.037[d]	1.74±0.16[b]	1.92±0.16[c]	1.25±0.078[a]	0.19	0.006
DGAT2	1.00±0.29[a]	0.38±0.078[c]	0.66±0.038[b]	0.40±0.21[c]	0.047	0.003
FASN	1.00±0.073[c]	1.20±0.093[b]	1.30±0.096[b]	1.76±0.055[a]	0.13	0.001
SREBP1c	1.00±0.0701[b]	1.90±0.21[a]	0.65±0.0044[c]	0.72±0.027 [c]	0.066	0.041
			Catabolic			
ACOT4	1.00±0.051[b]	1.62±0.11[a]	1.57±0.078[a]	1.56±0.12[a]	0.16	0.021
CPT1A	1.00±0.067[b]	1.20±0.072[a]	1.10±0.082[a]	0.64±0.042[c]	0.12	0.045
PLPP1	1.00±0.095[c]	1.93±0.22[b]	2.09±0.11[b]	7.3 ±1.38[a]	0.21	0.05
PPARA	1.00±0.024[a]	0.90±0.037[b]	0.63±0.034[d]	0.70±0.0.029[c]	0.064	0.033
			Conversion			
5-LOX	1.00±0.032[b]	2.03±0.185[a]	1.35±0.048[ab]	1.03 ±0.034[b]	0.10	0.050

DEGS1	1.00±0.049[b]	1.67±0.20[a]	0.98±0.032[b]	0.75±0.037[c]	0.098	0.022
ELOVL2	1.00±0.0.31[a]	0.75±0.031[b]	0.55±0.022[c]	0.51 ±0.018[c]	0.056	0.021
ELOVL5	1.00±0.044[a]	0.62±0.026[c]	0.59±0.025[c]	0.72 ±0.018[b]	0.072	0.007
FADS2	1.00±0.104[d]	2.55±0.25[a]	0.81±0022[c]	0.50 ±0.025[b]	0.050	0.049
			Absorption			
LPL	1.00±0.30[b]	0.84±0.16[b]	1.04±0.13[b]	13.55 ±4.87[a]	0.083	0.001
			Phospholipid Synthesis			
CHPT1	1.00±0.14[c]	2.90±0.14[b]	2.78±0.81[b]	11.47±1.46[a]	0.28	0.001
EPT1	1.00±0.072[b]	0.44±0.021[c]	1.67±0.17[a]	1.66 ±0.085[a]	0.17	0.005

*Data expressed as means (n=5).

Data analyzed via one-way ANOVA.

[a,b,c,d] Means in the same row without a common letter differ ($P < 0.05$).

Abbreviation: *ACC*, Acetyl-CoA carboxylase; *ACOT4*, Acyl-CoA thioesterase 4; *CHPT1*, Choline Phosphotransferase 1; *COX1*, Cyclooxygenase 1; *CPT1A*, Carnitine Palmitotransferase 1; *DEGS1*, Delta-4 Desaturase; *ELOVL2*, Elongase 2; *ELOVL5*, Elongase 5; *EPT1*, Ethanolaminephosphotransferase 1; *FADS2*, Delta-6 Desaturase; *FASN*, Fatty acid synthase; *LPL*, Lipoprotein Lipase; *PLPP1*, Phospholipid Phosphatase 1; *PPARA*, Peroxisome Proliferator Activator Receptor Alpha; *SREBP1c*, Sterol Regulatory Element Binding Protein 1c.

Adipose mRNA expression of anabolic lipid genes *SREBP1c* and *ACC* were downregulated ($P < 0.05$) while *FASN* was upregulated ($P < 0.05$) with algae supplementation (**Table 4.12**). Fat pad gene expression of lipid catabolic genes *CPT1* and *PPARA* were downregulated ($P < 0.05$) and *ACOT4* upregulated ($P < 0.05$) by dietary DHA algae. Expression of the shorter fatty acid chain desaturase *DEGS1*, elongase *ELOVL2*, and an indicator of prostaglandin synthesis, *COX1*, were suppressed ($P < 0.05$) with additional *Aurantiochytrium*. Adipose mRNA expression of enzyme responsible for lipoprotein absorption, *LPL*, and overall phosphatidycholine synthesis, *CHPT1*, were downregulated ($P < 0.05$) (**Figure 4.2**). Laying hen ovary mRNA expression of lipid metabolism related genes were almost all ubiquitously upregulated ($P < 0.05$) with *Aurantiochytrium* supplementation (**Table 4.13)** (**Figure 4.3**). Hepatic protein concentrations of both fatty acid synthase (FASN), and sterol regulatory binding element (SREBP1c), were decreased ($P < 0.05$) by microalgal DHA supplementations. Conversely, ovarian protein concentrations of FASN and SREBP1c were increased ($P < 0.05$) with this trend plateauing between 2 and 4% DHA supplementation **(Figure 4.1)**.

Table 4.12 Adipose gene expression of layers fed *Aurantiochytrium*

Treatment	Control	Treatment 1	Treatment 2	Treatment 3	SEM	P Value
DHA Algae (%)	0	1	2	4		
DHA (ppm)	0	1708	3415	6830		
			Anabolic			
ACC	1.00±0.035[a]	0.072±0.011[b]	0.037 ±0.0034[b]	N.D.	0.35	0.014
COX1	1.00±0.11	1.19±0.22	1.11±0.13	1.16±0.15	0.12	0.021
FASN	1.00±0.03[b]	1.37±0.13[a]	1.41±0.12[a]	1.47±0.077[a]	0.17	0.021
SREBP1c	1.00±0.095[a]	0.29±0.04[c]	0.93±0.096[b]	0.76±0.097[b]	0.021	0.001
			Catabolic			
ACOT4	1.00±0.12[b]	0.85±0.081[b]	1.02±0.079[b]	1.70±0.13[a]	0.086	0.036
CPT1A	1.00±0.12[a]	0.59±0.13[b]	0.43±0.08[bc]	0.35±0.077[c]	0.034	0.001
PPARA	1.00±0.082[a]	0.58±0.074[b]	0.73±0.13[b]	0.24±0.0.015[c]		0.001
			Conversion			
5-LOX	1.00±0.22[a]	0.34±0.042[b]	0.30±0.042[b]	0.31±0.034[b]	0.033	0.042
DEGS1	1.00±0.032[b]	1.56±0.15[a]	1.57±0.11[a]	1.26±0.14[ab]	0.14	0.042
ELOVL2	1.00±0.12[a]	0.34±0.055[b]	0.33±0.076[b]	0.28 ±0.028[b]	0.032	0.036

FADS2	1.00±0.18[a]	0.54±0.18[b]	0.59±0.047[b]	1.01±0.067[a]	0.052	0.036
			Absorption			
LPL	1.00±0.16[a]	0.28±0.046[b]	0.33±0.069[b]	0.32 ±0.066[b]	0.031	0.014
			Phospholipid Synthesis			
CHPT1	1.00±0.072[a]	0.76±0.0046[b]	0.42±0.008[c]	0.19 ±0.005[d]	0.017	0.043

*Data expressed as means (n=5).

Data analyzed via one-way ANOVA.

[a,b,c,d] Means in the same row without a common letter differ ($P < 0.05$).

Abbreviation: *ACC*, Acetyl-CoA carboxylase; *ACOT4*, Acyl-CoA thioesterase 4; *CHPT1*, Choline Phosphotransferase 1; *COX1*, Cyclooxygenase 1; *CPT1A*, Carnitine Palmitotransferase 1; *DEGS1*, Delta-4 Desaturase; *ELOVL2*, Elongase 2; *ELOVL5*, Elongase 5; *FADS2*, Delta-6 Desaturase; *FASN*, Fatty acid synthase; *LPL*, Lipoprotein Lipase; *PLPP1*, Phospholipid Phosphatase 1; *PPARA*, Peroxisome Proliferator Activator Receptor Alpha; *SREBP1c*, Sterol Regulatory Element Binding Protein 1c.

Table 4.13 Ovarian gene expression of layers fed *Aurantiochytrium*

Treatment	Control	Treatment 1	Treatment 2	Treatment 3	SEM	P Value
DHA Algae (%)	0	1	2	4		
DHA (ppm)	0	1708	3415	6830		
			Anabolic			
ACC	1.00±0.037^{d}	2.07±0.099^{c}	4.43±0.12^{b}	15.8±4.6^{a}	0.42	0.001
COX1	1.00±0.032^{b}	1.04±0.093^{b}	0.88±0.099^{b}	8.59±1.40^{a}	0.085	0.021
DGAT2	1.00±0.068^{a}	0.75±0.077^{b}	0.67±0.034^{b}	1.01±0.17^{a}	0.069	0.036
FASN	1.00±0.015^{b}	0.87±0.11^{b}	0.93±0.10^{b}	8.88±0.76^{a}	0.099	0.022
SREBP1c	1.00±0.039^{c}	0.89±0.11^{c}	1.41±0.16^{b}	6.62±0.73^{a}	0.017	0.004
			Catabolic			
ACOT4	1.00±0.035^{b}	1.24±0.044^{b}	1.15±0.15^{b}	12.0±1.60^{a}	0.14	0.001
CPT1A	1.00±0.11^{c}	1.55±0.07^{b}	1.38±0.11^{b}	3.55±1.02^{a}	0.12	0.028
PLPP1	1.00±0.04^{d}	4.99±0.38^{c}	9.46±1.4^{b}	11.8±2.2^{a}	0.046	0.021
PPARA	1.00±0.063^{c}	2.12±0.12^{b}	2.75±0.061^{b}	10.4±0.96^{a}	0.023	0.001
			Conversion			
5-LOX	1.00±0.037^{c}	0.94±0.49^{c}	1.66±0.13^{b}	10.4±0.96^{a}	0.17	0.034
DEGS1	1.00±0.030^{b}	1.27±0.15^{b}	1.03±0.10^{b}	12.8±1.70^{a}	0.13	0.023

ELOVL2	1.00±0.0.10[c]	1.08±0.20[c]	3.31±1.02[b]	9.99±2.80[a]	0.36	0.040
ELOVL5	1.00±0.059[c]	8.95±0.98[b]	8.17±0.097[b]	11.4±0.078[a]	0.89	0.041
FADS2	1.00±0.036[c]	0.88±0.089[c]	1.85±0.21[b]	4.36 ±0.41[a]	0.043	0.024
			Absorption			
LPL	1.00±0.060[b]	0.89 ±0.09[b]	0.94±0.029[b]	1.4 ±0.15[a]	0.083	0.035
			Phospholipid Synthesis			
CHPT1	1.00±0.073[c]	0.77 ±0.15[c]	1.33±0.037[b]	4.84 ±0.84[a]	0.17	0.001
EPT1	1.00±0.061[b]	0.91 ±0.061[b]	0.89±0.054[b]	1.93 ±0.079[a]	0.094	0.001

*Data expressed as means (n=5).

Data analyzed via one-way ANOVA.

[a,b,c] Means in the same row without a common letter differ ($P < 0.05$).

Abbreviation: *ACC,* Acetyl-CoA carboxylase; *ACOT4*, Acyl-CoA thioesterase 4; *CHPT1,* Choline Phosphotransferase 1; *COX1*, Cyclooxygenase 1; *CPT1A*, Carnitine Palmitotransferase 1; *DEGS1*, Delta-4 Desaturase; *ELOVL2*, Elongase 2; *ELOVL5*, Elongase 5; *EPT1*, Ethanolaminephosphotransferase 1; *FADS2*, Delta-6 Desaturase; *FASN*, Fatty acid synthase; *LPL*, Lipoprotein Lipase; *PLPP1*, Phospholipid Phosphatase 1; *PPARA*, Peroxisome Proliferator Activator Receptor Alpha; *SREBP1c*, Sterol Regulatory Element Binding Protein 1c.

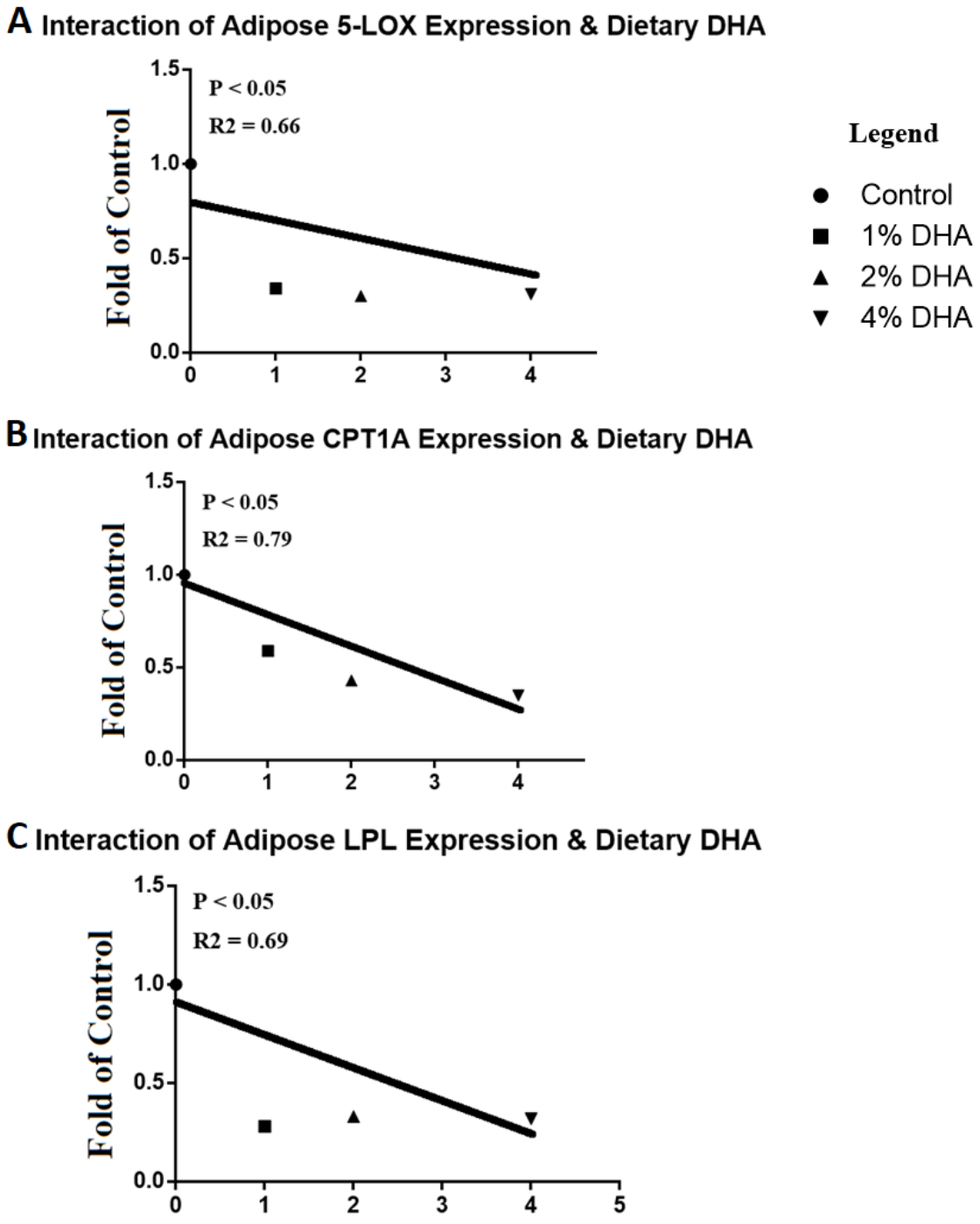


Figure 4.3 Effects of algal DHA on adipose gene expression of key regulators of lipid metabolism and conversion. Quantitive PCR was performed to measure levels of adipose 5-LOX (a), CPT1A (b), and LPL (c).

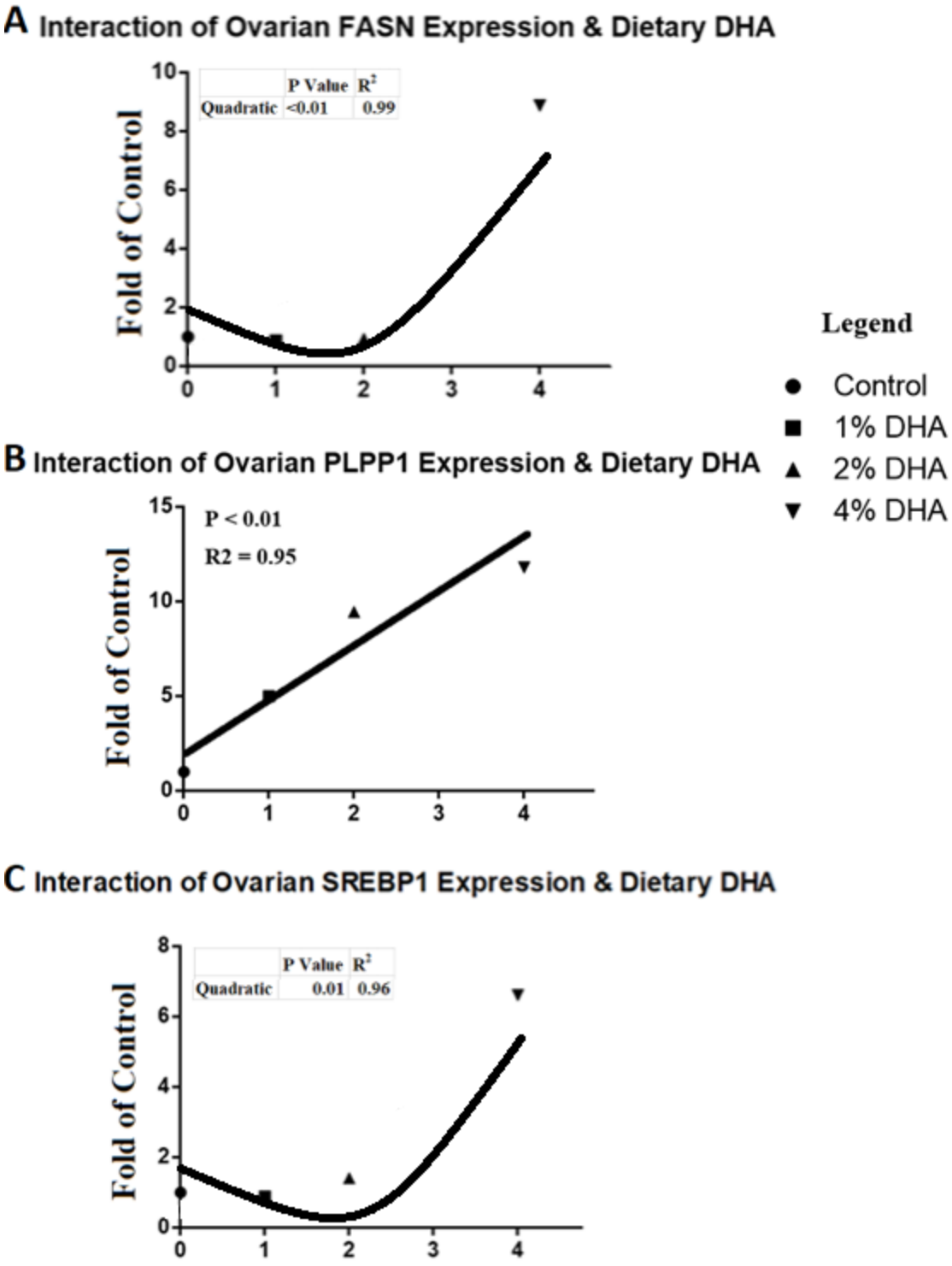


Figure 4.4 Effects of algal DHA on ovarian gene expression of key regulators of lipid metabolism and conversion. Quantitive PCR was performed to measure levels of ovarian FASN (a), PLPP1 (b), and SREBP1c (c).

Experiment 4.2 – Evaluating if dietary phospholipid components can enhance DHA deposition:

Additional choline, serine, and ethanolamine did not significantly change any production parameters or egg component weights aside from total egg shell which was significantly lowered ($P < 0.05$) by the serine treatment (**Table 4.15**). The full combination of serine, ethanolamine, and choline increased ($P < 0.05$) hepatic DHA, PUFA, and total n3 (**Table 4.16**). Plasma DHA was increased ($P < 0.05$) in the full combination group and the total n6/n3 ratio lowered. Additional serine, choline, and ethanolamine did not significantly change any plasma parameter measurements of health (**Table 4.17**).

Table 4.14 Effects of the novel phospholipid components & *Aurantiochytrium* on egg yolk fatty acid concentration and lipid profiles

Treatment	Treatment 1	Treatment 2	Treatment 3	Treatment 4	SEM	P Value
Fatty Acid, mg/egg						
Week 0						
C20:4	52.3	52.4	56.4	58.4	9.32	0.33
C22:6 (DHA[1])	179	166	170	172	15.4	0.75
SFA	1260	1240	1230	1220	79.8	0.46
MUFA	1270	1300	1280	1320	133	0.73
PUFA	722	668	679	712	90.3	0.61
n-3	179	166	170	172	16.4	0.47
n-6	543	502	509	540	90.3	0.61
n-6/n-3	3.03	3.03	2.86	3.09	0.31	0.69
Week 3						
C20:4	47.5^{b}	54.6^{a}	45.3^{b}	46.5^{b}	13.5	<0.001
C22:6 (DHA)	214^{b}	190^{b}	197^{b}	256^{a}	33.1	<0.001
Enrichment Efficiency (%)	31.7^{b}	28.1^{b}	29.1^{b}	37.9^{a}	12.0	<0.001
SFA[2]	1180	1150	1100	1190	108	0.26
MUFA[3]	1180	1270	1120	1150	135	0.95
PUFA[4]	680^{b}	700^{b}	608^{c}	789^{a}	27.5	0.01
n-3	214^{b}	190^{b}	197^{b}	256^{a}	22.1	<0.001
n-6	466^{ab}	506^{a}	410^{b}	534^{a}	78.5	0.04
n-6/n-3	2.18^{b}	2.66^{a}	2.08^{b}	2.09^{b}	0.18	<0.001
Lipid						
Total Phospholipid, g/dL						
Week 0	2.27	2.20	2.15	2.19	0.25	0.65
Week 3	2.05^{b}	2.15^{b}	1.63^{c}	2.46^{a}	0.15	<0.001

Total Cholesterol, mg/dL						
Week 0	364	375	363	370	28.4	0.54
Week 3	350	372	366	375	45.2	0.54
Triglycerides, g/dL						
Week 0	4.78	4.69	4.73	4.75	0.54	0.49
Week 3	4.45[a]	4.21[a]	2.65[c]	3.52[b]	0.49	<0.001
Non-esterified fatty acid, μmol/mL						
Week 0	8.55	8.68	8.70	8.58	0.46	0.64
Week 3	8.50[b]	8.62[b]	8.40[b]	9.00[a]	0.36	0.03

*Data expressed as means (n=5).

[a,b,c] Means in the same row without a common letter differ ($P < 0.05$).

[1]DHA, Docosahexaenoic Acid.

[2]SFA, saturated fatty acid; [3]MUFA, mono-unsaturated fatty acids; [4]PUFA, polyunsaturated fatty acids.

Table 4.15 Effects of the novel phospholipid components & *Aurantiochytrium* on body weight, feed intake, egg production, and egg components

Treatment	Treatment 1	Treatment 2	Treatment 3	Treatment 4	SEM	P Value
Initial BW[1], g	1610	1630	1600	1620	147	0.72
Final BW, g	1600	1620	1610	1600	165	0.62
ADFI[2], g/d	106	104	105	103	12.4	0.63
Egg Production, %	0.88	0.89	0.90	0.88	0.04	0.69
Week 0						
Total egg, g	56.5	57.7	57.1	54.1	3.10	0.47
Yolk, g	16.1	15.8	16.1	16.4	1.42	0.48
Albumen, g	33.1	31.2	33.5	30.1	1.90	0.45
Shell, g	4.64	5.05	4.52	5.64	0.35	0.34
Shell Thickness, mm	34.9	36.7	33.7	40.5	1.45	0.49
Week 3						
Total egg, g	54.3	53.1	56.5	54.9	3.18	0.34
Yolk, g	15.8	15.7	17.1	16.5	2.08	0.49
Albumen, g	31.1	30.2	31.9	31.4	2.68	0.37
Shell, g	5.19[a]	3.83[b]	5.3[a]	4.73[a]	0.809	0.04
Shell Thickness, mm	38.4	35.8	37.7	34.8	2.62	0.78

*Data expressed as means (n=5).

[a,b] Means in the same row without a common letter differ ($P < 0.05$).

[1]BW, Body Weight.

[2]ADFI, Average Daily Feed Intake.

Table 4.16 Effects of the novel phospholipid components & *Aurantiochytrium* on hepatic and egg yolk fatty acid distribution in different lipid species

Treatment	Treatment 1	Treatment 2	Treatment 3	Treatment 4	SEM	P Value
Liver, mg/g tissue						
Week 6						
C20:4						
PL[1]	114[a]	83.0[b]	59.4[c]	55.2[c]	5.47	0.04
TG[2]	2.56[a]	1.87[b]	1.33[c]	1.24[c]	0.17	0.03
NEFA[3]	11.5[a]	8.39[b]	6.00[c]	5.58[c]	0.64	0.01
C22:6 (DHA[4])						
PL	0.00[d]	103[c]	125[b]	187[a]	15.4	0.01
TG	0.00[d]	2.32[c]	5.76[b]	18.0[a]	14.3	0.01
NEFA	0.00[d]	10.4[c]	12.9[b]	20.3[a]	2.4	0.01
Week 6						
C20:4						
PL	106[a]	78.9[b]	58.4[c]	41.5[d]	4.65	0.01
TG	2.38[a]	1.77[b]	1.31[c]	0.93[d]	0.18	0.01
NEFA	10.7[a]	7.97[b]	5.90[c]	4.19[d]	0.65	0.01
C22:6 (DHA)						
PL	0.00[c]	109[b]	159[a]	163[a]	14.5	0.01
TG	0.00[d]	2.46[c]	7.32[b]	15.7[a]	1.60	0.01
NEFA	0.00[c]	11.1[b]	16.5[a]	17.6[a]	1.41	0.01

*Data expressed as means (n=5).

[a,b,c,d] Means in the same row without a common letter differ ($P < 0.05$).

[1]PL, Phospholipid.

[2]TG, Triglyceride.

[3]NEFA, Non-esterified Fatty Acid.

[4]DHA, Docosahexaenoic Acid.

Table 4.17 Effect of the novel phospholipid components & *Aurantiochytrium* on plasma lipid and health biomarkers in laying hen

Treatment	Treatment 1	Treatment 2	Treatment 3	Treatment 4	SEM	P Value
Enzyme						
ALT, U/L						
Week 0	136	127	141	135	18.3	0.74
Week 3	124	118	121	126	29.2	0.65
AKP, U/mL						
Week 0	52.3	56.3	49.3	54.3	10.2	0.92
Week 3	69.3	62.4	71.3	65.4	8.65	0.55
Inorganic phosphorus, mg/dL						
Week 0	37.8	32.4	31.4	34.7	9.40	0.65
Week 3	35.6	39.7	36.4	39.5	11.2	0.73
Lipid, g/dL						
Total Phospholipid						
Week 0	21.4	22.0	23.3	22.5	2.57	0.68
Week 3	23.5	20.5	21.5	23.8	3.10	0.43
Total cholesterol, mg/dL						
Week 0	75.3	72.3	76.9	74.3	7.52	0.56
Week 3	81.2	76.8	80.2	81.1	4.66	0.39
Triglycerides, g/dL						
Week 0	1.25	1.29	1.31	1.21	0.37	0.63
Week 3	1.29	1.36	1.35	1.32	0.47	0.24
Non-esterified fatty acid, μmol/mL						
Week 0	0.31	0.33	0.29	0.32	0.11	0.67
Week 3	0.28	0.28	0.31	0.28	0.13	0.28

Glucose, g/L						
Week 0	4.44	4.12	4.67	4.35	0.53	0.56
Week 3	3.84	3.67	3.95	4.05	0.49	0.39
Uric acid, mmol/L						
Week 0	0.65	0.64	0.66	0.64	0.45	0.71
Week 3	0.63	0.69	0.63	0.62	0.56	0.56

Table 4.18 Effects of the novel phospholipid components & *Aurantiochytrium* on hen liver and plasma fatty acid concentrations

Treatment	Treatment 1	Treatment 2	Treatment 3	Treatment 4	SEM	P Value
Liver, mg/g						
C20:4	2.15[a]	1.74[b]	1.35[c]	0.93[d]	0.281	<0.001
C22:6 (DHA[1])	0.474[c]	1.64[b]	2.06[ab]	2.38[a]	0.436	<0.001
SFA[2]	14.9[a]	15.6[a]	12.5[b]	11.0[c]	1.63	<0.001
MUFA[3]	14.2[a]	14.5[a]	10.7[b]	8.83[b]	2.25	<0.001
PUFA[4]	8.64[a]	8.56[a]	7.35[ab]	6.16[b]	1.22	0.0024
n-3	0.474[c]	1.64[b]	2.06[ab]	2.38[a]	0.436	<0.001
n-6	8.16[a]	6.92[a]	5.29[b]	3.78[c]	1.05	<0.001
n-6/n-3	17.1[a]	4.29[b]	2.60[b]	1.68[b]	3.52	<0.001
Plasma, mg/ml						
Week 3**						
C20:4	0.493	0.290	0.411	0.271	0.179	0.145
C22:6 (DHA)	0.175	0.367	0.428	0.260	0.216	0.472
SFA	4.86	5.33	5.20	3.45	1.60	0.191
MUFA	5.11	5.62	5.53	3.83	1.84	0.3
PUFA	2.88	3.16	3.02	1.96	0.865	0.111
n-3	0.175	0.367	0.428	0.260	0.216	0.472
n-6	2.71[ab]	2.80[a]	2.59[ab]	1.70[b]	0.750	0.046
n-6/n-3	16.2[a]	7.96[b]	9.07[b]	9.09[b]	5.03	0.041
Week 6***						
C20:4	0.410[a]	0.104[b]	0.353[ab]	N.D.[b]	0.207	0.021
C22:6 (DHA)	0.0709[b]	0.169[b]	0.88[a]	0.825[a]	0.363	<0.001
SFA	5.34	3.99	9.24	4.67	4.11	0.699
MUFA	6.01	4.10	9.36	4.89	3.92	0.812
PUFA	3.09[ab]	1.98[b]	4.46[a]	2.35[ab]	1.61	0.033

n-3	0.0709[b]	0.169[b]	0.702[a]	0.825[a]	0.363	<0.001
n-6	3.01[ab]	1.81[ab]	3.75[a]	1.53[b]	1.52	0.042
n-6/n-3	22.3[a]	4.41[b]	3.29[b]	1.89[b]	4.45	<0.001

*Data expressed as means (n=5).

**Hens were not fasted prior to blood collection.

***Hens were fasted prior to blood collection.

[a,b,c,d] Means in the same row without a common letter differ ($P < 0.05$).

[1]DHA, Docosahexaenoic Acid.

[2, 3, 4]SFA, saturated fatty acid; MUFA, mono-unsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Table 4.19 Effect of the novel phospholipid components & *Aurantiochytrium* on hen hepatic gene expression.

Treatment	Treatment 1	Treatment 2	Treatment 3	Treatment 4	SEM	p Value
			Anabolic			
ACC	1.00±0.049^{c}	0.80±0.018^{d}	1.18±0.079^{b}	1.43±0.070^{a}	0.047	0.041
COX1	1.00±0.15^{b}	0.61±0.069^{d}	0.81±0.074^{c}	1.45±0.30^{a}	0.082	0.002
DGAT2	1.00±0.31^{a}	1.34±0.28^{a}	0.44±0.073^{b}	1.07±0.46^{a}	0.015	0.005
FASN	1.00±0.072^{c}	1.09±0.04^{b}	1.17±0.046^{b}	1.52±0.104^{a}	0.015	0.021
SREBP1c	1.00±0.041^{c}	1.02±0.081^{c}	1.24±0.0024^{b}	1.78±0.13^{a}	0.14	0.001
			Catabolic			
ACOT4	1.00±0.23^{b}	0.74±0.13^{a}	1.29±0.26^{a}	1.07±0.33^{a}	0.025	0.035
CPT1A	1.00±0.11^{b}	1.72±0.18^{a}	1.77±0.20^{a}	9.90±0.72^{c}	0.16	0.003
PLPP1	1.00±0.077^{c}	7.11±0.19^{b}	8.04 ±0.52^{b}	14.3 ±1.40^{a}	0.13	0.001
PPARA	1.00±0.046^{b}	0.76±0.029^{d}	0.90±0.021^{c}	1.67±0.0.13^{a}	0.098	0.039
			Conversion			
5-LOX	1.00±0.15^{b}	0.55±0.028^{c}	1.64±0.31^{a}	1.59 ±0.323^{a}	0.063	0.021

DEGS1	1.00±0.069[d]	1.56±0.052[c]	1.74±0.13[b]	2.69±0.192[a]	0.017	0.005
ELOVL2	1.00±0.0.31[b]	0.86±0.05[c]	0.82±0.040[c]	1.67 ±0.12[a]	0.082	0.025
ELOVL5	1.00±0.044[d]	1.13±0.088[c]	1.33±0.084[b]	1.88±0.39[a]	0.016	0.005
FADS2	1.00±0.039[c]	0.92±0.038[c]	1.20±0.065[b]	2.14 ±0.099[a]	0.096	0.002
			Absorption			
LPL	1.00±0.138[b]	0.75±0.184[b]	0.83±0.191[b]	11.2[a]*	0.089	0.035
			Phospholipid Synthesis			
CHPT1	1.00±.27[a]	0.20±0.06[b]	0.37±0.097[b]	0.86±0.23[a]	0.048	0.001
EPT1	1.00±0.002[a]	0.19±0.037[c]	0.25±0.023[b]	0.12 ±0.041[c]	0.025	0.003

*Data expressed as means (n=5).

Data analyzed via one-way ANOVA.

[a,b] Means in the same row without a common letter differ ($P < 0.05$).

Abbreviation: *ACC*, Acetyl-CoA carboxylase; *ACOT4*, Acyl-CoA thioesterase 4; *CHPT1*, Choline Phosphotransferase 1; *COX1*, Cyclooxygenase 1; *CPT1A*, Carnitine Palmitotransferase 1; *DEGS1*, Delta-4 Desaturase; *ELOVL2*, Elongase 2; *ELOVL5*, Elongase 5; *EPT1*, Ethanolaminephosphotransferase 1; *FADS2*, Delta-6 Desaturase; *FASN*, Fatty acid synthase; *LPL*, Lipoprotein Lipase; *PLPP1*, Phospholipid Phosphatase 1; *PPARA*, Peroxisome Proliferator Activator Receptor Alpha; *SREBP1c*, Sterol Regulatory Element Binding Protein 1c.

Strikingly, additional serine increased total egg yolk n6 concentrations while ethanolamine decreased them, while these effects were on induced upon different n6 fatty acids linoleic acid and arachidonic acid for ethanolamine and serine respectively (**Table 4.18**). Total PUFA and n-3 content were increased ($P < 0.05$) by the full combination, while n6s were downregulated by ethanolamine, and n6/n3 the highest in the serine group. Total phospholipids and TG were decreased ($P < 0.05$) in the ethanolamine treatment group and total NEFAS increased ($P < 0.05$) in the full combination group. The full combination of serine, choline, and ethanolamine, enhanced ($P < 0.05$) *CPT1A*, *LPL*, and *PLPP1*, while continuing to suppress both *CHPT1* and *EPT1* (**Table 4.19**) (**Figure 4.4**).

4.5 Discussion

This study proved that laying hens can tolerate doses of the DHA rich microalgae Aurantiochytrium of up to 2% within their diets for 6 weeks without negative impacts on their health or egg production. Total egg and yolk weights were decreased by the highest concentration of the algae at 4% inclusion which may not be appealing to consumers, however, this difference was not seen at either 1 or 2% algae supplementation [116]. Plasma ALT began to increase in the 2% algae at the end of the study and while this was reversed in the 4% algae group these changes in liver function may suggest a tolerance level for DHA and going above this may be dangerous to animal health which has been seen before when feeding high levels of algae and omega 3 fatty acids [117] [118]. Strikingly there was a decrease in plasma glucose at week 3 which may signify enhanced insulin sensitivity from DHA or a change in energy demand [119]. Docosahexaenoic acid within the algae was both bioavailable and metabolizable by laying hens with it being deposited in a dose dependent manner in egg yolks at concentrations of 116, 144, 225 mg/g of egg for 1%, 2%, and 4% algae inclusion respectively. Enrichment efficiency of DHA from the diet to

what goes into the egg yolk decreases from 68%, 42%, and to 33% in the 1%, 2%, and 4% algae treatment groups respectively which is consistent with other types of n3 rich algae such as *phaeodactylum tricornutum*, *nannochloropsis oculata*, and *isochrysis galabana* [105] [111]. This implies that the range for most efficient incorporation of DHA in laying hens was between 116-144 mg/g of diet per day. The mechanism behind this decrease in efficiency is unknown but perhaps it could be due to a limiting factor in egg yolk formation. According to the Dietary Guidelines for Americans, 2015, the daily allowance of EPA+DHA is approximately 500 mg/day, which could be met by two of the eggs from the highest dose of algae in this study [41, 42]. As the average American consumes far more n6 fatty acids relative to n3, the egg serves as an excellent and very accessible carrier for these nutrients to correct this imbalance and improve human health including prevention of cancer, diabetes, and CVD [120] [121] [122] [123] [124] [125].

Both liver fatty acid profiles and hepatic mRNA gene expression of lipid related genes were substantially altered by *Aurantiochytrium* supplementation. The liver is the major organ for both processing, converting, and exporting lipids in the body for not only energy but egg yolk formation in laying hens. There was a negative correlation with the amount of DHA present in the diet and the amount of fatty acids, including SFA, MUFA, and n3 and n6 PUFA in the liver. The ability of DHA to suppress fat accumulation has been demonstrated before in both laying hens through other algae supplementation, however, when utilizing pure oils of DHA high concentrations can cause fatty liver disease and subsequent damage [126] [127] [128]. Expression of genes indicative of lipid anabolism were downregulated aside from *FASN* which may signify a long chain inhibition while short chain FAs are unaffected. While the effect of DHA suppressing laying hen hepatic *SREBP1c* and *ACC* expression has been seen before, there have been mixed

results with *FASN* which suggests this gene may be more sensitive to other factors aside from DHA [7] [129] [130]. Seemingly, high DHA concentrations enhanced the breakdown of the complexed forms of fatty acids including triglycerides and phospholipids signified through the upregulation of *ACOT4* and *PLPP1* respectively. Increased expression of *COX1* may suggest that the synthesis of eicosanoids was upregulated by dietary DHA, while this is associated with an increased risk for ovarian cancer this has only been demonstrated with the shorter chain n3 ALA [131]. As eicosanoids are hormones which are regulators of both inflammation, immune response, and are derived both from arachidonic acid or DHA when present, laying hens may synthesize these opportunistically when DHA is present and this has been used as a preventative of cancer [6] [132]. Creation of new long chain fatty acids including both desaturation, *DEGS1* and *FADS2*, and elongation, *ELOVL2* and *ELOVL5*, were downregulated by dietary DHA. The downregulation of fatty acid conversion is consistent with the tissue FA profiles and is controlled by negative feedback of DHA on these pathways which could explain why DHA reduces fatty liver incidence [133]. Hepatic absorption of lipids bound to lipoproteins being transported via the systemic circulatory system was enhanced as suggested by increased *LPL* expression which could be improving lipid turnover and transport [134]. Formation of phosphatidylcholine and phosphatidylethanolamine was upregulated signified by enhanced *CHPT1* and *EPT1* respectively which is positively correlated with chylomicron formation and transport [43] [135] [136]. Protein concentrations of *SREBP1c* and *FASN* demonstrate DHAs suppression of hepatic fatty acid anabolism, demonstrating directly that DHA can reduce lipid transport and turnover in layers.

Adipose tissue responded to high levels of circulating DHA in a similar fashion to liver tissue with regards to overall anabolism and catabolism of lipids. Unlike hepatocytes, adipocytes display a limited capacity to store and synthesis long chain fatty acids beyond carbon length 18

across almost every animal species [137-139]. Adipocytes have been shown to instead export EPA and DHA in an n-acyl ethanolamine form, a paracrine lipo-hormone established to reduce adiposity and reduce body fat [140]. Adipose de novo synthesis, *FASN*, was upregulated while *SREBP1c* and *ACC* was suppressed suggesting that lipid storage and formation was disrupted by DHA. As seen in the liver tissue, *ACOT4* and *PLPP1* were upregulated signifying enhanced triglyceride and phospholipid breakdown as well as decreased *CPT1* and *PPARA* suggesting depressed free fatty acid catabolism. Prostaglandin synthesis was seemingly impaired by *5LOX* downregulation which may demonstrate these hormones are used to communicate between adipose and ovary tissue for yolk formation [131]. Conversion and desaturation were mostly unaffected except for the enzyme dedicated to converting 20 carbon fatty acids to 22 carbon length, *ELOVL2*, which was suppressed. Lipoprotein lipase, *LPL*, mRNA expression was suppressed suggesting adipose tissue had impaired absorption of lipids from the blood supply which has been shown to aid in weight loss for humans [141]. Overall synthesis of phosphatidylcholine within the adipose tissue was depressed as shown by the downregulation of *CHPT1* which may signify reduced lipid turnover and export of very low density lipoproteins within adipocytes when DHA is present.

Laying hen's ovary tissue is unique in that it releases 1 follicle per day for many consecutive days for egg formation. These follicles comprise the egg yolk which is made up of an outer phospholipid membrane made up of mainly phosphatidylcholine and phosphatidylethanolamine species with free fatty acids and triglycerides internally [142]. Ovary tissue relies on circulating lipids and incorporates them from lipoprotein complexes, particularly very low density lipoproteins [143]. Strikingly, lipid metabolism-related genes were almost all ubiquitously upregulated by the highest dose of dietary DHA which may suggest that ovary action

may have a unique mechanism tied to this fatty acid. As this upregulation was not consistent with liver or adipose tissue the response of ovary to *Aurantiochytrium* may be related to the demand to create an egg yolk while there is a dramatic change in lipid metabolism. Egg yolks became smaller with higher levels of DHA which may be due to the changes in eicosanoids and prostaglandins [144]. Elongases and markers of lipid catabolism were the most responsive to DHA across treatments which may suggest the ovary was trying to create a lipid which was limiting in yolk formation. Both phospholipid breakdown and synthesis were upregulated via their genes *PLPP1*, *EPT1*, and *CHPT1* indicating yolk membrane formation was enhanced. Interestingly, *EPT1* is shown to promote estrogen sensitivity in ovarian tissue which is necessary for follicle development and release in layers [145]. Ovarian protein concentrations of *SREBP1c* and *FASN* were upregulated which was consistent with the change in gene expression. While this was the reverse of what was seen in the liver perhaps there are additional regulators in ovarian tissue for fatty acid synthesis as total yolk formation was decreased by DHA and perhaps this increase in protein concentration was meant to offset this.

Additional dietary phospholipid components when fed in tandem with the highest level of microalgal DHA enabled more DHA to be deposited into the egg yolks. Oddly, total phospholipid and TG content of the eggs were lowered implying that other fatty acids in the phospholipid form were removed when additional DHA containing phospholipids were synthesized. This is supported by the change in hepatic gene expression with a downregulation in both *CHPT1* and *EPT1* by the full combination of serine, ethanolamine, and choline. While the exact mechanism behind how DHA influences other fatty acid species when it is stored this data strongly implies that when DHA is incorporated into the phospholipid form this effect is greatly enhanced as opposed to its free fatty acid form

4.6 Conclusion

In summary, these two studies demonstrated that feeding up to 2% dietary *Aurantiochytrium* to laying hens enriched DHA in their liver, adipose, and egg yolks without adversely effecting performance or health, as well as that additional choline and ethanolamine can further promote DHA storage and enrichment efficiency. Hen fatty acid metabolism was significantly modulated by high concentrations of DHA with upregulation of key genes for triglyceride and phospholipid catabolism, *ACOT4* and *PLPP1*, in liver, adipose, and ovary tissue. While genes indicative of general fatty acid anabolism were suppressed in all tissues including *SREBP1c*, *ACC*, and *DGAT2*, de novo synthesis was upregulated by the enhanced expression of *FASN*. Protein concentrations of *FASN* and *SREBP1c* in the liver and ovarian tissue imply that DHA suppresses fatty acid anabolism in the liver with a reversed effect in the ovary. Conversion and synthesis was long chain fatty acids was disrupted in liver and adipose tissue by the downregulation of *DESGS1*, *FADS2*, *ELOVL2*, and *ELOVL5* while this was reversed in ovary tissue which had enhanced expression. Formation of phospholipids including phosphatidylcholine and phosphatidylethanolamine genes *CHPT1* and *EPT1* were upregulated in liver and ovary suggesting DHA promotes more lipid droplet and chylomicron formation. The concentration of DHA in egg yolks reached up to 225 mg/egg which would fulfill the American Heart Association daily allowance of n3 fatty acids at 2 of these eggs per day, this has not been achieved before. While there were no significant impacts hen health or performance at 4% of *Aurantiochytrium* for 6 weeks we would suggest that 2% would seem to be the wiser choice for long term production as there we significant alterations in lipid metabolism which may not be sustainable for longer time periods.

Chapter Five

Simultaneous Enrichment of the Three Health Promoting Compounds: Docosahexaenoic Acid, Astaxanthin, and Calcifediol

5.1 Summary

Eggs serve as an excellent carrier of lipophilic compounds including AST and DHA, two compounds typically only found in marine products which are scarce in the United States diet. An additional micronutrient of interest, vitamin D, is difficult to obtain aside from milk which has been fortified with it. While lactose intolerance is somewhat prevalent in the US, it is much higher in eastern Asia which makes finding alternative food choices to milk for obtaining vitamin D a necessity. The focus of this experiment was to explore the potential to enrich eggs simultaneously with all three health promoting agents, AST, DHA, and calcifediol to improve human health by providing a nutritionally sound and fortified animal product.

5.2 Introduction

While our two previous studies focused on the marine AST and DHA for improving human health, there is an additional micronutrient which the average American is insufficient in which is vitamin D. Currently within the United States it is difficult for the average consumer to meet the USDA recommended daily intake of vitamin D without consuming dairy products or fortified orange juice and grains [146]. Deficiency of vitamin D is correlated with higher risks of cancer, multiple sclerosis, diabetes, elevated blood pressure, stroke, and poor cognition [147]. People who do not get much sun exposure or who are older are more susceptible to vitamin D deficiency and consume supplements to compensate for this [148]. Very few foods are enriched

with vitamin D which leads to a desire to enrich commonly consumed foods with this nutrient [149]. While eggs naturally serve as a source of vitamin D there is potential to further increase the concentration of this nutrient and its hydroxylated form calcifediol [150] [151]. There are different forms of vitamin D including vitamin D_3 and its more activated form 25 OH-vitamin D_3 with the latter demonstrating 3-5 times more potency for human and animal health [152].

The recommended daily requirement of vitamin D_3 for adults is approximately 600 IU [153]. Approximately 100g of egg yolk contains between 1 to 3 *u*g of vitamin D_3 [5]. Little research has been conducted to determine the efficacy of enriching eggs with vitamin D3, however, it has been demonstrated that yolks were capable of be enriched with levels of up to 7 *u*g/100 g and 4 *u*g/100 g of cholecalciferol and calcifediol respectively [106] [154] [53] [109]. Vitamin D supplementation in layers has been studied for its benefit to improve egg shell quality and immune function in order to maximize performance potential and minimize egg breaking which is a concern in the layer industry. The NRC requirement of vitamin D for layers is set to 300 IU/kg while in industry diets containing up levels up 2 to 3 times this are utilized [155]. Dietary levels of up to 4000 IU have been reached without any adverse effect on layer performance and egg shell strength improved with this dietary inclusion [6].

Generally, nutrient incorporation into animal products has been shown to improve their respective bioavailability including the absorption and retention as opposed to taking the nutrients as an isolated supplement [156]. While milk serves as an excellent carrier for vitamin D, considering its fat and calcium content, there are many Americans who cannot consume dairy due to allergies to either whey or lactose [157] [158]. Supplementation of calcium in either a pill or powder form is taken by approximately 43 percent of all people and by 70 percent of

postmenopausal woman which could be reduced by further incorporation of vitamin D into foods [159].

5.3 Materials and Methods

Protocols were approved by the Institutional Animal Care and Use Committee of Cornell University (Ithaca, NY). Supplemental microalgae, *Haematococcus pluvialis,* were provided by Heliae Development, LLC (Gilbert, Arizona, USA), DHA Oil by Archer Daniels-Midland (Chicago, Illinois, USA), and calcifediol by DSM (Heerlen, Netherlands).

Shaver-White laying hens (20-wk old, donated by Kreher Farmers, Clarence, NY, USA) were divided into 5 dietary treatments based on egg production and body weight (n=10). Hens were maintained on dietary treatments for 6 wk. Diet 1 consisted of a corn-soy basal, diet 2 = diet 1 + 0.8% DHA oil, diet 3 = diet 2 + 200 ug/kg calcifediol, diet 4 = diet 2 + 0.16% AST, and diet 5 = diet 3 + 0.16% AST.

Hens were individually caged in 0.44 m high x 0.30 m wide x 0.45 m deep units inside an environmentally controlled room maintained at 25°C, 55% relative humidity. Hens had free access to feed and water and exposed to 8:16 dark:light cycles. Vitamins, minerals, and crystalline amino acids were added to meet laying hen nutrient requirements (NRC, 1994) and diets were formulated to be isocaloric and isonitrogenous [71]. Proximate and mineral analyses were completed by Dairy One, Inc. (Ithaca, NY). Diet formulations are presented in **Table 5.1,** and diet fatty acid profiles in **Table 5.2**. Body weights were measured weekly, feed intake biweekly, and egg production daily. Eggs from each treatment were collected at wk 3 and 6 to be evaluated for egg component weights and biochemical composition. Blood was drawn from wing veins at wk 0, 3, and 6 to

prepare plasma samples which were subsequently stored at -20°C until analyses. At week 6, 5 representative birds per diet (selected based on average egg production and body weight) were euthanized by carbon dioxide asphyxiation. Subsamples of liver, breast muscle, adipose, and ovary were harvested and immediately frozen in liquid nitrogen and stored at -80^{o}C for gene expression analyses. Additionally, frozen liver, breast muscle, adipose, and ovary tissue samples stored at -20^{o}C to be used for compositional analyses.

Egg components were measured using the same protocol as described by Kim et al. [109] Blood was drawn from wing veins at wk 0, 3, and 6 and was held on ice during collection, centrifuged at 2,000 g for 20 min at 4oC, and stored at -20oC until analyses. Plasma glucose was determined spectrophotometrically with a glucose assay kit (GAG020, Sigma-Aldrich, Sigma Chemical Co., St. Louis, MO). Plasma uric acid was analyzed with Infinity Uric Acid Liquid Stable Reagent (Thermo-Fisher Scientific, Inc., MA). Plasma non-esterified fatty acids, triglyceride, total phospholipid, and total cholesterol were analyzed using commercial enzymatic kits following manufacturer's protocols (Wako Pure Chemical Industries, Ltd., Richmond, VA). All samples were analyzed in duplicate. Lipids were extracted from egg yolk, plasma, and tissue utilizing a modified methodology created by Folch et al. as described by Manor et al. [105] which involved homogenizing samples in TRIZMA-EDTA followed by two extractions in Folch solution, 2:1 chloroform-methanol (v/v), and 4:1 chloroform-methanol. Chloroform layers were separated via centrifugation at 3000 RPM for 15 minutes at 10 °C. Prior to methylation the fatty acids concentrated in the chloroform were evaporated using industrial N_2 gas in a water bath at 60 °C. Fatty acids were methylated with methanolic sulfuric acid (1%) and tritridecanoin was used as an internal standard. Fatty acids were identified by their respective retention time of the fatty acid methyl ester standard (Sigma-Aldrich Co., St. Loius MO). Methyl esters of fatty acids were

analyzed within 24 hours of methylation using a gas chromatography (Agilent 6890N, Agilent Technologies, Santa Clara, CA) fitted with a flame-ionization detector. A fused-silica capillary column coated with CP-SIL 88 for fatty acid methyl esters (1.00 m x 0.25 mm i.d., 0.2 mm film thickness) was used (Varian Inc., Lake Forest, Ca). Oven temperature was programmed to be held for 4 min at 140 °C, increased by 4 °C per minute to 220 °C, and then held for 5 min. Carrier gas was N_2 with a constant flow rate of 45 mL/min. The injector temperature was 230 °C and the detector temperature was 280 °C. For analysis of individual lipid species, prior to methylation a separate extraction procedure was utilized following the methodology was described by Corl et al. [114] which involved extraction of lipids followed by passing them through a solid phase extraction column to isolate triglyceride, cholesterol, and phospholipid components. Total concentrations of vitamin D3 and calcifediol were measured utilizing an adapted method developed for egg yolks and meat by Mattila et al [38].

Software R (version 3.3.1, R Foundation for Statistical Computing, Vienna, Austria) was used for the data analyses. Each hen or cage was the experimental unit. Main effects of the treatments were analyzed by one-way ANOVA and the mean comparisons were conducted with Duncan's multiple-ranged method. Data were presented as means ± SEM, and the significance level for differences was $P < 0.05$. The P value was adjusted using a *Bonferroni* correction procedure for multiple regression analyses, with the significance level at $P \leq 0.01$.

Table 5.1 Ingredient composition of multi-enrichment layer study experimental diets

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
Ingredient, %					
Corn	64.8	64.8	64.8	64.8	64.8
Soybean Meal	23.7	23.7	23.7	23.7	23.7
DHA Oil	0.00	0.80	0.80	0.80	0.80
Calcifediol (ug/kg) (Calculated)	0.00	0.00	200	0.00	200
Calcifediol (ug/kg) (Analyzed)	0.00	0.00	194	0.00	208
AST (mg/kg) (Calculated)	0.00	0.00	0.00	0.16	0.16
AST (mg/kg) (Analyzed)	0.00	0.00	0.00	0.06	0.07
Corn Oil	1.50	1.50	1.50	1.50	1.50
Dicalcium Phosphate	0.94	0.94	0.94	0.94	0.94
Limestone	7.89	7.89	7.89	7.89	7.89
NaCl	0.35	0.35	0.35	0.35	0.35
Methionine	0.08	0.08	0.08	0.08	0.08
Isoleucine	0.09	0.09	0.09	0.09	0.09
Cellite	0.30	0.30	0.30	0.30	0.30
Vitamin/Mineral Mix[1]	0.35	0.35	0.35	0.35	0.35

[1]The vitamin/mineral mix contained vitamin A 4550 IU/kg, vitamin D 450 IU/kg, vitamin E 7.5 IU/kg, vitamin K 0.752 mg/kg, biotin 0.152 mg/kg, choline 1575 mg/kg, folacin 0.376, niacin 15.147 mg/kg, pantothenic acid 3 mg/kg, riboflavin 3.76 mg/kg, thiamine 1.07 mg/kg, vitamin B63.78 mg/kg, vitamin B12 0.006 mg/kg, copper 12 mg/kg, iodine 0.05 mg/kg, 30.23 mg/kg, manganese 53 mg/kg zinc, selenium 0.09 mg/kg, and iron 67.82 mg/kg.

Table 5.2 Fatty acid profiles and astaxanthin concentrations of laying hen diets, DHA Oil, and full fatted *Haematococcus pluvialis*

Treatment	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	DHA Oil	*Haematococcus pluvialis*
Fatty Acid, g/kg							
C14	0.00	0.49	0.42	0.43	0.42	232	N/A
C16	2.20	2.05	1.92	1.94	1.96	109	N/A
C18	0.31	0.26	0.27	0.26	0.24	0.00	N/A
C18:1	3.26	2.70	2.80	2.76	3.06	0.00	N/A
C18:2	7.55	6.20	6.40	6.49	6.65	19.0	N/A
C18:3	0.38	0.34	0.37	0.37	0.31	0.00	N/A
C20:5	0.00	0.00	0.00	0.00	0.00	126	N/A
C22:6 (DHA[1])	0.00	3.60	3.50	3.60	3.60	445	N/A
SFA	2.51	2.8	2.61	2.63	2.62	341	N/A
MUFA	3.26	2.7	2.8	2.76	3.06	0.00	N/A
PUFA	7.93	7.21	7.43	7.54	7.62	590	N/A
n-3	0.38	1.01	1.03	1.05	0.97	571	N/A
n-6	7.55	6.2	6.4	6.49	6.65	19.0	N/A
n-6/n-3	19.87	6.14	6.21	6.18	6.86	0.03	N/A
AST[2] (mg/kg)	0.00	0.00	0.00	0.06	0.07	0.00	6.70

[1]DHA, Docosahexaenoic Acid.

[2]AST, Astaxanthin.

5.4 Results

Neither AST, DHA, or calcifediol negatively impacted body weight gain, average daily feed intake, or egg production of hens throughout the 6 week period (**Table 5.4**). Additional calcifediol significantly increased the total egg shell weight and shell thickness in both diet 3 and 5 at week 3 and 6. Hepatic and yolk arachidonic acid was decreased in all DHA oil containing diets with stronger effects in the PL fraction (**Table 5.3**). Total DHA was elevated in every diet with DHA oil for both liver an yolk but with the majority of said enrichment happening within PL. Astaxanthin was detected in both liver and egg yolk for diets 4 and 5 but with noticeably lower concentrations than the previous study. Calcifediol was capable of being enriched up to 9.3 ug/100 g yolk at week 3 and 6.0 ug/100 g yolk at week 6. Concentrations of d, l-α-tocopherol were significantly lower in DHA oil containing diets for both egg yolk and liver. Plasma ALT was significantly higher across all DHA-oil added diets (**Table 5.5**). Conversely, plasma inorganic phosphorus was significantly lower in the full combination group fed diet 5. Plasma total phospholipid, TG, and NEFA were lowered by dietary DHA oil. Vitamin D metabolism related gene expression was generally increased by dietary calcifediol whereas there were mixed effects upon lipid metabolism by all three compounds (**Table 5.6**).

Table 5.3 Effects of dietary DHA oil, astaxanthin, and calcifediol on hepatic and egg yolk fatty acid distribution in different lipid species, and astaxanthin, calcifediol, and d, l-α-tocopherol concentrations

Treatment	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	SEM	P Value
Liver, mg/g tissue							
Week 6							
C20:4							
PL[1]	2.01[a]	1.70[b]	1.72[b]	1.69[b]	1.78	0.10	0.05
TG[2]	0.05	0.04	0.04	0.04	0.04	0.001	0.20
NEFA[3]	0.20	0.17	0.17	0.17	0.18	0.05	0.25
C22:6 (DHA[4])							
PL	0.44[b]	1.61[a]	1.62[a]	1.63[a]	1.72[a]	0.30	0.01
TG	0.010[b]	0.036[a]	0.033[a]	0.035[a]	0.038[a]	0.15	0.01
NEFA	0.04[b]	0.16[a]	0.16[a]	0.16[a]	0.17[a]	0.08	0.01
AST[5] (mg/g)	0.00[b]	0.00[b]	0.00[b]	1.81[a]	1.75[a]	0.96	0.01
d, l-α-tocopherol (ug/g)	23.5[a]	17.6[b]	21.8[a]	17.1[b]	15.6[b]	2.20	0.01
Egg Yolk, mg/g							
Week 3							
Calcifediol (ug/100 g yolk)	0.88[b]	0.55[b]	7.62[a]	0.84[b]	9.25[a]	2.54	0.01
Week 6							
C20:4							
PL	6.50[a]	3.32[b]	3.25[b]	3.82[b]	3.40[b]	1.5	0.02
TG	0.146[a]	0.075[b]	0.073[b]	0.086[b]	0.08[b]	0.60	0.03
NEFA	0.66[a]	0.34[b]	0.33[b]	0.39[b]	0.34[b]	0.25	0.02
C22:6 (DHA)							
PL	0.00[b]	6.86[a]	6.54[a]	6.56[a]	6.45[a]	3.7	0.01
TG	0.00[c]	0.15[b]	0.30[a]	0.63[a]	0.62[a]	0.14	0.01
NEFA	0.00[b]	0.69[a]	0.68[a]	0.71[a]	0.70[a]	0.36	0.01

Calcifediol (ug/100 g yolk)	1.27[b]	1.15[b]	6.04[a]	2.33[b]	4.86[a]	2.23	0.01
AST (mg/g)	0.00[b]	0.00[b]	0.00[b]	5.50[a]	5.30[a]	1.56	0.01
d, l-α-tocopherol (ug/g)	100.1[a]	91.2[ab]	97.5[a]	89.6[ab]	85.4[b]	8.60	0.03

*Data expressed as means (n=5).

[a,b]Means in the same row without a common letter differ ($P < 0.05$).

[1]PL, Phospholipid.

[2]TG, Triglyceride.

[3]NEFA, Non-esterified Fatty Acid.

[4]DHA, Docosahexaenoic Acid.

[5]AST, Astaxanthin.

Table 5.4 Effects of dietary DHA oil, astaxanthin, and calcifediol on body weight, feed intake, egg production, and egg components

Treatment	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	SEM	P Value
Initial BW[1], g	1540	1550	1530	1560	1530	137	0.84
Final BW, g	1610	1620	1600	1630	1610	142	0.42
Egg Production, %	0.92	0.90	0.91	0.89	0.90	0.09	0.78
Week 0							
Total egg, g	58.3	60.2	59.1	57.6	58.6	2.60	0.55
Yolk, g	16.8	16.7	16.3	16.4	16.0	1.22	0.62
Albumen, g	34.2	32.6	35.4	34.2	31.5	2.30	0.75
Shell, g	5.34	5.50	5.53	5.47	5.73	0.32	0.35
Shell Thickness, mm	40.4	40.8	41.5	40.9	40.8	1.75	0.45
Week 3							0.55
Total egg, g	60.2	57.4	59.8	58.20	60.3	5.14	0.34
Yolk, g	16.1	15.4	15.1	15.3	15.6	2.67	0.28
Albumen, g	33.1	35.6	32.3	33.5	33.5	2.63	0.33
Shell, g	5.49^{b}	5.41^{b}	6.30^{a}	5.39^{b}	6.40^{a}	0.58	0.01
Shell Thickness, mm	36.5^{b}	37.8^{b}	45.5^{a}	38.4^{b}	44.9^{a}	2.66	0.01
Week 6							
Total egg, g	63.1^{a}	58.5^{b}	61.5^{a}	57.9^{b}	62.1^{a}	2.56	0.02
Yolk, g	16.5^{a}	15.1^{b}	15.3^{b}	15.1^{b}	15.3^{b}	1.03	0.01
Albumen, g	36.3	35.4	35.6	35.4	35.7	1.61	0.09
Shell, g	5.51^{b}	5.46^{b}	6.43^{a}	5.54^{b}	6.42^{a}	0.51	0.01
Shell Thickness, mm	38.8^{b}	38.6^{b}	46.2^{a}	38.4^{b}	45.7^{a}	2.62	0.01

*Data expressed as means (n=5).

[a,b] Means in the same row without a common letter differ ($P < 0.05$).

[1]BW, Body Weight.

Table 5.5 Effects of dietary DHA oil, astaxanthin, and calcifediol on plasma lipid and health biomarkers in laying hen

Treatment	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	SEM	P Value
ALT[1], U/L							
Week 0	130	129	140	134	132	17.3	0.54
Week 6	124[b]	144[a]	146[a]	143[a]	172[a]	26.2	0.01
AKP[2], U/mL							
Week 0	54.3	55.3	52.3	53.4	51.3	8.2	0.72
Week 6	69.3	62.4	71.3	68.2	65.4	6.65	0.45
Inorganic phosphorus, mg/dL							
Week 0	37.8	32.4	31.4	33.8	34.7	7.40	0.55
Week 6	34.5[a]	27.2[a]	27.1[a]	27.4[a]	21.6[b]	8.2	0.01
Lipid, g/dL							
Total Phospholipid							
Week 0	21.4	22.0	23.3	23.0	21.9	2.43	0.56
Week 6	21.5[a]	14.5[b]	13.7[b]	15.6[b]	12.8[b]	2.10	0.47
Total cholesterol, mg/dL							
Week 0	75.9	74.6	75.7	73.9	74.1	7.20	0.69
Week 6	80.2	78.8	80.6	78.4	81.2	4.63	0.65
Triglycerides, g/dL							
Week 0	1.26	1.29	1.36	1.35	1.28	0.35	0.52
Week 6	1.74[a]	1.46[b]	1.52[b]	1.49[b]	1.39[b]	0.15	0.04
Non-esterified fatty acid, μmol/mL							
Week 0	0.32	0.35	0.31	0.30	0.32	0.15	0.69
Week 6	0.33[a]	0.30 [ab]	0.31 [ab]	0.29 [ab]	0.27[b]	0.09	0.05

Glucose, g/L							
Week 0	4.17	4.18	4.25	4.31	4.40	0.59	0.45
Week 6	4.10[a]	3.67[b]	3.75[b]	3.69[b]	3.63[b]	0.35	0.05
Uric acid, mmol/L							
Week 0	0.69	0.65	0.64	0.63	0.63	0.27	0.75
Week 6	0.96[ab]	1.36[a]	1.27[a]	1.41[a]	0.46[b]	0.67	0.02
AST[3] (ug/ml)	0.00[b]	0.00[b]	0.00[b]	1.10[a]	1.25[a]	0.54	0.01

*Data expressed as means (n=5).

Data analyzed via one-way ANOVA.

[a,b] Means in the same row without a common letter differ ($P < 0.05$).

[1]ALT, Alanine aminotransferase, U/L: activity defined as the rate of decreasing in absorbance at 340nm due to the oxidation of NADH.

[2]AKP, Alkaline phosphatase, U/mL: activity defined as the amount of p-nitrophenol product formed per minute when alkaline phosphatase exposed to phosphatase substrate.

[3]AST, Astaxanthin.

Table 5.6 Effects of dietary DHA oil, calcifediol, and algal AST on layer hepatic gene expression.

Treatment	Control	DHA Oil	DHA Oil + Calcifediol	DHA Oil + AST	Combination	P Value
			Vitamin D Metabolism			
CYP2R1	1.00±0.12[b]	2.09±0.45[a]	1.93±0.41[a]	1.92±0.15[a]	1.58±0.41[a]	0.02
CYP24A1	1.00±0.04[b]	0.79±0.04[b]	2.47±0.30[a]	1.00±0.07[b]	0.93±0.19[b]	0.01
VDR	1.00±0.09[b]	0.82±0.26[b]	1.09±0.17[b]	1.79±0.12[a]	1.81±0.09[a]	0.01
			Catabolic			
CPT1A	1.00±0.12[b]	2.39±0.24[a]	0.58±0.15[c]	0.46±0.21[c]	0.46±0.05[c]	0.01
			Anabolic			
DGAT1	1.00±0.33[b]	1.36±0.22[a]	1.41±0.32[a]	1.33±0.13[a]	1.32±0.28[a]	0.05
FASN	1.00±0.05	0.87±0.30	0.93±0.35	0.91±0.40	0.96±0.41	0.45
SREBP	1.00±0.06[a]	0.78±0.15[b]	0.49±0.12[b]	0.62±0.33[b]	0.48±0.20[b]	0.01
LPL	1.00±0.04[a]	1.05±0.23[a]	0.67±0.15[b]	0.78±0.15[b]	0.71 ±0.11[b]	0.01
			Phospholipid Synthesis			
CHPT1	1.00±0.15	1.24±0.17	1.23±0.14	1.29±0.26	1.22±0.13	0.33
EPT1	1.00±0.15[ab]	1.22±0.40[a]	0.70±0.10[b]	0.83±0.39[ab]	0.66±0.19[b]	0.01

Abbreviation: *ACC*, Acetyl-CoA carboxylase; *ACOT4*, Acyl-CoA thioesterase 4; *CHPT1*, Choline Phosphotransferase 1; *COX1*, Cyclooxygenase 1; *CPT1A*, Carnitine Palmitotransferase 1; *DEGS1*, Delta-

4 Desaturase; *ELOVL2*, Elongase 2; *ELOVL5*, Elongase 5; *EPT1*, Ethanolaminephosphotransferase 1; *FADS2*, Delta-6 Desaturase; *FASN*, Fatty acid synthase; *LPL*, Lipoprotein Lipase; *PLPP1*, Phospholipid Phosphatase 1; *PPARA*, Peroxisome Proliferator Activator Receptor Alpha; *SREBP1c*, Sterol Regulatory Element Binding Protein 1c.

5.5 Discussion

In agreement with the two previous studies involving the AST rich *Haemotoccocus pluvialis* and the DHA rich *Aurantiochytrium*, neither compound adversely affected performance parameters including body weight gain, feed intake, or egg production. Strikingly, supplemental calcifediol improved both the egg shell thickness and weight of layers eggs independently of AST and DHA oil. This is consistent in previous studies, however, this is the first study to demonstrate that layers can tolerate up to 200 ug/kg of calcifediol without adverse effects with the previous being up to 122 ug/kg (Matilla). Currently the lowest level of calcifediol which demonstrated a toxicity effect was 400 ug/kg. Yolk enrichments of calcifediol reached up to 9 ug/100 g yolk at week 3, a concentration capable of meeting the human daily required intake of this nutrient in approximately 10 to 15 eggs. Yolk calcifediol enrichment decreased at week 6 reaching up to 6 ug/100 g yolk with negative feedback like this having been seen before Total DHA enrichments from the 0.8% DHA oil were comparable to that of 2% *Aurantiochytrium*, reaching levels up to 100 mg DHA/egg. Despite reductions in total PL content in the liver, egg yolk, and plasma from dietary DHA inclusion there were elevations of DHA within this type of lipid. Total arachidonic acid was lowered by DHA supplementations which may explain this decrease in PL while there was an increase in DHA in this form. While AST was enriched in both egg yolk and liver tissue the amount of said enrichment was approximately 30% of the previous study, this decrease in AST carried over to the algae itself which may be due to the

material oxidizing from age. As AST is an excellent electron donor and anti-oxidant it may also be sensitive to oxidation and may decay rapidly unless stored in very cold and dark environments. The decrease in d, l-α-tocopherol may have been primarily attributed to the anti-lipogenic effects of DHA as d, l-α-tocopherol is lipophilic and may lose potential sites of storage as total lipid decreases. While increases in ALT have been correlated with dietary AST in previous studies, we did not see this phenomenon with DHA which could imply there were other compounds in the DHA-oil which could be the cause. Strikingly, plasma inorganic phosphorus was decreased in the full combination group, a marker of increased or decreased bone mobilization in hens. While a decrease in inorganic phosphorus may suggest decreased shell formation due to lack of medullary bone resorption, as egg shells were become thicker this may suggest that the vitamin D in combination with AST and DHA may have been changing renal filtration or blood ion balance. As demonstrated before, DHA decreases all lipid species ubiquitously across many tissues including egg yolk, however, the mechanism behind this sharp decrease in PL has yet to be fully explored.

5.6 Conclusion

Dietary supplementations of 0.16% full fatted *Haematococcus pluvialis*, 0.8% DHA-oil, and 200 ug/kg of calcifediol were able capable of enriching these compounds individually into the tissues and eggs of layers without reducing the enrichment of AST or DHA. Calcifediol at 200 ug/kg did not produce a toxicity, was enriched at levels up to 9 ug/100 g yolk, and was able to exert potent effects upon egg shell weight and thickness.

Chapter Six

Evaluating the Potential Human Health Benefit of Enriched Eggs via Murine Models

6.1 Summary

Mice can serve as an excellent model for human nutrition and metabolic pathways due to the genetic homology between our species. Utilizing the enriched egg yolks containing DHA, AST, and calcifediol from the previous layer studies, we studied the potential and efficacy of eating these enriched eggs for human health. Two studies were conducted testing 1) individually enriched DHA and AST containing egg yolks and 2) multi enriched egg yolks with either or DHA, AST, and calcifediol.

6.2 Introduction

Astaxanthin is a lipophilic compound with potent anti-oxidant capabilities which is highly enriched within the algae species *Haematococcus pluvialis*. Work done by our lab has shown that the AST in this algae can be readily incorporated into egg yolks when fed laying hens in both fatted and defatted forms. When said egg yolks were subsequently fed to mice at 2 mg/kg of their diet there was a reduction in liver TC, TG, and NEFA, muscle NEFA, and plasma TGs. Dietary supplementation of these enriched eggs had upregulated genes in the liver associated with b-oxidation and enhanced lipid catabolism including CPT1 and PPAR. For a majority of these effects the eggs derived from the full fatted version of the algae (product 2) showed a more powerful response.

Docosahexaenoic acid (DHA) is a long and highly unsaturated omega-3 fatty acid which can be produced in large quantities by the algal species *Aurantiochytrium*. The DHA from this

algae has been shown to be readily absorbed and deposited into the egg yolks of commercial laying hens. When these DHA enriched eggs were fed to ~6 week old GPX1 OE mice at 2.9 g/kg of the diet there was a significant decrease in plasma TG, plasma glucose, week 3 plasma NEFA, and liver TC. Dietary DHA enriched eggs also improved the liver fatty acid n3/n6 profile from 0.23 to 0.28 in the control and experimental groups respectively. The concentration of DHA in the liver was elevated from 2.09 to 2.16 mg/g (3.3%) and total n6 concentration decreased from 9.40 to 7.72 mg/g with regards to the control and experimental groups.

Vitamin D3 and its prehormone hydroxylated form, calcifediol, are naturally only found within animal fat including meat and egg yolks. Our group has yet to conduct a trial involving vitamin D enriched egg yolk, however, our research has demonstrated the potential of vitamin D3OH supplementation at levels of 800 IU/day can prevent diaebetes formation in non-obese diabetic mice, and improve bone strength, but worsens heart condition by increasing blood pressure by causing hypertension.

In order to follow up the previous AST & DHA yolk related studies, this study incorporated the multi enriched egg yolk products based upon the relative DHA concentration to match the former studies. Based upon a human equivalent dose of 200 mg DHA/day, approximately 0.49 mg DHA/day in mice, roughly 99 mg DHA/kg of diet was added in prior studies, with or without 14.3 ug of AST/kg of diet. As the egg yolk of this study has a lower DHA concentration relative to the prior egg yolk material (new yolk DHA = 20.4 mg/g yolk, old yolk DHA 34 mg DHA/g yolk), more egg yolk will be incorporated reaching up to 5.5 g/kg of diet. The previous DHA/AST mouse study utilized up to 5 g/kg of total yolk so this is not far off and should not induce adverse effects. Normal control yolk will be supplemented if less yolk is added so all total amounts of yolk match.

6.3 Materials and Methods

Experiment 1 – DHA & AST Enriched Egg Yolks: Male 10 week old C3H wild type mice were offered sucrose yeast based diets which were supplemented with either DHA or AST enriched egg yolks produced from Experiments 1 and 2. Four diets in total were utilized: a control containing 0.5% of its composition as a regular control egg yolk, an experimental group with 0.5% of the total diet containing DHA enriched egg yolk, a second experimental group containing 0.2% of its total diet as AST enriched egg yolk, and a third experimental group containing both 0.5% DHA egg yolk and 0.2% AST egg yolk (**Table 6.1**).

Experiment 2 – DHA, AST, and Calcifediol Enriched Egg Yolks –

An distribution of male and female C3H wild type mice ranging from 20-30 weeks old were offered sucrose yeast based diets which were supplemented with either DHA, AST, and or calcifediol enriched egg yolks produced from the multi enrichment layer study Five diets in total were utilized: a control containing 0.5% of its composition as a regular control egg yolk, or 0.5% of experimental egg yolks with diet 2 containing DHA enriched yolk, diet 3 DHA & calcifediol enriched yolk, diet 4 DHA & AST enriched yolk, and diet 5 containing DHA, AST, and calcifediol enriched yolk.

Mice were housed in cages in up to groups of 3 and exposed to a 12 hour light:dark cycle with free access to food and water for 8 weeks. Body weights and blood was collected via tail snip weekly. At the end of week 8 mice were fasted for 8 hours and euthanized for liver, muscle, heart, and adipose tissue collection. Fatty acid profiles, lipid composition, plasma glucose concentrations, and lipid related gene expression was measured.

Table 6.1 Composition (g/kg) of experimental diets for mouse studies 1 & 2

Ingredients, g/kg	Experiment 1				Experiment 2				
	Control	**T2**	**T3**	**T4**	**Control**	**T2**	**T3**	**T4**	**T5**
Yeast	335	335	335	335	326	326	326	326	326
Sucrose	609	609	609	609	599	599	599	599	599
Control Egg Yolk	4.90	2.00	2.90	0.00	5.50	0.00	0.00	0.00	0.00
AST Enriched Egg Yolk	0.00	0.00	2.00	2.00	0.00	0.00	0.00	0.00	0.00
DHA Enriched Egg Yolk	0.00	2.90	0.00	2.90	0.00	5.00	0.00	0.00	0.00
DHA + Calcifediol** Enriched Egg Yolk	0.00	0.00	0.00	0.00	0.00	0.00	5.00	0.00	0.00
DHA + AST Enriched Egg Yolk**	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.00	0.00
DHA + AST + Calcifediol Enriched Egg Yolk**	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	5.00
DL-Methionine	1.07	1.07	1.07	1.07	0.96	0.96	0.96	0.96	0.96
Corn Oil	40.8	40.8	40.8	40.8	41.1	41.1	41.1	41.1	41.1
Sodium Chloride	0.67	0.67	0.67	0.67	0.62	0.62	0.62	0.62	0.62
Calcium Carbonate	7.64	7.64	7.64	7.64	7.76	7.76	7.76	7.76	7.76
Vitamin and Mineral Premix*	4.36	4.36	4.36	4.36	4.36	4.36	4.36	4.36	4.36
Nutritive value									
Me, kcal/kg	3900	3900	3900	3900	3900	3900	3900	3900	3900
Crude protein%	18	18	18	18	18	18	18	18	18
Crud fat%	5	5	5	5	5	5	5	5	5
Phosphorus, g/kg	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Calcium, g/kg	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50

*[1]The vitamin/mineral mix contained vitamin A 4550 IU/kg, vitamin D 450 IU/kg, vitamin E 7.5 IU/kg, vitamin K 0.752 mg/kg, biotin 0.152 mg/kg, choline 1575 mg/kg, folacin 0.376, niacin 15.147 mg/kg, pantothenic acid 3 mg/kg, riboflavin 3.76 mg/kg, thiamine 1.07 mg/kg, vitamin B63.78 mg/kg, vitamin B12 0.006 mg/kg, copper 12 mg/kg, iodine 0.05 mg/kg, 30.23 mg/kg, manganese 53 mg/kg zinc, selenium 0.09 mg/kg, and iron 67.82 mg/kg.

**Egg yolk amount fed based upon DHA concentration (20.4 mg DHA/g yolk)

6.4 Results

Experiment 1 -

At the end of the study plasma glucose concentrations were non-significantly decreased by both DHA and AST egg yolks (**Table 6.2**). Plasma total cholesterol was significantly decreased in the AST groups while NEFA was increased. Strikingly within heart tissue there was a sharp decrease in TC and TGs in both the AST and combination groups (**Table 6.3**). Genes related to lipid anabolism, *DGAT1*, *DGAT2*, *SREBP1c*, *FASN*, lipid catabolism, *PPARα*, and lipid transport *LPL* were down regulated by the DHA and AST yolks (**Table 6.4**). Conversely, heart tissue expression of the lipid catabolic gene CPT1 was greatly enhanced in the AST group. Hepatic expression of these lipid metabolism genes was not as consistent however there were decreased mainly in the lipid anabolism genes *DGAT1*, *DGAT2* and transport *LPL* in the experimental treatment groups. Hepatic expression of *CPT1* was elevated in the DHA group and then suppressed in both AST groups.

Experiment 2 –

None of the enriched egg yolks caused any change in body weight, food consumption, or mortality. All of the DHA enriched egg products lowered plasma glucose levels, TG, and NEFA similar to what has been seen before. While total cholesterol was unaffected, total phospholipid content of plasma was reduced to 0.5 mg/dL across treatment groups, a 40% reduction from the baseline 0.81 mg/dL of the control. Similar to what was observed in plasma, hepatic and heart concentrations of TG, NEFA, and PL were ubiquitously reduced in DHA enriched egg yolk containing diets while TC remained unaffected. Egg yolks containing AST dramatically

increased hepatic gerne expression of CPT1A. All groups fed yolks containing DHA had general increases in lipid metabolism related genes. Calcifediol from egg yolks was capable of being enriched into the liver of mice in treatment groups 3 and 5.

Table 6.2 Effect of different enriched egg yolk supplemented diets on body weight, plasma glucose, and lipid profiles.

	Experiment 1				Experiment 2				
Ingredients, g/kg	**Control**	**T2**	**T3**	**T4**	**Control**	**T2**	**T3**	**T4**	**T5**
Body Weight, g									
0 wk	27.9	26.2	27.7	26.8	28.5	29.4	29.1	27.6	28.4
8 wk	30.7^{a}	29.3^{ab}	26.0^{ab}	24.5^{b}	33.5	31.2	35.6	32.1	32.5
Glucose, mg/dl									
0 wk	158	154	177	167	156	149	162	138	154
8 wk	83.2^{ab}	90.7^{a}	83.1^{ab}	71.3^{b}	122^{a}	79.0^{b}	82.0^{b}	80.0^{b}	78.0^{b}
TC[1], mg/dl									
0 wk	78.1	80.7	86.1	81.4	80.2	83.1	80.5	81.1	82.2
8 wk	87.7	91.5	102	93.4	85.6	87.5	84.2	82.9	84.6
TG[2] mg/dl									
0 wk	27.9	21.1	22.7	28.0	44.2	46.7	48.2	41.5	45.3
8 wk	44.7^{ab}	33.5^{ab}	54.0^{a}	20.1^{b}	40.1^{a}	35.4^{b}	34.7^{b}	33.5^{b}	31.5^{b}
NEFA[3], mol/L									
0 wk	0.24	0.27	0.32	0.31	0.35	0.33	0.37	0.32	0.34
8 wk	0.39^{b}	0.24^{b}	0.37^{b}	0.62^{a}	0.38^{a}	0.25^{b}	0.28^{b}	0.24^{b}	0.26^{b}
PL[4], mg/dL									
0 wk	N/A	N/A	N/A	N/A	0.87	0.82	0.91	0.84	0.83
8 wk	N/A	N/A	N/A	N/A	0.81^{a}	0.51^{b}	0.53^{b}	0.49^{b}	0.46^{b}

*Data expressed as means (n=5).

Data analyzed via one-way ANOVA.

^{a,b} Means in the same row without a common letter differ ($P < 0.05$).

[1]TC, Total Cholesterol.

[2]TG, Triglyceride.

[3]NEFA, Non-esterified Fatty Acid.

[4]PL, Phospholipid.

Table 6.3 Effect of different enriched egg yolk supplemented diets on tissue lipid profiles & calcifediol at week 8.

Item	Experiment 1				Experiment 2				
	Control	**T2**	**T3**	**T4**	**Control**	**T2**	**T3**	**T4**	**T5**
Liver									
[1]TC, mg/g protein	20.6	22.9	22.4	20.6	22.5	23.4	21.8	22.4	23.2
[2]TG, mg/g protein	230[a]	213[b]	220[ab]	204[b]	210[a]	198[ab]	190[b]	192[b]	193[b]
[3]NEFA, µmol/g protein	33.2[a]	33.0[a]	28.5[b]	28.3[b]	30.2[a]	27.8[b]	26.9[b]	26.8[b]	27.2[b]
Calcifediol, ng/g	N/A	N/A	N/A	N/A	13.7[b]	11.9[b]	41.0[a]	15.3[b]	54.0[a]
Heart									
TC, mg/g protein	14.9[a]	11.9[a b]	10.1[b]	9.51[b]	12.5	13.0	12.1	12.2	11.5
TG, mg/g protein	36.2[a]	18.9[b]	18.5[b]	19.4[b]	38.5[a]	25.4[b]	17.6[b]	26.2[a]	17.8[b]
NEFA, µmol/g protein	31.1	26.8	25.0	28.9	32.4[a]	28.5[b]	24.6[b]	29.6[a]	25.1[b]
PL, mg/dL	N/A	N/A	N/A	N/A	70.5[a]	42.5[b]	46.2[b]	38.6[b]	41.2[b]

*Data expressed as means (n=5).

Data analyzed via one-way ANOVA.

[a,b] Means in the same row without a common letter differ ($P < 0.05$).

[1]TC, Total Cholesterol.

[2]TG, Triglyceride.

[3]NEFA, Non-esterified Fatty Acid.

Table 6.4 Effect of different enriched egg yolk supplemented diets on mRNA concentrations in the heart and liver at week 8 for Experiment 1.

Gene name	Control	T2	T3	T4
Liver				
CPT1A	1^{ab}	1.50^{a}	0.78^{b}	0.70^{b}
DGAT1	1^{a}	0.26^{b}	0.10^{b}	0.08^{b}
DGAT2	1^{a}	0.40^{b}	0.36^{b}	0.29^{b}
FASN	1	1.02	0.91	0.80
LPL	1^{a}	0.38^{b}	0.28^{b}	0.22^{b}
PPARA	1	1.09	0.99	1.04
SCD1	1^{ab}	1.26^{a}	0.87^{ab}	0.59^{b}
SREBP1c	1	0.83	0.88	0.69
Heart				
CPT1A	1^{c}	1.68^{bc}	2.59^{ab}	3.50^{a}
DGAT1	1^{a}	0.26^{b}	0.10^{b}	0.08^{b}
DGAT2	1^{a}	0.40^{b}	0.36^{b}	0.29^{b}
LPL	1^{a}	0.59^{b}	0.45^{b}	0.47^{b}
PPARA	1^{a}	0.51^{b}	0.37^{b}	0.42^{b}
SCD1	1^{a}	0.69^{ab}	0.55^{b}	0.41^{b}
SREBP1c	1^{a}	0.28^{ab}	0.09^{b}	0.08^{b}

*Data expressed as means (n=5).

Data analyzed via one-way ANOVA.

^{a,b} Means in the same row without a common letter differ ($P < 0.05$).

Abbreviation: *CPT1A*, Carnitine Palmitotransferase 1; *DGAT1*, Diacylglycerol O-acyltransferase 1; *DGAT2*, Diacylglycerol O-acyltransferase 2; *FASN*, Fatty Acid Synthase; *LPL*, Lipoprotein Lipase; *PPARA*, Peroxisome Proliferator Activator Receptor Alpha; *SCD1*, Stearoyl-CoA desaturase 1; *SREBP1c*, Sterol Regulatory Element Binding Protein 1c.

Table 6.5 Effects of dietary DHA oil, calcifediol, and algal AST enriched egg yolks on mouse hepatic gene expression in Experiment 2.

Treatment	Control	DHA Oil	DHA Oil + Calcifediol	DHA Oil + AST	Combination	P Value
			Catabolic			
CPT1A	1.00±0.55^{d}	2.01±0.77^{c}	0.99±0.13^{d}	11.1±1.13^{b}	25.0±2.05^{a}	0.01
			Anabolic			
DGAT1	1.00±0.08^{c}	1.22±0.22^{c}	0.96±0.36^{c}	21.3±0.29^{a}	7.53±0.93^{b}	0.01
FASN	1.00±0.40^{c}	2.10±0.20^{b}	1.40±0.10^{c}	2.36±0.36^{b}	3.49±0.67^{a}	0.01
SREBP	1.00±0.25^{c}	1.00±0.09^{c}	1.33±0.14^{c}	4.92±0.26^{a}	3.18±0.23^{b}	0.01
			Absorption			
LPL	1.00±0.07^{b}	1.60±0.28^{a}	1.71±0.31^{a}	1.55 ±0.29^{a}	1.65 ±0.27^{a}	0.01
PLPP1	1.00±0.69^{b}	1.04±0.67^{b}	1.23±0.38^{b}	0.84±0.19^{b}	2.60±0.60^{a}	0.01
			Phospholipid Synthesis			
CHPT1	1.00±0.15^{a}	0.69±0.14^{b}	0.80±0.11^{ab}	0.62±0.20^{b}	0.68±0.15^{b}	0.01
EPT1	1.00±0.06^{c}	1.09±0.31^{c}	1.03±0.29^{c}	3.62±0.19^{b}	5.50±0.51^{a}	0.01

*Data expressed as means (n=5).

Data analyzed via one-way ANOVA.

^{a,b,c} Means in the same row without a common letter differ ($P < 0.05$).

Abbreviation: *CHPT1*, Choline Phosphotransferase 1; *CPT1A*, Carnitine Palmitotransferase 1; *DGAT1*, Diacylglycerol O-acyltransferase 1; *EPT1*, Ethanolaminephosphotransferase 1; *FASN*, Fatty Acid Synthase; *LPL*, Lipoprotein Lipase; *PLPP1*, Phospholipid Phosphatase 1; *PPARA*, Peroxisome Proliferator Activator Receptor Alpha; *SREBP1c*, Sterol Regulatory Element Binding Protein 1c.

6.5 Discussion

Both DHA and AST enriched egg yolks were capable of modulating the lipid metabolism of mice as well as changing glucose regulation. While the hepatic responses were not as clear with there being less pronounced changes in liver lipid composition and gene expression there was a very pronounced effect on cardiac tissue. The heart muscle relies primarily on fat as its source of fuel to generate ATP and both AST and DHA from egg yolks changed not only the lipid composition by sharply decreasing TC and TG there were also a very powerful suppressive effect on lipid metabolism related genes. Particularly within the heart tissue *LPL* was downregulated greatly, this gene codes for lipoprotein lipase which is an enzyme responsible for internalizing and breaking down lipoproteins in the blood in order to facilitate their transport into the cell. Expression of *CPT1*, a gene which codes for carnitine palmitotransferase, an enzyme responsible for fatty acid catabolism within the mitochondria, was enhanced by both treatment groups in a synergistic effect which suggests AST and DHA improve lipid breakdown within the heart. A reduction of lipid accumulation within heart tissue is correlated with improved heart function and health although the mechanism behind how DHA and AST egg yolks exert these effects still needs to be further studied.

6.6 Conclusion

Consumption of fortified egg yolks with DHA, AST, and calcifediol produced rather striking changes within the heart tissue of mice with substantial changes in phospholipid related gene expression and lipoprotein metabolism. While DHA is known for its anti-lipogenic effects it has yet to be fully studied how this compound can modulate heart tissue and particularly when DHA is incorporated into egg yolk through layers. There is potential for these eggs to improve human health by combating metabolic disorders which occur due to excess lipid storage and decreased insulin sensitivity, both of which can be partially reversed by DHA intake.

Chapter Seven

Conclusion

Due to a lack of balance in intake of omega-3 to omega-6 fatty acids, fruits and vegetables which contain bioactive compounds, and vitamin D necessary for bone and cardiovascular health, there has been a sharp increase in the incidence of non-communicable diseases in the US. By utilizing eggs as a carrier for these three-choice health promoting compounds it may be possible to reverse the incidence of these diseases and improve societal nutrition. The daily required intake for calcifediol, DHA, and carotenoids is 10 ug, 400 mg, and 15 mg respectively, concentrations which can realistically be reached by consuming 2-3 of the enriched eggs produced in these studies. In addition to AST being a compound which can be enriched into the yolks of hens at up to 2 mg/egg, it also improved the anti-oxidative status of the eggs and tissues of hens. As AST can reduce the formation of MDA this may have implications when this compound alongside omega-3 fatty acids which are especially susceptible to oxidation. It was also demonstrated that DHA can be enriched up to 225 mg/egg when fed from the algal source *Aurantiochytrium* in combination with dietary supplemental choline, ethanolamine, and serine. Although it is possible to enrich this much DHA into the yolks of hens it is not advised to utilize concentrations greater than 3.4 g DHA/kg diet as feeding levels higher than this cause adverse effects on hen performance and egg parameters. When AST, DHA, and supplemental calcifediol were fed simultaneously to hens at 0.16%, 0.8%, and 200 ug/kg of the diet respectively it was possible to create eggs which were multi enriched with all three of these compounds. Particularly calcifediol was capable of being enriched at levels up to 9 ug/100 g yolk, a concentration which could meet the daily requirement of this nutrient in as little as 2 eggs. When eggs fortified with these compounds were fed to murine models there were substantial changes associated in lipid

metabolism, specifically cardiac tissue of mice fed AST enriched yolks. While it remains to be explored there is potential for these enriched eggs to attenuate non-communicable disorders in humans based upon these results from mice feeding trials.

Our research has demonstrated the capacity of eggs to serve as a readily accessible and commercially convenient site of storage for DHA, AST, and calcifediol. Both DHA and AST when stored in egg yolks exerted potent effects upon cardiac tissue of mice models which has implications for helping to offset and act as a preventative for CVD. While it is possible to enrich AST, DHA, and calcifediol simultaneously there may be limitations upon how long laying hens can enrich these compounds beyond six weeks without an attenuation or decrease in enrichment efficiency. Further research is required to fully understand how this modulates cardiac tissue health and what daily concentrations of DHA and AST enriched yolk could optimize this.

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