AN ABSTRACT OF THE THESIS OF

Joseph A. Yonke for the degree of Master of Science in Animal Science presented on May 24, 2018.

Title: Effects of High-docosahexaenoic Acid Microalgae and Choline in the Hen Diet on Egg Production and Lipid Metabolism.

Abstract approved: _____

Gita Cherian

The objectives of the current study were to 1) investigate the effect a high-docosahexaenoic acid (C22:6 n-3, DHA) microalgae product (MAL), and choline dosage in diets containing MAL, on egg production performance, physical egg quality, and nutritional parameters, and 2) determine whether choline dosage influences hepatic lipid metabolism of hens fed MAL. Fifty six, 26-week-old, White Leghorn hens were kept in individual cages and randomly allocated to one of the four dietary treatments, each with seven replicates of two hens (n=7 per treatment). All diets were corn and soybean meal based, isocaloric, isonitrogenous, and contained the same amount of vitamin and mineral premix, which provided 551 mg/kg choline in the diets. Experimental diets contained 0% MAL and no supplemental choline (Control), 1% MAL and no additional choline chloride (Alg), 1% MAL and 0.1% supplemental choline chloride (Ch0.1), and 1% MAL and 0.2% supplemental choline chloride (Ch0.2). The feeding trial lasted 16 weeks after a two week adaptation period. Eggs were collected, counted and weighed daily, feed intake and egg quality were measured every two weeks, and fatty acid (FA) composition was

analyzed every four weeks. Birds were euthanized for tissue collection at the end of the trial. The effects of MAL and supplemental choline on all response variables was analyzed as a general linear mixed model with repeated measures using SAS software (version 9.4) (SAS Institute). All results from this analysis were considered significant at $p \leq 0.05$. Using orthogonal contrasts, Alg was compared to Control to calculate the effect of MAL, and Ch0.1 and Ch0.2 were compared separately to Alg to calculate the effect of choline chloride dosages in diets containing MAL. Compared to Control, Alg increased egg yolk content of DHA ($p<0.0001$), phosphatidylethanolamine (PE) ($p=0.02$), and phosphatidylcholine (PC) ($p<0.0001$). In the liver, Alg similarly increased PE ($p=0.0003$) and PC ($p=0.0003$) concentrations, but also increased lipid peroxidation products compared to Control ($p=0.01$). Compared to Alg, Ch0.1 increased hen day egg production ($p=0.03$), daily egg mass ($p=0.02$), Haugh unit ($p=0.04$), total lipids ($p=0.04$) and γ-tocopherol (γT) ($p=0.05$) concentrations in egg yolks. Additionally, Ch0.1 increased hepatic concentrations of α-tocopherol (αT) ($p=0.03$) and γT ($p=0.005$), and decreased feed conversion ratio ($p=0.005$) and hepatic lipid peroxidation products ($p=0.005$) compared to Alg. Ch0.2 likewise increased hepatic γT ($p=0.0002$) and αT ($p=0.001$) concentrations, but did not produce any of the other changes associated with Ch0.1.

These results indicate that DHA content of eggs can be greatly increased by supplementing MAL in the diets of laying hens. Furthermore, supplementing 0.1% choline chloride in hen diets containing MAL can improve production performance and egg quality, and protect the liver from oxidative stress.

Effects of High-docosahexaenoic Acid Microalgae and Choline in the Hen Diet on Egg Production and Lipid Metabolism

by
Joseph A. Yonke

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Joseph A. Yonke, Author

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LIST OF ABBREVIATIONS

AA	Ascorbic acid
AHA	American Heart Association
AI	Adequate intake
ALA	α-linolenic acid (C18:3 n-3)
Alg	Diet supplemented with 1% microalgae and no choline chloride
ApoB	Apolipoprotein B_{100}
ApoII	Apolipoprotein-VLDL-II
ARA	Arachidonic acid (C20:4 n-6)
CDP	Cytidine diphosphate
Ch0.1	Diet supplemented with 1% microalgae and 0.1% choline chloride
Ch0.2	Diet supplemented with 1% microalgae and 0.2% choline chloride
CK	Choline kinase
CPT	CDP-choline:1,2-diacylglycerol choline phosphotransferase
CT	CTP:phosphocholine cytidylyltransferase
CTP	Cytidine triphosphate
DAG	Diacylglycerol
DHA	Docosahexaenoic acid (C22:6 n-3)
EM	Hen day egg mass
EP	Hen day egg production
EPA	Eicosapentaenoic acid (C20:5 n-3)
ER	Endoplasmic Reticulum

LIST OF ABBREVIATIONS (Continued)

ET	CTP:phosphoethanolamine cytidylyltransferase
EW	Average egg weight
FA	Fatty acid
FAME	Fatty acid methyl esters
FCR	Feed conversion ratio
FI	Feed intake
FLHS	Fatty liver hemorrhagic syndrome
FMO3	Flavin containing monooxygenase 3
FSH	Follicle stimulating hormone
GC	Gas chromatograph
GnRH	Gonadotropin releasing hormone
HDL	High density lipoprotein
HPLC	High pressure liquid chromatograph
IOM	Institute of Medicine
LA	Linoleic acid (C18:2 n-6)
LC	Long chain (≥20 carbons)
LDL	Low density lipoprotein
LH	Luteinizing hormone
LPL	Lipoprotein Lipase
MAL	High-docosahexaenoic acid microalgae supplement
MDA	Malondialdehyde

LIST OF ABBREVIATIONS (Continued)

MUFA	Monounsaturated fatty acid
n-3	Omega-3
n-6	Omega-6
NASH	Nonalcoholic steatohepatitis
ND	Not detected
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHANES	National Health and Nutrition Examination Survey
NRC	National Research Council
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEMT	Phosphatidylethanolamine N-methyltransferase
PL	Phospholipid
PPAR	Peroxisome proliferator activated receptor
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
SAM	S-adenosylmethionine
SEM	Standard error of the mean
SFA	Saturated fatty acid
SREBP-1	Sterol regulatory element binding protein
TAG	Triacylglycerol
TBARS	Thiobarbituric acid reactive substance

TLR4	Toll-like receptor 4
TMA	Trimethylamine
TRL	Triglyceride rich lipoproteins
USDA	U.S. Department of Agriculture
VLDL	Very low density lipoprotein
VLDLy	Yolk specific very low density lipoprotein
αT	α-Tocopherol
γT	γ-Tocopherol

1 INTRODUCTION

Eggs are an excellent source of dietary protein, essential fatty acids (FA) and vitamins. They are not only naturally nutritious, but widely consumed because of their convenience and affordability. Furthermore, they can easily be enriched with certain nutrients by including them in the rations of laying hens (Cherian, 2011). The egg has the potential to be an effective functional food to help balance the diets of Americans. The average American, or "Western," diet tends to be high in saturated fats (SFA), which leads to a host of chronic metabolic diseases (Doll, et al., 2009). Replacing conventional eggs and meats with eggs enriched with omega-3 (n-3) polyunsaturated FA (PUFA), and reduced omega-6 (n-6) PUFA and SFA can ameliorate this issue. Consumption of n-3 PUFA can improve fat burning metabolism and decrease the incidence of cardiovascular and metabolic disease (Jump et al., 2012). Poultry products have been enriched with n-3 PUFA for many years, but there may still be room for improvement in PUFA quality and other factors in the hen diet to maximize enrichment efficiency.

Eggs are commonly enriched with n-3 PUFA by supplementing flaxseed or fish oils in the laying hen diet, but strains of microalgae are an emerging alternative. The current study investigates the use of a microalgae supplement because it is rich in the long chain (≥20 carbons, LC) n-3 PUFA docosahexaenoic acid (C22:6 n-3, DHA). DHA is the longest and most biologically active n-3 PUFA, as opposed to the shorter, plant derived n-3 found in flaxseed, α-linolenic acid (C18:3 n-3, ALA) (Hishikawa et al., 2017).

While the choline requirement in the laying hen diet remains under investigation, its biological effects hint that it may alter the n-3 PUFA content of eggs if supplemented

to commercial hens. Although not a lipid itself, choline effects lipid metabolism as a substrate of phosphatidylcholine (PC) synthesis and a donor of methyl groups ($R\text{-}CH_3$). As the epicenter of lipid metabolism in the laying hen, the liver serves as the major site of FA synthesis, and is the site where all dietary lipids are processed prior to delivery to developing yolk follicles. PC synthesized in the hen liver is secreted in bile, aiding the absorption of dietary lipids, and is also incorporated in lipoproteins that export lipids away from the liver. This helps prevent hepatic fat accumulation (Griffith et al., 1969; Ruiz et al., 1983) and may alter the lipids, including DHA, incorporated in egg yolks (Wang et al., 2017).

This thesis entails one feeding trial on laying hens divided into two experiments based on objectives. The objective of the first experiment was to investigate the effect of a high-DHA microalgae supplement (MAL), and choline dosage in diets containing MAL, on production performance, physical egg quality, and nutritional parameters. The objective of the second experiment was to determine whether choline dosage influences hepatic lipid metabolism of hens fed MAL.

2 HEN REPRODUCTIVE BIOLOGY

Hens bred for egg production produce one egg nearly every day. Sexual maturity occurs around 18 weeks of age, but the exact age of maturity is dependent on genetics and, most importantly, a stimulatory lighting schedule. Birds sense light not only with their eyes, but with photoreceptors located directly on the hypothalamus, which also regulates their physiological response to daylight hours (Benoit, 1935). After the first long day a hen experiences, her levels of gonadotropin releasing hormone (GnRH) and the gonadotropins luteinizing hormone and follicle stimulating hormone (FSH) increase that night (Etches, 1996). Luteinizing hormone (LH) is a misnomer in birds because no true corpus luteum is formed or required to maintain pregnancy as in mammals. In birds, LH functions to regulate follicular growth, maintain large yolk-filled follicles, stimulate steroid production from them, and induce ovulation. GnRH stimulates the pulsatile release of LH from the anterior pituitary every one or two hours in the mature hen (Etches, 1996). FSH, although structurally similar to mammalian FSH, has no known stimulatory function in birds.

From the time of hatch, the hen has thousands of follicles to recruit from on the left ovary (the right ovary does not mature and is nonfunctional). Follicles under one millimeter in diameter are classified as small white follicles, large white follicles are between two and four millimeters, small yellow follicles five to 10 millimeters, and the largest are known as yellow yolky follicles, which are around six in number and reach the size of an egg yolk. LH stimulates follicles to mature, and the follicles begin producing estrogen. Follicular estrogen creates a positive feedback loop with LH secretion, until a

threshold level results in ovulation of the largest follicle. The oocyte is released into the oviduct, where it will become the yolk of a new egg that forms around it.

As the oocyte matures into a yolk inside the ovarian follicle, nutrients are deposited in a process known as vitellogenesis. FA are delivered as triacylglycerol (TAG) and phospholipids (PL), primarily in yolk specific very low density lipoprotein (VLDLy) particles originating in the liver, which is further described in Chapter 3. VLDLy travels through the bloodstream to the highly vascular outermost layer of the follicle, the theca layer. The theca layer, divided into the outer theca externa and the inner theca interna, blankets the surface of the follicle, with the exception of a line at the apex of the follicle where rupture occurs during ovulation (the stigma). Fine branches of arteries reach through the inner layers of the theca interna, contacting the basement membrane. This is the point at which all yolk precursor nutrients transfer into the oocyte. In order to continue past the basement membrane to the vitelline, particles must fit between granulosa cells, which are 40 nm apart. This filtration is crucial to selectivity of yolk precursor particles (Alvarenga et al., 2011).

Lipoproteins that reach the vitelline membrane of the developing oocyte begin the process of yolk incorporation. Shallow depressions in this membrane contain the very low density lipoprotein (VLDL) receptor LR8, which binds Apolipoprotein B_{100} (ApoB) and vitellogenin apolipoproteins, (Nimpf and Schneider, 1998). Once a depression in the membrane is sufficiently loaded with VLDLy and vitellogenin, that entire section pinches off into a membrane-bound vesicle that moves into the developing yolk (Perry and Gilbert, 1979) (Figure 2.1). Lipoproteins immediately dissociate from the membrane as

their bound apolipoproteins, ApoB and vitellogenin, are degraded into the smaller proteins phosvitin and lipovitellin by the enzyme cathepsin-D. With their bound apolipoproteins removed, the VLDL particles merge together into larger particles called yolk spheres, which are up to 150 μm in diameter and encapsulated by the segment of vitelline membrane. The accumulation of these VLDL yolk spheres results in the growth of the egg yolk.

As a single large cell, the egg yolk is a liquid emulsion of the yolk spheres in an aqueous medium. The yolk is about 50% water, and contains almost as much protein as the albumen, in addition to housing essentially all of the lipids and vitamins in the egg (Stadelman and Coterill, 1977). The yolk is the primary source of nutrition for developing embryo. VLDL particles inside the yolk spheres contain 65% of yolk solids, containing approximately 12% protein and 88% lipids (Walzem, 2012). Lipids comprise about 32% of the egg yolk. Of these, about 64% are TAG, 31% are PL, and 5% are cholesterol, with palmitic (C16:0) and oleic (C18:1) acids being the most common FA components (Cherian, 2005). Percentage of individual FA are greatly affected by feedstuffs consumed by the hen. Due to the large lipid content of yolks and the high rate of production in commercial hens, lipid synthesis is increased about 14-fold in response to estrogen stimulation and associated egg production in the laying hen (Etches, 1996).

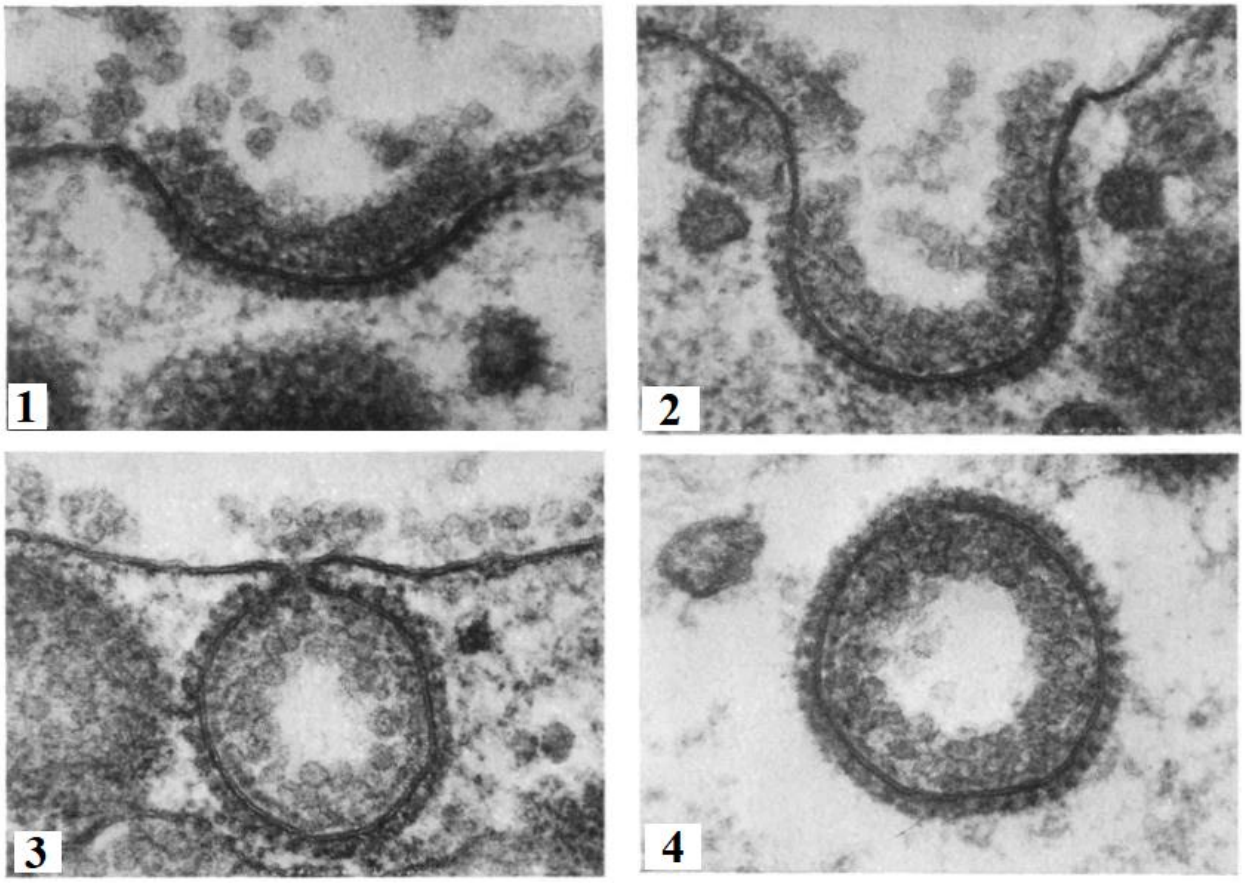


Figure 2.1. Endocytosis of whole lipoproteins into yolk follicle. The section of vitelline membrane is coated with VLDLy and vitellogenin (1) before sealing off and forming yolk spheres (3). From Perry and Gilbert (1979).

3 LIPOPROTEIN METABOLISM

As hydrophobic compounds, lipids require active transport through the aqueous environments of the body. FA are typically transported acylated to TAG and PL and packaged inside spherical particles called lipoproteins. Lipoproteins can move lipids through aqueous solutions because their outer surface can form hydrogen bonds with water, but their interior is hydrophobic. Lipoproteins are surrounded by the polar ends of amphipathic lipids including PL and unesterified cholesterol, and apolipoproteins with polar amino acid residues exposed outwards (Figure 3.1). Inside this monolayer membrane, lipoproteins contain a payload of non-polar lipids including TAG, cholesterol esters, and fat soluble vitamins.

Lipoproteins are classified by their density (Table 3.1). Denser lipoproteins have smaller non-polar cores. Lipoproteins are also distinguished by the proteins embedded in their membranes, called apolipoproteins. Each lipoprotein plays a unique role in lipid transport. For example, high density lipoprotein (HDL) generally removes lipids from peripheral tissue, earning its contents the reputation of “good cholesterol,” and low density lipoprotein (LDL) delivers lipids to peripheral tissues, earning its contents the “bad cholesterol” designation in our high fat consuming society.

Lipoprotein metabolism varies significantly between birds and mammals. Dietary lipids encounter their first lipoprotein in the enterocytes of the small intestine after absorption. In laying hens, these lipoproteins are portomicrons, which transport dietary lipids from the enterocyte to the liver. Portomicrons exit the cell via intracytoplasmic vesicles and enter the hepatic portal vein. Mammals, in contrast, export dietary lipids

from enterocytes as chylomicrons, which first enter lymphatic circulation prior to being deposited into the bloodstream via the thoracic duct. Chylomicrons then deliver dietary lipids to tissues throughout the body. As birds have a less developed lymphatic system, the chylomicron-lymphatic transport system of mammals is not utilized. A small fraction of portomicrons do escape immediate absorption by the liver, however, into the coccygeomesenteric vein, and will reach other tissues before transport to hepatic tissue (Fraser et al., 1986).

From the liver, chickens deliver lipids to other tissues as VLDL. Outside of the liver, the kidney and yolk sac membrane also serve as sites of VLDL synthesis, though these are minor in comparison (Tarugi e al., 1998). Each VLDL particle contains one unit of ApoB. ApoB is a protein embedded in the PL membrane of triglyceride rich lipoproteins (TRL) including portomicrons, VLDL, LDL, and IDL. They serve as ligands for lipoprotein lipase (LPL) receptors, allowing tissue to recognize and bind TRL, and absorb their contents. Hens exclusively produce $ApoB_{100}$, as opposed to in humans and other mammals which primarily produce $ApoB_{48}$ (Nakamuta et al., 1999). In hens, ApoB production is stimulated greatly by estrogen to increase transport of yolk lipids, but it is also present in non-layers for normal function.

Laying hens produce specialized VLDL to transport lipids specifically from the liver to the oocytes, VLDLy (Walzem et al., 1999). They are distinguished from normal VLDL by up to 25 units of the estrogen induced apolipoprotein apolipoprotein VLDL-II (ApoII). ApoII functions to inhibit LPL from acting on VLDLy, which prevents the loss of lipids to other tissues, and allows it to be taken into the developing yolk. ApoII reduces

the particle size of VLDLy to about half the diameter of VLDL, facilitating its passage through the theca interna of ovarian follicles. VLDLy are also rich in carotenoids that give yolk their yellow to red pigmentation (Alvarenga et al., 2011). Sexually mature hens also produce vitellogenin, another yolk specific lipoprotein activated by estrogen. In contrast to VLDLy, vitellogenin are very dense, and rich in PL.

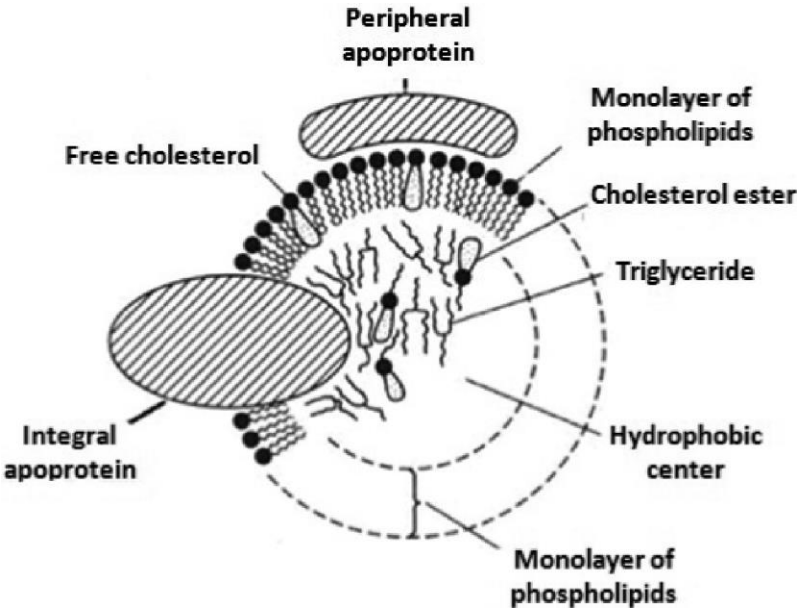


Figure 3.1. Basic structure and characteristics of a lipoprotein. From Alvarenga et al. (2011).

Table 3.1. Characterization of different types of lipoprotein particles in the laying hen. Adapted from Walzem (2012).

Particle	Origin	Functions	Diameter (nm)	Major Apolipoproteins
Portomicrons	Intestines	Absorption of dietary fat	100-400	ApoB, A-I, A-IV, C-II
Porto Remnants	Plasma	Deliver dietary fat to liver	≤100	
VLDL	Liver, Kidney, Heart, Yolk sac	Deliver TAG from liver to other tissues	50-100	ApoB, C-I, C-II, A-I, A-IV
VLDLy	Liver	Deliver TAG to yolk follicle	25-50	ApoB, ApoII
IDL	Plasma	Initial product of VLDL catabolism	30-50	
LDL	Plasma	Cholesterol transport	20-24	ApoB
HDL	Liver, Intestines	Removal of excess cholesterol from tissues, lipoproteins, and antioxidant actions	8-17	A-I, A-II, A-II
Vitellogenin (VHDL)	Liver	Deliver PL to yolk follicle	<17	Vitellogenin

VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; TAG, triacylglycerol; PL, phospholipids; ApoB, apolipoprotein B_{100}; A-I to A-IV and C-I and C-II, apolipoprotein A1 to A4 and C1 and C2; ApoII, apolipoprotein VLDL-II.

4 LIPIDS

4.1 Overview

Lipids are a large family of small, nonpolar or amphipathic organic molecules that are soluble in organic solvents. More specifically, they originate in part or entirely from the condensation of thioesters and/or isoprene units. They are commonly recognized as fats and oils. Lipids represent many compounds which are classified by their diverse structures and functions as FA, glycerolipids, PL, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides (Fahy et al., 2009). In the diet, fats are a dense source of energy and several serve as essential nutrients. For example, the fat soluble vitamins A, E, and K are prenol lipids, and vitamin D is a sterol lipid. The PUFA linoleic acid (C18:2 n-6, LA) and ALA are also essential nutrients for humans. Lipids play a wide variety of physiological roles including energy storage, membrane structure, lubrication, conditioning of body surfaces, intra- and inter-cellular signaling, as receptors, antigens, sensors, electrical insulators, biological detergents, and membrane anchors for proteins (Brenna and Sacks, 2013). FA and PL are the subjects of the current study.

4.2 Fatty acids

4.2.1 Structure and terminology

FA are carboxylic acids (COOH-) with an aliphatic hydrocarbon. The carboxylate end is known as the delta (Δ) end, and the methyl end is referred to as the omega (ω or n)

end. Two major distinctions of different FA found in animal tissues are length and degree of carbon chain hydrogen saturation. The chemical abbreviation for SFA, therefore, is always $COOH(CH_2)_nCH_3$. FA in chicken tissue and egg yolk usually vary from 14 to 22 carbons in length. Unsaturated FA have one or more carbon-carbon double bond. Those with one are called monounsaturated FA (MUFA) and those with two or more are called polyunsaturated PUFA. These double bonds can occur in the cis configuration – with the hydrogens on double bonded carbons facing the same direction, or the trans configuration – with said hydrogens opposite each other (Ratnayake and Galli, 2009). In poultry tissue, only cis bonds are synthesized. Cis double bonds create bends in the otherwise straight hydrocarbon chains, which impart them with unique properties critical to their function in specialized tissues.

Specific FA can be referred to with a notation conveying how many carbons (x) and how many double bonds (y) they contain: Cx:y. The location of the first double bond can also be included, and if so, is counted from the methyl end (e.g. LA, 18:2 n-6).

4.2.2 Synthesis

FA, in the form of TAG, are the most abundant form of stored energy in the body. In chickens, 70% of FA synthesis occurs in the cytosol of cells in the liver (Griffin and Hermier, 1988). Through conversion to acetyl coenzyme A, dietary carbohydrates and proteins can be utilized for FA generation during energy storage. In animals, FA synthesis utilizes fatty acid synthase which terminates when the FA reaches 16 carbons in length, strictly producing palmitic acid (C16:0). FA can be shortened by being partially

oxidized in β-oxidation. Further elongation and desaturation events occur in the mitochondria and endoplasmic reticulum (ER), where carbon-carbon bonds are desaturated by three membrane-bound desaturases. As FA enter acyl-CoA desaturases carboxylate end first, the furthest bond accessible is the Δ-9 carbon (Sul, 2013). Additional double bonds are made by the other desaturases in the direction back towards the carboxylate end, and are always spaced every three carbons. The physical limitation of animal acyl-CoA desaturases not to desaturate bonds past the Δ-9 position is very important. Because the shortest FA they can desaturate is 16 carbons in length, they can create double bonds no closer to the methyl end than n-7. Thus, 18-carbon n-6 and n-3 PUFA must originate from plants or microalgae (Ratnayake and Galli, 2009).

4.3 Docosahexaenoic acid

4.3.1 Definition

DHA is a 22 carbon n-3 PUFA, with six carbon-carbon double bonds (Figure 4.1). It is the most biologically active n-3 PUFA, and critical to brain development and neurological health (Hashikawa et al., 2017). As long as animals consume ALA, DHA can be synthesized through a series of elongations, desaturations, and oxidations (Figure 4.2). It is the longest n-3 PUFA commonly found in poultry and human tissues, and the most highly unsaturated. DHA is preferentially incorporated into PL, where it become a functional component of membranes, as opposed to being stored in TAG (Neijat et al., 2017).

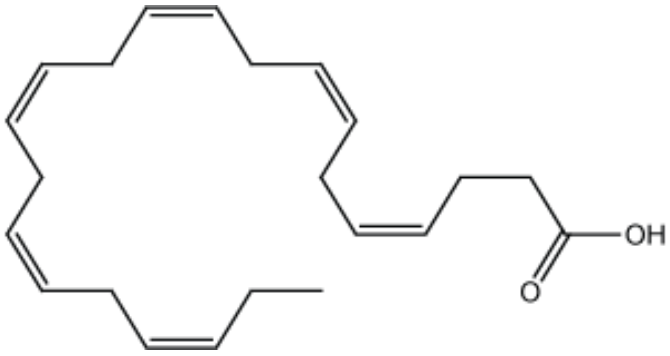


Figure 4.1. Structure of docosahexaenoic acid (C22:6 n-3, DHA).

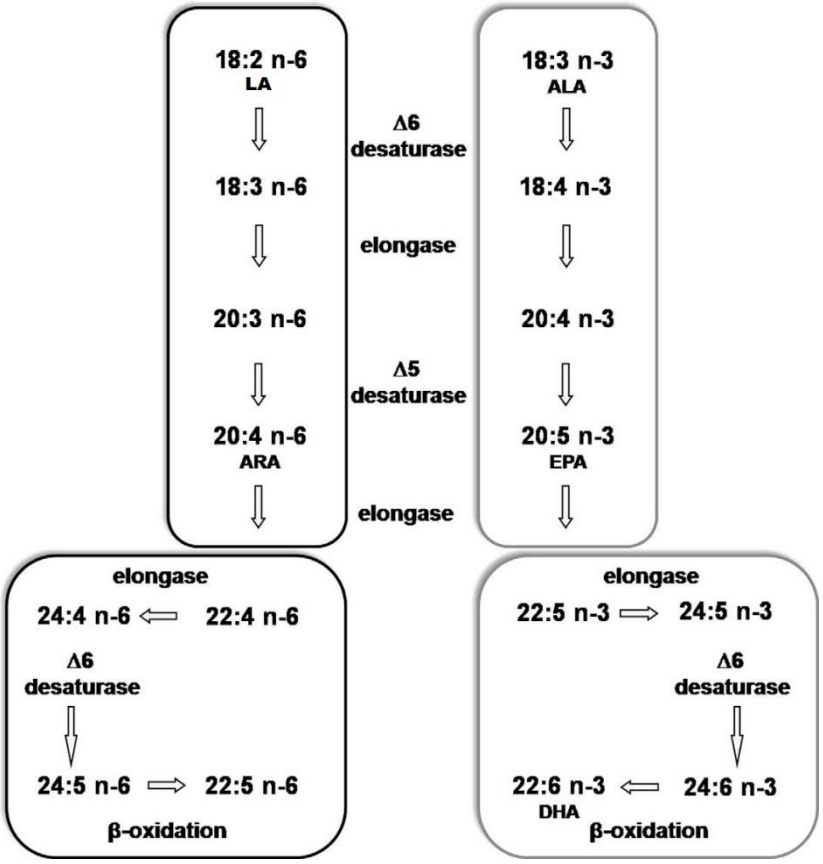


Figure 4.2. The reactions required to synthesize long chain polyunsaturated fatty acids from linoleic acid (LA) and α-linolenic acid (ALA) in hen tissues. From Barcelo-Coblijin and Murphy (2009).

4.3.2 Importance

DHA is most concentrated in the brain and the retinas. The vast neural network of the central nervous system (CNS) utilizes blood glucose as a primary energy source, thus few energy storage deposit structures are included in the CNS. FA in the CNS are therefore found in PL. Cerebral DHA is primarily concentrated in phosphatidylethanolamine (PE) of the gray matter (Svennerholm, 1968).

The curled shape of DHA does not allow it to align with adjacent FA, lowering its melting point and increasing membrane fluidity. In the CNS, these structural properties of

DHA allow increased flexibility, speed of signal transduction and neurotransmission, formation of lipid rafts and direct interaction with membrane proteins (Lund et al., 1999). DHA is also metabolized to neuroprotective metabolites (docosanoids) and is protective of the aging brain (Calon et al., 2004). The flexibility of DHA rich membranes allow membrane proteins to quickly execute their conformational changes, which can have positive health effects for a variety of organ systems (Leaf et al., 2003).

DHA is especially important in the developing CNS of infants. Infant diets supplemented with DHA are associated with improved visual acuity, psychomotor/mental development index, vocabulary score, learning, and behavioral performance (Innis, 2014a). Breast milk is commonly deficient in DHA as humans convert less than 1% of dietary ALA to DHA, and fats are usually added to infant formula as corn oil, with few LC PUFA (Carlson et al., 1986). Therefore, DHA supplementation with enriched animal products during pregnancy and lactation is ideal for increased tissue incorporation in the fetus or infant (Innis, 2014a, b)

4.3.3 Biochemical roles of DHA

In body tissues, DHA is primarily found in the inner membrane lipid bilayer as a component of PL, especially PE, phosphatidylserine (PS), and ethanolamine plasmalogens (PL with alkenyl group after ether bond at sn-1 position in place of alkyl group) (Sul, 2013).

Oxylipins

Oxylipins are bioactive, hydroxylated derivatives of selective PUFA hydrolysis from membrane PL. They are excreted from cells and exert autocrine and paracrine effects on other cells by binding to G protein-coupled receptors. Oxylipins generated from n-6 PUFA are pro-inflammatory, while those generated from n-3 PUFA are considered anti-inflammatory (Calder, 2015). Oxylipins derived from DHA are known as docosanoids, while those derived from eicosapentaenoic acid (C20:5 n-3, EPA) and arachidonic acid (C20:4 n-6, ARA) are known as eicosanoids. EPA and DHA compete with ARA for incorporation into membranes and with all oxylipin generating enzymes. They also tend to have opposing functions, and for these reasons it is important to maintain a low n-6 to n-3 ratio in the diet.

Regulation of gene expression

The genes regulated by PUFA are involved in inflammation and FA and glucose metabolism. For PUFA to be transcriptionally active, they must be desaturated at the Δ6 position, which excludes the essential FA LA and ALA. Activation of several nuclear receptors by PUFA facilitates the production of mRNA of genes associated with regulation of inflammation and lipid metabolism (Komprda, 2012).

The western diet, which is high in SFA, increases risk of fatty liver and hepatic inflammation through nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and similar signaling cascades. On the other hand, DHA reduces activation of NF-κB both directly and indirectly. By increasing fluidity of lipid rafts, DHA decreases the

signal strength of toll-like receptor 4 (TLR4), reducing the pro-inflammatory consequences of NF-κB. DHA also decreases nuclear abundance of NF-κB subunits and its mRNA. Decreasing NF-κB activity decreases inflammation in the liver, preventing oxidative damage that could lead to fibrosis (Selvaraj, 2012).

Activation by proteolytic cleavage of the sterol regulatory element binding protein (SREBP-1) increases FA biosynthesis for energy storage in response to glucose intake. SREBP-1 promotes the transcription of FA synthesizing enzymes including fatty acid synthase, acetyl-CoA carboxylase α, and elongases and desaturases. The newly synthesized SFA and MUFA in the liver are incorporated in TAG for storage, contributing to hepatic steatosis and the diseases that progress from it. DHA inhibits SREBP-1 by increasing its proteasomal degradation and increasing turnover of its protein subunits and mRNA (Jump, 2008).

In addition to NF-κB and SREBP-1, DHA may promote normal lipid regulation through the peroxisome proliferator activated receptor (PPAR) nuclear receptor family. PPARα, the primary isoform of PPAR found in the liver, reduces fat stores in the liver by inducing expression of FA transport enzymes including carnitine palmitoyl transferase 1 (CPT1A), ApoB, microsomal triglyceride transfer protein (MTTP), and fatty acid binding protein (FABP) when activated. CPT1A reduces hepatic FA by transporting FA into mitochondria for oxidation, while ApoB is a component of lipoproteins that export TAG from hepatocytes. Although ApoB synthesis is increased by PPAR activation, dietary n-3 PUFA also decrease TAG accumulation, and may therefore result in reduced VLDL secretion from the liver (Botham et al., 2003).

4.3.4 Current intake of DHA

DHA itself is not currently considered an essential nutrient because it can be synthesized from other n-3 PUFA. The rate of conversion from shorter chain n-3 PUFA are inefficient, however (Bowen and Clandinin, 2005). In rats, only 6% of dietary ALA is elongated to LC n-3 PUFA, with the majority catabolized for energy and carbon recycling (Lin and Salem Jr., 2007). ALA also competes with LA for elongases and desaturases, and LA is typically much more abundant in the diet. Directly consuming DHA is therefore a more efficient way to increase deposition in the body tissues, including the CNS.

Several organizations have released dietary recommendations for DHA or total LC n-3 PUFA based on reducing risk of cardiovascular disease. The Institute of Medicine (IOM) has established Adequate Intake (AI) levels for LA and ALA, with daily n-3 PUFA requirement of 1,100 mg for women over 19 years of age, and 1,300 mg and 1,400 mg when pregnant and lactating, respectively (Brenna and Sachs, 2013). Alternatively, The Office of Disease Prevention and Health Promotion of the U.S. Department of Agriculture (USDA) recommend that adults consume 250 mg of EPA and DHA daily in the 2015–2020 Dietary Guidelines for Americans (USDA, 2015). The American Heart Association (AHA) recommends consuming two servings of fish each week, which adds up to about 500 mg of EPA and DHA each day (AHA, 2016). These recommendations are consistent with the range published by most European organizations (Barcelo-Coblijin and Murphy, 2009).

Richter et al. (2017) analyzed data from the National Health and Nutrition Examination Survey 2003-2008 (NHANES) to assess LC n-3 PUFA intake among American subpopulations. They found that 90% of the population consumed less than the 500 mg/day recommended by the AHA, with the median American consuming an average of 110 mg/day (Richter et al., 2017). Although this is well short of recommendations, it is surprisingly high considering the limited sources available. Most LC n-3 PUFA are consumed as fish, and only 6.2% of Americans take supplements. Availability may contribute to the particularly low LC n-3 PUFA intakes by individuals of lower economic status, children, and pregnant women. Individuals of low economic status, however, eat more eggs on average (Conrad et al., 2017), and egg consumption is already recommended for pregnant women and young children. This simple yet effective approach to dietary DHA supplementation highlights the potential impact of DHA enriched eggs.

4.3.5 Sources of DHA in human diet

DHA is only synthesized in organisms that can elongate it from ALA or have a separate pathway for DHA synthesis (such as microalgae). Terrestrial plants are an important source of ALA, but never DHA, as they lack the necessary enzymes for these pathways. Fish are the most common source of DHA in the human diet. Aquatic algae produce LC n-3 PUFA, which is consumed by plankton, and passed up the food chain to fish. No terrestrial food sources have naturally comparable levels of LC n-3 PUFA. In fact, eggs and chicken meat are the next best sources (Table 4.1). Toxic mercury content

in seafood is a growing concern, to the extent that the U.S. Food and Drug Administration and the U.S. Environmental Protection Agency have published detailed guidelines of how much of which kinds of fish are safe for women of childbearing age to eat without risking mercury toxicity (U.S. Environmental Protection Agency, 2017).

USDA certified eggs may not be advertised as a "good source of," or "rich in," EPA or DHA specifically because they have no established percent daily value (USDA, 2016). Alternatively, quantitative statements such as "contains 80 mg of docosahexaenoic acid per egg," are allowed for advertising DHA, EPA, or total n-3 PUFA, and may be utilized by producers to increase retail value of enriched poultry products (USDA, 2016).

Table 4.1. Food sources and content of n-3 polyunsaturated fatty acids. From USDA (2018) and Neijat et al. (2016)

Food	Serving Size	ALA (g)	EPA (g)	DHA (g)
Flaxseed, whole[1]	1 tbsp.	2.35	0	0
Salmon, Atlantic, farmed, cooked[1]	3 oz.	0	1.24	0.59
Tuna, light, canned in water, drained[1]	3 oz.	0	0.17	0.02
Chicken, breast, roasted[1]	3 oz.	0	0.02	0.01
Beef, ground, 85% lean, cooked[1]	3 oz.	0.04	0	0
Milk, low-fat (1%)[1]	1 cup	0.01	0	0
Egg, raw, standard[2]	2 eggs	0.02	0	0.08
Egg, raw, enriched using MAL[2]	2 eggs	0.03	0.01	0.37

ALA, α-linolenic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.
[1]Accessed from https://ndb.nal.usda.gov/ndb/.
[2]Neijat et al. (2016)

4.3.6 Sources of DHA in laying hen diet

Feedstuffs used to enrich eggs with n-3 PUFA include flaxseed, fish oil, and MAL. Each of these sources are have different effects on egg FA composition, feed palatability to the hens, storage stability, egg quality and production, and egg taste to consumers. The efficiency of n-3 PUFA deposition in eggs of hens fed flaxseed, fish oil, MAL (DHAgold, as used in the current study), and the autotrophic microalgae *I. galbana*, were compared by Lemahieu et al. (2015). Each of these supplements were balanced to contribute equal n-3 PUFA content to separate diets. Fish oil was the most efficient at enriching with LC n-3 PUFA (Table 4.2). The author suggests that its advantage over MAL was that oils are more biologically available than solids. MAL was the second most efficient. Because DHA is preferentially incorporated into the yolk compared to EPA or other intermediates (Fredriksson et al., 2006; Nitsan et al., 1999), MAL, with almost all of its n-3 PUFA as DHA, is a more efficient yolk n-3 PUFA enrichment tool than flaxseed.

Table 4.2. Efficiency of four sources of n-3 polyunsaturated fatty acids at enriching eggs with long chain and total n-3 polyunsaturated fatty acids. The enrichment efficiency was calculated by taking the ratio of the n-3 LC PUFA (mg) in the egg to the actual n-3 PUFA intake (g), multiplied by 100. From Lemahieu et al. (2015). Mean comparisons not provided for total n-3 PUFA.

Supplement	Efficiency LC n-3 PUFA	Efficiency total n-3 PUFA
Flaxseed	6[a]	10
Autotrophic microalgae	30[b]	30
Fish oil	55[d]	54
MAL	45[c]	45

LC n-3 PUFA, long chain n-3 polyunsaturated fatty acid (≥C20)

Flaxseed

Flaxseed is the most commonly used ingredient commercially for n-3 PUFA enrichment of table eggs. Advantages include reduced cost relative to the other options, and the protection of PUFA from rapid peroxidation by seed coatings. ALA is the longest n-3 PUFA in flaxseed, so eggs of hens fed flaxseed are mostly enriched with ALA. When flaxseed is fed at 15%, it is possible to achieve very high n-3 PUFA content in eggs, and even DHA levels comparable to those from fish oil fed at 1.5% (Aymond & Van Elswyk, 1995). Research by Hayat et al. (2010) reported that off-flavor can be detected by trained panelists but not by untrained consumers in eggs from hens fed 10% flaxseed.

The effect of flaxseed on egg production performance is unclear, as studies report a decrease (Aymond and Van Elswyk, 1995), increase (Scheideler & Froning, 1996), and no change (Bean & Leeson, 2003). Flaxseed supplementation has been found to decrease egg weight, and is commonly associated with digestive upset when fed at higher levels (Caston et al., 1994).

The antinutritive factors of most concern in flaxseed are non-starch polysaccharides (NSP). Composed of cellulose and lignin, NSP are indigestible, increase intestinal viscosity, and decrease the digestibility of other nutrients including lipids by interfering with bile acid metabolism and micelle formation (Kristensen et al., 2012). Mucilage, a glue-like substance also found in flaxseed, causes similar problems by thickening digesta. Mucilage components have been associated with inhibiting growth performance in poultry (Rebole et al., 2002). Flaxseed also contains trypsin inhibitors, and cyanogenic glycosides. Trypsin inhibitors reduce protein digestion, and cyanogenic

glycosides inhibit cytochrome oxidase and become toxic when ingested, though neither are concentrated enough in flaxseed to be of much concern.

Fish oil

Fish oil is a rich source of LC n-3 PUFA that efficiently enriches chicken eggs. In addition to the increased cost over flaxseed, fish oil presents challenges for feed storage, egg taste, and ethics of sustainability. The FA composition is also highly variable depending on the fish sourced, though it is generally high in EPA and DHA. Very little EPA is deposited in egg yolks because, unlike ALA, the hen will readily convert EPA to DHA. Consequently, the EPA content of fish oil can be considered a DHA equivalent to poultry producers and human health officials. To slow lipid peroxidation during storage, fish oil can be microencapsulated, and is also available deodorized. Both of these processed feed supplements, however, still taint eggs with fish flavor when included over 1.5% (Gonzalex-Esquerra and Leeson, 2000). Gonzalez-Esquerra and Leeson (2000) also observed decreased feed intake (FI) in laying hens fed 1.5% fish oil. Decreased FI may be the reason that fish oil, similar to flaxseed, can lead to decreased yolk and egg weight (Lawlor et al., 2010). Another possible explanation is that because LC n-3 PUFA decrease blood, less total fat reaches the developing yolk follicles (Van Elswyk et al., 1994).

Microalgae

Development of highly specialized strains of MAL has become a frontier in biochemical research and development. MAL can be grown in contained facilities nearly anywhere in the world. Strains that are very rich in LC n-3 PUFA are now being produced in mass quantities sold for enrichment of pet and agricultural animal feeds, and are a promising, highly sustainable alternative to fish oil. In fact, wild marine MAL synthesize the LC n-3 PUFA that make their way up the food chain to ocean fish, and are the root source of the PUFA content found in fish oils and other fish products. Additionally, eggs from hens fed MAL can be marketed as vegetarian, whereas those from hens fed fish oil cannot.

MAL that synthesize their own energy source are called autotrophic MAL. Because they require less nutrient input, these MAL are the most sustainable species to produce. Lemahieu et al. (2013) identified *Isochrysis galbana* Park as the most effective commercially available autotrophic MAL for enriching eggs with LC n-3 PUFA. *I. galbana* enriched eggs with up to 81 mg per yolk without affecting production performance or egg quality (Lemahieu et al., 2013). While *I. galbana* and several other strains of autotrophic MAL are high in DHA, they are not as rich as heterotrophic MAL, which must consume energy and nutrients, but are more commonly chosen by poultry producers (Lemahieu et al., 2015).

The heterotrophic genus of MAL most chosen for enrichment of animal feed is *Schizochytrium*. *Schizochytrium* are single cell eukaryotic MAL originating from the ocean. They are naturally high in DHA, which they synthesize by an entirely different

process than animals (Huang et al., 2008). Rather than using elongases and desaturases, these organisms assemble PUFA with polyketide synthases and isomerases (Metz, et al., 2001). The MAL product used in the current study is DHAgold S17-B, (DSM Nutrition Products, Heerlen, Netherlands). In addition to being more sustainable than fish oil, it has a shelf life of 18 months and can be included at 4.8% of hen diets without any detectable off taste in eggs (Herber-McNeill and Van Elswyk, 1998). DHAgold is preserved with lecithin, rosemary extract, mono- and diglycerides, citric acid, β-carotene and other carotenoids. Feeding MAL to chickens decreases their serum TAG and cholesterol, but no effects on production performance are typically reported (Park et al., 2015).

5 CHOLINE

5.1 Structure and functions

Choline is a small, nitrogen containing organic molecule (Figure 5.1). It is often grouped with the B vitamins, as it is a water soluble and an essential nutrient with a role in one carbon metabolism. It is not, however, formally classified as a B vitamin because it is required in higher amounts than vitamins and does not have any coenzyme function. Choline is normally supplemented to poultry diets in the form of choline chloride, the salt formed between choline's positively charged ammonium group and a chloride anion. Based on the molecular weights of the two ions, choline chloride is 74.61% choline by weight. The concentration of choline chloride supplements used in poultry research varies between 50 and 98%.

Three methyl groups bound to the nitrogen atom impart choline with its function as a methyl donor for various metabolic processes. Choline is also necessary for neurotransmission, lipid digestion, cellular membrane structure, and lipoprotein metabolism. With only a slight structural modification, choline becomes the neurotransmitter acetylcholine, which send motor signals to muscles. Choline is an important component of the PC and sphingomyelin PL (Figure 5.2). PC is the primary PL secreted in bile, facilitating lipid emulsion during digestion. PC is also found in cellular lipid bilayer membranes throughout the body, and in the monolayer membranes of lipoproteins such as portomicrons and VLDL, which carry lipids to and from tissues throughout the body, including to the ovarian follicles of the hen as VLDLy. In this way,

choline facilitates lipid absorption and distribution, and is the driving factor behind the prevention of fatty liver disease as an outcome of choline deficiency. In egg yolks, PL make up 32% of total lipids, and 78% of those are normally PC (Cherian, 2005). Eggs are therefore a rich source of choline for human consumption, with approximately 140 mg in a regular large egg (Patterson et al., 2008). Excluding the vitelline membranes, all choline in eggs is localized in the yolk.

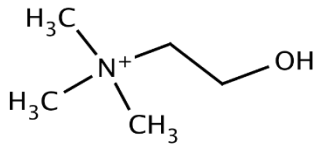


Figure 5.1. Choline structure.

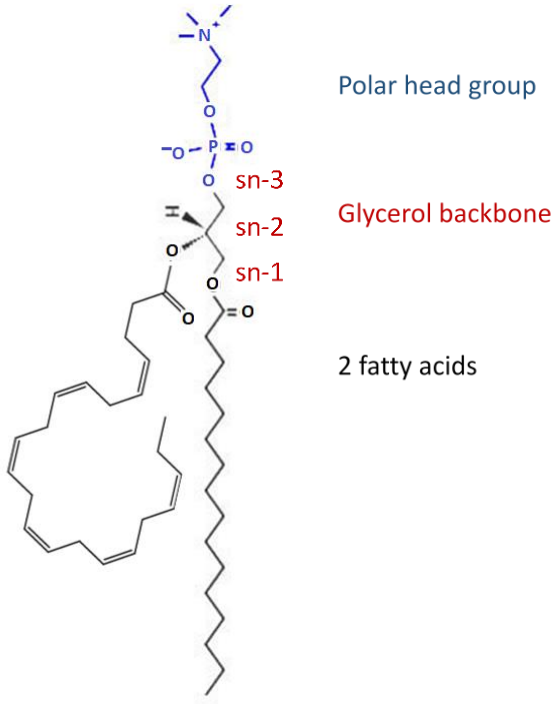


Figure 5.2. Structure of phosphatidylcholine (PC). The components of a glycerophospholipids include the polar head group, in blue, which is choline in PC, attached to the glycerol backbone by a phosphate group. The glycerol backbone has three carbons, which share ester bonds with the polar head group and two fatty acids. In this depiction, DHA is esterified at the sn-2 position and palmitic acid at the sn-1 position.

5.2 Phospholipid synthesis

PC and PE are the first and second most abundant PL in animal tissues, respectively. Both PC and PE can be synthesized de novo and from other preexisting PL, primarily by hepatocytes. PC is synthesized exclusively in the ER. Two pathways are known for choline synthesis: the CDP-choline pathway and the PEMT pathway (Figure 5.3).

Free choline is used to synthesize PC de novo via the CDP-choline pathway, also known as the Kennedy pathway. Phosphorylation of free choline to phosphocholine by choline kinase (CK) is the first of three enzymatic steps in this pathway. This

phosphocholine then reacts with cytidine triphosphate (CTP) to form cytidine diphosphate (CDP)-choline, a step which is catalyzed by CTP:phosphocholine cytidylyltransferase (CT). CT activity forming CDP-choline is the main regulatory step in the pathway, and CT activity is notably upregulated by diacylglycerol (DAG) and downregulated by PC. In the final step, CDP-choline:1,2-diacylglycerol choline phosphotransferase (CPT) catalyzes the combination of CDP-choline and DAG to form PC.

Catabolism of PE serves as an alternate pathway for PC synthesis, in a mechanism known as the PEMT pathway. Phosphatidylethanolamine N-methyltransferase (PEMT) joins three methyl groups from S-adenosylmethionine (SAM) to PE, generating PC. Oxidized choline (betaine) ties back into this pathway as a donor of groups to SAM. PEMT derived PC incorporates more DHA than PC from the Kennedy pathway, which tends to incorporate more LA (Delong et al., 1999; Pynn et al., 2011). After PE becomes PC, it can generate de novo choline when degraded into free choline and DAG. Chickens only synthesize a small amount of choline, so it is an essential dietary nutrient.

Similar to PC, PE can be synthesized in three ways. The most common is by the Kennedy pathway, by the same mechanisms as de novo PC synthesis (Figure 5.4). The first step, phosphorylating ethanolamine, uses the same enzyme, CK, while the remaining enzymes are ethanolamine-specific equivalents of those used in the CDP-choline pathway. In contrast to the PC pathway, the PE Kennedy pathway is not inhibited by the final substrate (PE).

Additionally, PS can exchange its serine for ethanolamine, transitioning it to PE, a reaction catalyzed by phosphatidylserine synthase, and taking place in the ER. In the mitochondria, PS can be decarboxylated to PE by phosphatidylserine decarboxylase. PS is a potential link between PC and PE synthesis, as PC can be converted to PS.

In poultry research, Tsiagbe et al. (1988) demonstrated that 1000 mg/kg of supplemental choline in laying hen diets increased total PL, PC, and PC to PE ratio in eggs, but decreased PE. In this same work, supplemental methionine also increased total PL but had no effect on PC or PE. Tsiagbe et al. proposed that the large increase of PC relative to the small decrease in PE was a result of PC synthesis via the CDP-choline pathway. This is consistent with the current understanding of choline's preferential use in this pathway over one carbon metabolism in hepatocytes. Similarly, supplemental choline, folic acid, and vitamin B12 all increased PC, total PL, and PC to PE ratio, and decreased PE (Rajalekshmy, 2010). The fact that these B vitamins had the same effects as choline on PC and PE synthesis, however, suggests that the PEMT pathway was responsible for increased PC synthesis.

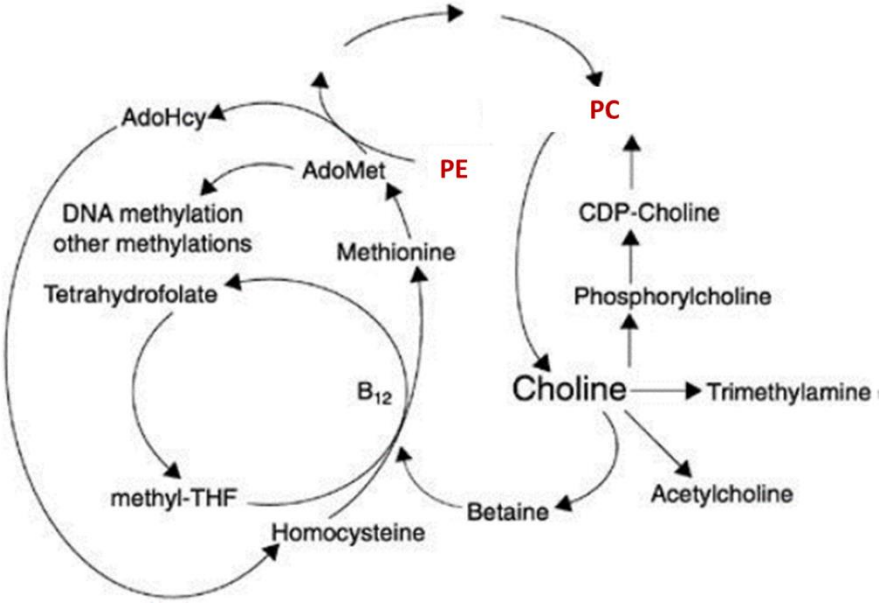


Figure 5.3. Phosphatidylcholine (PC) synthesis pathways. The CDP-choline pathway can be seen on the right, between choline and PC. The PEMT pathway can be seen between PE and PC. Choline is involved in both pathways, as it is oxidized to betaine and donates methyl groups to homocysteine. From Zeisel and Blusztajan (1994).
THF, tetrahydrofolate; AdoMet, S-adenosyl methionine (SAM); AdoHcy, S-adenosyl-L-homocysteine.

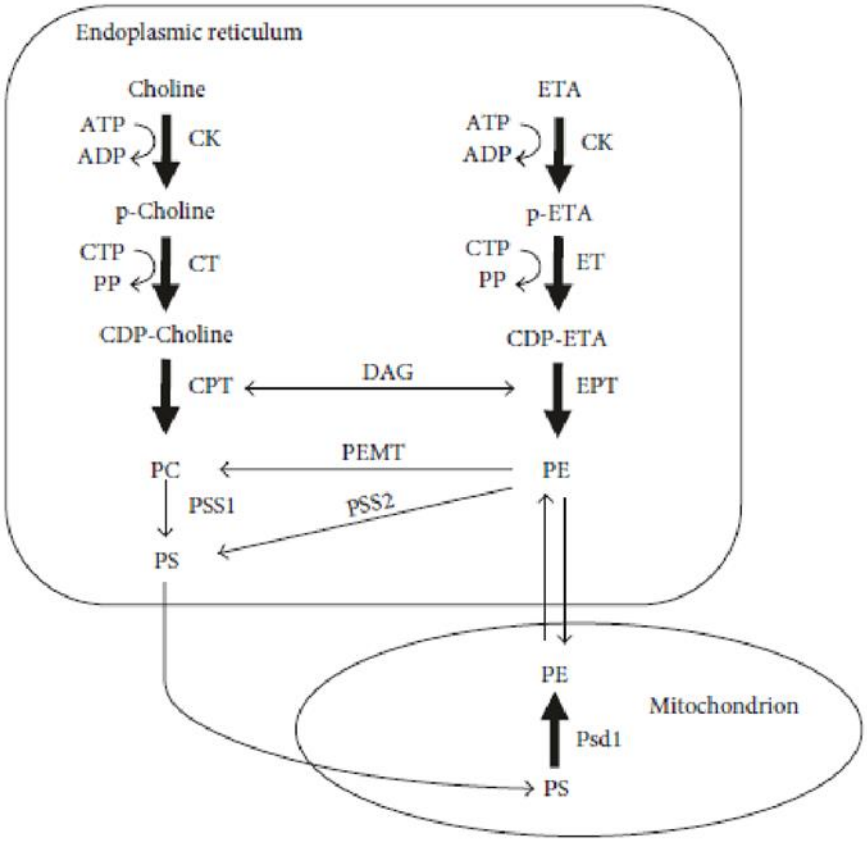


Figure 5.4. Relationships between phosphatidylcholine (PC) and phosphatidylethanolamine (PE) synthesis. Adapted from Patel and Witt (2017). ATP, adenosine triphosphate; ADP, adenosine diphosphate; CTP, cytidine triphosphate; PP, pyrophosphate; PC, phosphatidylcholine; PS, phosphatidylserine; CK, choline kinase; CT, CTP:phosphocholine cytidylyltransferase; CPT, CDP-choline:1,2-diacylglycerol choline phosphotransferase; PSS1 and PSS2, phosphatidylserine synthase; ETA, ethanolamine; ET, CTP:phosphoethanolamine cytidylyltransferase; EPT, CDP-ethanolamine:1,2-diacylglycerol ethanolamine phosphotransferase; PE, phosphatidylethanolamine; Psd1, phosphatidylserine decarboxylase 1; DAG, diacylglycerol; PEMT, phosphatidylethanolamine N-methyltransferase.

5.3 One carbon metabolism

Acquiring a single carbon, or methyl group, for biosynthesis is a complex process. Methyl groups are used for synthesizing nucleotides, methionine, and PC, and for methylating DNA, altering gene expression. Choline must first be oxidized to betaine by

the enzyme choline oxidase to participate in transmethylation reactions (du Vigneaud et al., 1946). Betaine can transfer a methyl group to homocysteine, forming methionine, catalyzed by betaine-homocystine methyltransferase (Figure 5.3). In this setting, methionine acts as a methyl transporter as part of the SAM complex, critical to PC and PE formation through the PEMT pathway. The conversion of choline to betaine is an irreversible step, and betaine is further degraded to form formate, ammonia and CO_2.

Choline also plays a role in the antioxidant network through its activities in one carbon metabolism. The oxidation of methyl-tetrahydrofolate (THF) in the carbon cycle generates significant quantities of NADPH for use in anabolic and antioxidant processes. NADPH is used to regenerate oxidized glutathione (GSSH) back to reduced glutathione (GSH), which regenerates vitamin C and vitamin E radicals. Therefore, by methylating THF, choline can potentially improve antioxidant status (Hsieh et al., 2014). Antioxidants are important for hens fed high quantities of PUFA, as these lipids are subject to peroxidation. Dietary choline has been shown to increase hepatic SAM and subsequently glutathione in fish fed high fat diets, linking choline to antioxidant capacity and downstream positive health effects (Li et al., 2014).

5.4 Choline requirement for laying hens

Starchy cereal grains are generally considered poor sources of choline. While corn has only 620 mg/kg of choline on an as fed basis, soybean meal is rich in PC and, subsequently, choline with 2,790 mg/kg. Other oil seeds including peanuts (2,400 mg/kg) and cottonseed (2,690 mg/kg) are also good sources. The typical commercial laying hen

diet that utilizes soybean meal, therefore, already has a substantial source of choline through the soybean component. Unfortunately, the choline in soybean meal based diets has oral bioavailability of around 83% (Emmert and Baker, 1997). Choline deficiency has been documented in chickens even when fed at National Research Council (NRC) recommended dietary levels (Berry et al., 1943; Marvel et al., 1943). Due to the hen's ability to synthesize choline in their liver, clinical signs of choline deficiency only occur when there are also insufficient substrates. Recommendations for laying hens are therefore based on achieving maximum production performance, as opposed to avoiding gross disease conditions.

Eggs of hens supplemented with choline have been associated with a fishy odor, which results from the generation of trimethylamine (TMA). TMA is a product of choline degradation by enteric bacteria. Healthy commercial strains of white egg laying hens utilize the hepatic enzyme flavin containing monooxygenase 3 (FMO3) to metabolize TMA to the odorless TMA oxide, which is then eliminated in excreta (Bain et al., 2005). Fortunately, hens with functional FMO3 can be supplemented with over 4,600 mg of choline chloride without raising TMA levels in their egg yolks (Kretzschmar et al., 2009).

The choline requirement for laying hens remains controversial. White Leghorn type laying hens eating 100g of feed per day should be fed 1,050 mg/kg of choline in their diet as fed, and require little to no additional supplementation according to NRC guidelines (NRC, 1994). Management guidelines from Hy-Line for their W-36 hens recommend adding 110 mg/kg of choline to diets during the laying period for ideal performance (Hy-Line, 2016). Up to 2,313 mg/kg of total choline in the diets of brown

egg laying hens has been shown to maintain adequate production (Zhai et al., 2013). Over-supplementation of these same hens was achieved at 4,078 mg/kg, which caused egg production to drop but improved Haugh units (Zhai et al., 2013). Tsiagbe et al. (1988) found that increasing choline from 1040 to 2040 mg/kg through supplementation in layer diets increased egg and yolks weights. Together, the literature suggests that up to about 2,000 mg/kg of choline in the diet may have overall positive effects, but supplementation beyond that may be detrimental.

As dietary choline has been shown to increase PC content in chicken eggs, and PC preferentially incorporates LC PUFA, it can be hypothesized that supplementing choline in laying hen diets will increase LC PUFA content in their eggs. Wang et al. (2017) tested this hypothesis, and found that adding 980 mg/kg of choline chloride to corn and soybean meal based diets containing MAL increased DHA incorporation in egg yolks ($p<0.05$).

5.5 Choline in the human diet

The daily adequate intake of choline established by the IOM is 550 and 425 mg for adult males and females, respectively. However, according to 2013–2014 NHANES data, Americans are consuming much less choline than recommended, with men consuming only 402 mg on average and women 278 mg (USDA, 2016). Recommendations for pregnant and lactating women are increased to 450 and 550 mg, respectively. As with DHA, Choline is important for cell proliferation and neural differentiation because of its role in PL membrane formation and maintenance. Very high

concentrations of choline are delivered to the human fetus across placenta, providing further support for its importance in embryo development (Sweiry et al., 1986; Ozard et al., 2002).

Beside chicken eggs, liver and fish are among the richest dietary sources of choline. It is also found widely in other meats, vegetables, and legumes (Patterson, 2008). The enrichment of table eggs with additional choline may serve as a mechanism by which to increase dietary choline to acceptable levels in the Western diet, especially for pregnant or nursing women and young children.

6 FATTY LIVER DISEASE

Lipid accumulation in the liver is a metabolic issue that affects both people and livestock, and may be modulated by diet. In people, the progressive form is called nonalcoholic steatohepatitis (NASH). Excess fat accumulation in the liver sensitizes it to inflammation, oxidative stress, liver damage, and fibrosis. NASH affects 18 million Americans, and these people have 10-fold higher risk of hepatic failure and death, and two-fold higher risk of cardiovascular-related mortality (Kapeller, 2015).

Laying hens are prone to accumulating hepatic lipids because of their extremely high rate of lipogenesis, stimulated by estrogen at the onset of lay. The hen's liver enlarges to three times its normal size at sexual maturity, in preparedness for the metabolic demands of the reproductive effort. Commercial hens are also confined to small cages where caloric expenditure is limited. Fatty hen livers can become brittle and tear, characterizing the metabolic disease fatty liver hemorrhagic syndrome (FLHS), resulting in sudden death and economic losses (Khosravinia et al., 2015).

Dietary choline supplementation may reduce hepatic fat content and associated disorders through the lipid transport and metabolism pathways outlined above. Supplementing 284 and 567 mg/kg of choline in the diets of broiler breeder hens reduced liver fat concentration (Rao et al., 2001). Liver fat was similarly decreased in brown laying hens supplemented with 1000 and 4000 mg/kg of choline, but not 500 mg/kg (Danicke et al., 2006). When more than 1000 mg/kg of choline is supplemented, the benefit may not continue to increase. No decrease in hepatic fat was observed in laying hens when diets already containing supplemental choline at a rate of 900 mg/kg were

provided with another 980 mg choline/kg feed (Schumann et al., 2003). Addition of 10% flaxseed, however, did decrease liver fat significantly, indicating that choline supplementation of 900 mg/kg was sufficient to support the health benefits of flax supplementation (Schumann et al., 2003).

N-3 PUFA may ameliorate the liver health consequences characterizing NASH in laying hens (Schumann et al., 2010). LC PUFA are associated with anti-inflammation, inhibiting excessive FA synthesis, increasing lipolysis and β-oxidation, and lowering blood TAG. In rats, dietary DHA reversed western diet induced NASH (Depner et al., 2013). This study also showed that DHA is more effective than EPA at preventing hepatic lipid oxidation and gene expression markers of NASH. In laying hens however, fish oil has increased hepatic lipid accumulation (Van Elswyk et al., 1994). The author of that study suggested that hens may respond differently than mammals to n-3 PUFA due to interactions with estrogen, which has strong lipogenic action in laying hens.

7 PHYSICAL EGG QUALITY CHARACTERISTICS

Physical egg quality refers to all of the visual and tactile aspects of the egg that influence its acceptability to consumers. Taste and smell are not accounted for in the current study but may influence consumer preferences. Egg quality can be affected by hen nutrition, genetics, age and oviposition, environmental temperature, housing type, and egg storage (Ketta and Tůmová, 2016; Roberts, 2004). Physical egg quality is judged on the three major components of the egg: the outer shell, the albumen, and the yolk. The characteristics of each of these components are of critical importance for proper marketability and profitability of table eggs.

Egg shells have the biological roles of providing protection, appropriating gas exchange, and serving as calcium source for the developing embryo. Shells also protect the egg as a food product from spoilage, and thicker shells are advantageous for breakage prevention. The quality of a shell is measured by its weight, thickness, and percentage of the total egg by weight. Larger eggs are associated with thinner shells, as approximately the same amount of calcium is distributed over a larger area. Commercially, eggs are also graded for texture and color uniformity, though the pigmentation of egg shells is not an indicator of nutritional quality. Nutritional factors in the hen diet which impact shell quality include calcium, vitamin D, non-starch polysaccharides, and phytases (Roberts, 2004). Including MAL in hen diets may also increase shell thickness without decreasing egg size (Park et al., 2015).

The albumen, or egg white, provides nutrition and anti-microbial protection for the developing embryo, and is of importance in the food and baking industries. Albumen

quality is judged by its height, weight, and percentage of egg weight. Haugh units are a common way to express albumen height as a function of egg weight which accounts for the misrepresentative albumen height of smaller eggs. Supplemental choline in hen diets has been found to increase Haugh units (Zhai et al., 2013).

Egg yolks, which provide the majority of nutrition for developing avian embryos, are similarly judged by their height, weight, width, and percentage of egg weight. The ratio of yolk height to width, or yolk index, may be used to account for the integrity of the vitelline membrane which helps the yolk hold its shape when fresh and from hens of an appropriate nutritional status. The color of egg yolks is also of interest to consumers, though color preference varies on an individual basis. Yolk color can be manipulated by including carotenoids, especially xanthophylls, in the hen diet. Some MAL, including *Schizochytrium*, have been shown to increase the red pigmentation of egg yolks (Herber-McNeill and Van Elswyk, 1998; Lemahieu et al., 2014). DHAgold is advertised with 29.8 mg/kg of β-carotene and 0.6 mg/kg of canthaxanthin, among other carotenoids, but does not affect yolk color.

Egg quality is also negatively affected by portions of blood or reproductive tissue known as blood and meat spots, respectively. Not only may these lead to consumer refusals, but meat spots larger than one eighth of an inch are grounds for downgrading of table eggs by the USDA. Deposition of other foreign materials, such as parasites or enveloped smaller eggs, is a rare but unfortunate occurrence which results in total refusal of affected eggs. As with yolk, shell and albumen characteristics, these negative quality

parameters may be influenced by hen genetics, management and diet (Ketta and Tůmová, 2016; Roberts, 2004).

8 LIPID PEROXIDATION

8.1 Overview

Lipid peroxidation causes several undesirable changes in food quality (Arab-Tehrany et al., 2012). The nutritional quality of foods decreases with the loss of essential FA, vitamins, and calorie content. Off flavor, odor, and color are negative sensory changes. In addition, shelf life, consumer acceptability, food safety, and economic loss are important concerns in commercial situations.

Lipid peroxidation is a challenge concomitant with PUFA, as the numerous carbon-carbon double bonds in PUFA are more prone to this form of spoilage than SFA. This process can lead to the degradation of the FA and others around it. Lipid peroxidation can be described in three phases: initiation, propagation, and termination.

Lipid peroxidation is usually initiated by a molecule with an unpaired electron, known as a free radical. Free radicals react readily to regain stability by gaining another electron from nearby molecules. In cellular systems, most free radicals originate naturally as reactive oxygen species from mitochondria and peroxisomes. Many have higher electronegativity than carbon, and thus re-stabilize by gaining the hydrogen and electrons from a nearby carbon, creating a carbon centered radical (R·). Transition metal ions like Fe^{2+} and Cu^{2+}, ultraviolet light, and ionizing radiation can also have the same effect. Carbon radicals readily react with O_2, creating a peroxyl radical (ROO·). If the carbon radical is on a lipid, such as the lipid component of the cellular membrane, this radical is now known as a lipid peroxyl radical.

The lipid peroxyl radical can react with other FA in an attempt to stabilize itself, propogating a chain reaction. If a lipid peroxyl radical gains a hydrogen and electrons from the carbon of a neighboring FA, it now becomes the more stable FA hydroperoxide (ROOH). The newly generated lipid peroxyl radicals will continue to react with neighboring FA until they react with another radical or an antioxidant terminates the chain reaction.

Termination of the lipid peroxidation chain reaction requires that the lipid radical engage in a reaction with stable products. In one such reaction, two unpaired electrons become one set of paired electrons when the lipid radical reacts with another lipid radical or peroxyl radical, bonding them together (ROOR' or RR'). Antioxidants are the other option for breaking the oxidation cycle. Antioxidants can donate an electron, becoming a relatively benign antioxidant radical (A·) which can usually be reduced and regenerated in the antioxidant cycle. In highly oxidized tissues, antioxidants can be depleted due to the continuous need to regenerate stable lipid species and other molecules. ROOH can also be converted to a less toxic hydroxyl FA (ROH) by the selenium-dependent enzyme phospholipid hydroperoxide glutathione peroxidase (GPx4).

Although PUFA are subject to peroxidation, they may also have antioxidant effects. In broiler chickens, supplementation with MAL increases total antioxidant capacity and antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, catalase (Long et al., 2018). This increase in antioxidant capacity was associated with a decrease in biomarkers of oxidative stress in breast and thigh tissues, which may improve food quality and shelf stability of products from these animals (Long et al., 2018).

8.2 Vitamin E

Vitamin E is an essential, fat soluble vitamin synthesized by plants which includes 8 similar, naturally occurring molecules: α-, β-, γ-, and δ- tocopherol and α-, β-, γ-, and δ- tocotrienol (Mene-Saffrane and DellaPenna, 2010). Each of these molecules has some antioxidant capability, but α-tocopherol (αT) has substantially more vitamin E activity in humans and animals, and is the only form that satisfies dietary requirements. The increased bioavailability of αT is due to the binding pocket of αT transfer protein, critical for transport of vitamin E to the systemic circulation, strictly fits d-α-tocopherol (the natural phytyl tail conformation). γ-Tocopherol (γT), on the other hand, is the most abundant form in the hen and human diets, but it is cleared quickly from circulation and not well retained in tissues. All forms of vitamin E preserve lipids in feed during storage, and may protect the integrity of food ingredients high in PUFA prior to ingestion. In the body, vitamin E is a component of PL membranes and serves to protect PUFA from oxidation. It has much higher affinity for reducing FA peroxyl radicals than FA do, so is capable of effectively interrupting the cycle of lipid peroxidation. Egg yolks are a rich source of vitamin E, which is an essential nutrient for developing avian embryos. Normal large table eggs contain about 590 μg of αT (USDA, 2018). Supplemental vitamin E in the laying hen diet preserves PUFA and decreases the peroxidation products in eggs (Cherian et al., 1996). In the liver, unfortunately, αT is degraded more quickly when diets high in EPA or DHA are consumed as it is oxidized in the process of salvaging PUFA (Depner et al., 2013).

Objectives of this thesis are:

1. To investigate the effect MAL, and choline dosage in diets containing MAL, on egg production performance, physical egg quality, and nutritional parameters.
2. To determine whether choline dosage influences hepatic lipid metabolism of hens fed MAL.

Hypotheses of this thesis:

1. Addition of dietary MAL would alter egg FA composition without affecting egg production or quality.
2. Supplementing diets containing MAL with choline chloride would change egg PC, FA, and choline content without affecting egg production or quality.
3. Dietary choline would alter PL synthesis, hepatic fat accumulation, and oxidative status.

9 EXPERIMENT 1. EFFECT OF DIETARY MICROALGAE AND CHOLINE ON LAYING HEN PERFORMANCE AND EGG QUALITY

9.1 Introduction

N-3 PUFA are essential nutrients in the human diet, and their biological roles in cell function and inflammation decrease the risk of heart disease (Jump et al., 2012). The body requires LC n-3 PUFA such as DHA to perform these functions, but only ALA is considered essential by the IOM. The conversion of dietary ALA to DHA is inefficient, thus direct consumption of DHA is more effective. Unfortunately, Americans fall well short of the 500 mg of LC n-3 PUFA recommended by the AHA (Richter et al., 2017). This is largely because the limited fish consumption accounts for almost all dietary LC n-3 PUFA, and other protein sources are more widely utilized. One way to distribute n-3 PUFA in foods for human consumption is to allow livestock to consume n-3 PUFA, creating enriched products. Eggs are one such product which is readily available, affordable, and enriched with dietary supplementation to the layer hen.

MAL is an efficient supplement option for enrichment of egg yolks with DHA without negative production or product sensory quality effects (Herber-McNeill and Van Elswyk, 1998). Eggs enriched with n-3 PUFA sell for more at retail, but the supplemental feedstuffs also raise costs for egg producers. There are other dietary factors to consider in order to maximize n-3 PUFA enrichment efficiency. Alternatives to MAL supplementation include flaxseed, which has several drawbacks, including the necessity of carbohydrase enzymes to overcome the antinutritive properties for optimal production. Diets high in PUFA from any source can benefit from antioxidants, such as tocopherols,

to prevent the lipid peroxidation and negative sensory characteristics associated with PUFA enriched eggs. There has been little research, however, on non-vitamin or enzyme supplements to improve PUFA enrichment and quality in egg products. In the current study, choline is investigated as a nutritional supplement to compliment a PUFA enriched diet.

The 1,050 mg/kg of choline recommended by the NRC (1994) is nearly satisfied by standard corn and soybean meal based diets, as soybean meal is rich in PC, a natural source of choline. Consequently, all diets formulated in the current study to meet Hy-Line W-36 recommendations were calculated to contain sufficient choline at 1,050 mg/kg without vitamin supplementation. The Hy-Line W-36 manual recommends adding an additional 110 mg/kg in the form of a choline chloride supplement. Evidence exists that supplementation can be beneficial for egg quality, production, and PC enrichment of egg yolks, but that over-supplementation can reduce egg production (Tsiagbe et al., 1988; Zhai et al., 2013).

The purpose of this study was to investigate the effect a MAL, and choline dosage in diets containing MAL, on egg production performance, physical egg quality, and nutritional parameters. It was hypothesized that MAL would alter egg FA composition without affecting egg production or quality, and supplementing diets containing MAL with choline chloride would change egg PL, FA, and choline content without affecting egg production or quality.

9.2 Materials and Methods

All animal work was approved by the Oregon State University Institutional Animal Care and Use Committee (ACUP #4913). Animals were housed indoors at the Oregon State University Harrison Poultry Farm in Corvallis, Oregon for the duration of this work.

9.2.1 Birds, diet and management

A total of 56 white leghorn hens (24 weeks age) were kept in individual cages (46 cm x 53 cm x 58 cm (l·w·h)) and randomly assigned to one of the four treatments. Hens were fed experimental diets during a two week adaptation period prior to physiological measurements beginning at 26 weeks of age. Each treatment had 14 hens kept in seven replicate groups with two hens per group (n=7 per treatment). The experimental trial period lasted 16 weeks. Birds were weighed before and after the end of the feeding trial. Feed and water were provided ad libitum.

Choline content of MAL was measured in house, while choline content of other feed components, including the vitamin-mineral premix, were taken from manufacturer literature or NRC values (NRC, 1994). Gross energy, crude protein, and ash content of the experimental diets were analyzed at the Center of Excellence for Poultry Science Central Analytical Laboratory of the University of Arkansas (Fayetteville, AR). All diets were formulated using Windows User-Friendly Feed Formulation (WUFFDA), v2.0 (Pesti et al. 2015) to be corn-soybean meal based, isocaloric, isonitrogenous, and

containing 551 mg supplemental choline per kg diet via a commercial vitamin-mineral premix.

Diets Alg, Ch0.1, and Ch0.2 were supplemented with 1% MAL (DHAgold S17-B, DSM Nutritional Products, Heerlen, Netherlands) as recommended by the manufacturer. In addition to the choline present in the vitamin and mineral premix, choline chloride was supplemented at 0.1% in Ch0.1 and 0.2% in Ch0.2. The control diet (Control) contained no supplemental MAL or choline chloride (Table 9.1). Feed was mixed every two weeks to maintain freshness and minimize PUFA loss to oxidation. Light and heat were provided according to Oregon State University Poultry Center standard procedures for laying hens. The house was heated and the daily photoperiod was 16 hours.

Table 9.1. Summary of supplements added to experimental diets.

Diet	Control	Alg	Ch0.1	Ch0.2
Microalgae Supplement (%)	0	1	1	1
Choline Chloride (%)	0	0	0.1	0.2

Control = Corn and soybean meal diet; Alg = Control plus 1% MAL; Ch0.1 = Alg plus 0.1% choline chloride; Ch0.2 = Alg plus 0.2% choline chloride.

9.2.2 Analysis of performance data

The number and weight of eggs laid by each replicate were recorded daily throughout the trial. Each replicate was fed from its own container of feed. Hen production performance was quantified by calculating hen day egg production (EP, %), average egg weight (EW, g/egg), hen day egg mass (EM, g/hen/day), feed intake (FI,

g/hen/day), and feed conversion ratio (FCR) as shown below. EP, EW, and EM were averaged for each replicate over each week. FI and FCR were calculated for each replicate every 2 weeks. Although experimental replicates consisted of 2 hens, values were averaged over each animal to present a per hen basis. The formulas used are as follows:

$$EP = \frac{n\ eggs\ laid}{n\ hens\ \times\ n\ days} \times 100\%$$

$$EW = \frac{sum\ egg\ weights\ (g)}{n\ eggs\ laid}$$

$$EM = \frac{sum\ egg\ weights\ (g)}{n\ hens\ \times\ n\ days}$$

$$FI = \frac{\left(feed\ remaining\ prevous\ wk.\ (kg) + feed\ added\ previous\ wk.\ (kg) - feed\ remaining\ current\ wk.\ (kg)\right) \times 1000\ g}{n\ hens\ \times\ n\ days}$$

$$FCR = \frac{FI\ (g)}{EM\ (g)}$$

9.2.3 Physical Egg Quality Characteristics

One egg from each replicate, for a total of seven eggs per diet, was collected every two weeks for assessment of physical quality characteristics. All eggs were analyzed on the same day that they were laid. Each egg was weighed intact, and then broken onto a level breakout table, on which yolk and albumen height were measured with a Haugh meter and yolk width was measured using a digital caliper. Yolk color was recorded using a Roche yolk color fan. Yolks were then separated from the albumen, weighed individually, and frozen at -20°C until further analysis. Egg shells were rinsed clean with distilled water and dried in an oven before weighing and measurement of

thickness twice on opposite sides of the midline with a digital micrometer. Calculated quality parameters were derived from the following equations:

$$Albumen\ wt.(g) = egg\ wt.(g) - (yolk\ wt\ (g) + shell\ wt.(g))$$

$$Yolk\ index = yolk\ ht.(mm) \div yolk\ width\ (mm)$$

$$Haugh\ Unit\ = 100 \times log(h - 1.7w^{0.37} + 7.6)$$

when h = albumen height (mm) and w = egg weight (g) (Haugh, 1937).

9.2.4 Analysis of egg nutritional parameters

Lipid extraction and analysis

At every four weeks of the feeding trial, lipids were extracted from each of the 28 egg yolks that had been previously analyzed for physical quality using the method of Folch et al. (1957). Lipids were similarly extracted from feed every two weeks and once from MAL. Two grams of sample were homogenized in 18 mL of chloroform:methanol (2:1, vol/vol) and refrigerated overnight. Samples were then filtered and mixed with 0.88% NaCl solution to separate hydrophilic compounds. After separation, the volume of the hydrophobic chloroform layer was recorded and stored at -20°C for further analysis. Total lipid content of each egg yolk was measured gravimetrically by evaporating a known volume of lipid extract from each sample onto pre-weighed aluminum trays.

Fatty Acid Analysis

The previously generated lipid extracts were used for analysis of FA using an HP 6890 gas chromatograph (GC) (Hewlett-Packard Co., Wilmington, DE) equipped with an autosampler, flame ionization detector, and SP-2330 fused silica capillary column. Methyl docosanoate (C22:0, cat. no. 1036, Matreya, LLC, 500 mg) was used as an internal standard. FA methyl esters (FAME) of lipid extract with internal standard were generated by reconstituting lipids in two milliliters of methylating reagent (45% BF_3, 35% methanol, 20% hexane) and heating for 60 minutes at 100°C. After cooling, two milliliters of hexane and two milliliters distilled water were added and tubes shaken vigorously by hand for 90 seconds. Once the solvents had separated, the hexane layer was removed and diluted 75:400, 100:300, or 50:450 with hexane for yolk, feed, and MAL samples, respectively. Injection volume used was one microliter, with helium as a carrier gas. Initial column temperature was set at 150°C, held for 1.5 minutes, then increased to 190°C at a rate of 15°C/minute, held for 20 minutes, then increased to a final temperature 230°C at a rate of 30°C/minute, and held at this final temperature for three minutes. Inlet and detector temperatures were set at 250°C. Individual FAME were identified by matching peak elution time with those of pure standards (Nuchek Prep, Elysian, MN). Peak areas were calculated using Hewlett-Packard ChemStation software (Agilent Technologies Inc., Wilmington, DE), and concentration of each FA calculated using the internal standard and the following formula:

$$[FA]\ (mg/g) \ = \frac{sample\ peak\ area \div int.standard\ peak\ area}{original\ sample\ (g) \div chloroform\ (mL) \times sample\ extract\ methylated\ (mL)}$$

Tocopherol analysis

αT and γT were quantified in feed and MAL using the HPLC procedures described in detail in Chapter 10, with minor changes. Feed and MAL samples were run in triplicate. Initial sample sizes were 50 and 25 mg of feed and MAL, respectively, and saponification reagents were doubled for MAL sample preparation. Dry sample of both analytes were resuspended in 0.2 mL of ethanol:methanol mixture.

Phospholipid analysis

Egg yolks collected at week 12 of the trial were also analyzed for PL classes by a procedure previously described in Chen et al. (1982), with modifications described in Balazs et al. (1996). PC and PE were separated and quantified with the same HPLC described in vitamin E procedures, but with a Cosmosil 5SL-II, 250 × 4.6 mm packed column and guard column (Nacalai Tesque Inc., Japan). Mobile phase was acetonitrile:methanol:phosphoric acid (100:30:0.05 v/v/v), oven temperature set at 40°C, the flow rate one mL/min., and wavelength 205 nm. Sample preparation started with lipid extract prepared as for FAME analysis, above. Twenty five microliters of lipid extract for yolk and MAL and 50 μL for feed samples was evaporated under nitrogen gas and resuspended in 800, 400, or 300 μL of methanol for egg, MAL, and feed samples, respectively. Samples were passed through 0.45 μm nylon membrane syringe filters and 10 μL injected for analysis. Concentrations of PC and PE were calculated by comparing elution peak areas to standard curves. A polar lipid mixture containing 25 mg/mL of equal parts cholesterol, PE, PC, and lysoPC (cat. no. 1127, Matreya, LLC) was diluted to

one mg/mL and used with increasing injection volumes to calculate a standard curve. As the specific FA composition of PL fractions were not measured, PC and PE concentrations were calculated using the molecular weight of the pure standards used for standard curve construction (which were also extracted from egg yolk per the manufacturer).

Total choline analysis

Chemicals and reagents: Total choline was measured in samples using an enzymatic, colorimetric assay. All compounds used in this assay were purchased from Sigma-Aldrich Corp., St. Louis, MO.

Total choline content of egg yolks, feed, and Alg were measured using the Official Methods of Analysis by the Association of Analytical Communities (AOAC, 2006), with modifications for egg yolk as reported by Sun et al. (2013). To hydrolyze choline, one gram samples were homogenized and heated in 15 mL of 1 N NaOH (≥98%, S5881) at 70°C. A chromogenic reagent was prepared using 300 mL of 0.05 M Trizma buffer (≥99.9%, T1503), five microliters of phospholipase D from *Streptomyces chromofuscus* (≥50,000 units/mL, P0065), 28 mg choline oxidase from *Alcaligenes sp.* (≥10 units/mg, C5896), six milligrams peroxidase from horseradish (type I, 50-150 units/mg, P8125), 45 mg 4-aminoantipyrine (≥99%, A4384), and 150 mg phenol (BioXtra, ≥99.5%, P5566). After adjusting samples to a pH of 3.5-4 with six N HCl solution (37%, 320331), adjusting sample volumes to 25 mL with water, and filtering, three milliliters of this chromogenic reagent was added to 0.1 mL of each sample and

three milliliters of water. All samples were incubated in 37°C water bath and absorbance was measured at 505 nm with a spectrophotometer (UNICO, Dayton, NJ). Choline concentration was calculated by comparing sample absorbance to a standard curve of choline bitarate (cat. no. C1629) prepared in the same manner as biological samples.

Thiobarbituric acid reactive substances

Lipid peroxidation in egg yolks was evaluated by estimating malondialdehyde (MDA) concentration, a highly thiobarbituric acid reactive substance (TBARS). This was done using a colorimetric assay previously described in Salih et al. (1987), with modifications built on those described in Cherian et al. (2002). Two gram samples of egg yolks were homogenized in 18 mL of 3.86% perchloric acid solution and 50 µL of butylated hydroxyanisole (0.045 g/mL). The homogenate was filtered and divided into two milliliter duplicates for each sample. Two mL of 20 mM TBA solution in water was added, and samples heated to 100°C for 30 minutes and allowed to react overnight at 4°C. The absorbance of samples was measured using a spectrophotometer (UNICO, Dayton, NJ) at 531 nm and compared to a standard curve made with graded concentrations of 1,1,3,3-tetraethoxypropane (T-9889, Sigma-Aldrich Corp., St. Louis, MO), which had been prepared in a similar manner as biological samples. The two duplicates of each sample were averaged for data analysis. Values represent milligrams of MDA equivalents per gram of egg yolk.

9.2.10 Statistical analysis

The effect of dietary treatment on all response variables was analyzed with a general linear mixed model using the Mixed procedure of SAS software (version 9.4) (SAS Institute). The experimental unit was replicate group in all models. Week and diet by week interaction were covariates in models with measurements from each replicate analyzed as repeated measures. Because a dosage effect of choline was observed between the two dietary levels of choline, comparisons between treatment means were made by orthogonal contrast. For all response variables, the effect of Alg vs Control was calculated, and Ch0.1 and Ch0.2 were compared separately to Alg to quantify the effect of adding choline chloride to diets containing MAL. P-values were considered significant at ≤ 0.05 and trending at $0.05 \leq p \leq 0.10$. Least square means and pooled standard error of the means (SEM) are reported.

9.3 Results

9.3.1 Feed analysis

The ingredient composition and calculated nutrient content of experimental diets are shown in Table 9.2. The amount of choline contributed by each ingredient is shown in Table 9.3. The measured choline content of diets was only about half of what was calculated based on database values (Table 9.4). Choline measurements in egg yolks and liver (in Experiment 2) were consistent with those previously recorded by others, 10.5 and 1.4 vs. 6.8 and 1.9 (Patterson, 2008). The disparity may be due to differences between nutrient content of feedstuff samples used in the current experiment and those used for database generation. Alternatively, finely ground feed samples caused filtering complications during the procedure, which may have affecting quantification.

MAL brought the DHA content of feed from undetectable to one mg/kg (Table 9.4). Total n-3 PUFA concentration doubled, cutting the n-6 to n-3 ratio in half. Analysis of the MAL confirmed that it contains above 40% crude fat. It is also a rich source of choline, greatly exceeding what could be provided by PC (Table 9.5). The MAL had only a small concentration of αT, suggesting that αT and γT were not used as antioxidant preservatives in this supplement.

Table 9.2. Ingredient composition of experimental diets and calculated nutrient content.

Ingredient, %	Dietary Treatment			
	Control	Alg	Ch0.1	Ch0.2
Corn	59.76	59.05	58.68	58.41
Soybean Meal	24.25	24.13	24.18	24.22
Limestone	10.35	10.35	10.35	10.35
Corn Oil	2.50	2.33	2.48	2.55
Dicalcium Phosphate	2.10	2.10	2.10	2.10
Premix[1]	0.50	0.50	0.50	0.50
Salt	0.41	0.41	0.41	0.41
D,L-Methionine	0.13	0.13	0.13	0.13
Microalgae	-	1.00	1.00	1.00
Choline Chloride (60%)	-	-	0.17	0.33
Calculated Nutrient composition (%)				
ME (kcal/kg)	2,840	2,840	2,840	2,840
Crude protein	16.15	16.15	16.15	16.15
Ether extract	4.81	5.01	5.15	5.21
Calcium	4.56	4.56	4.56	4.56
Sodium	0.18	0.21	0.21	0.21
Potassium	0.66	0.67	0.67	0.67
Chloride	0.29	0.29	0.31	0.34
$(Na^+ + K^+):Cl^-$	2.90	3.03	2.84	2.59
Available phosphorus	0.49	0.49	0.49	0.49
Methionine	0.38	0.38	0.38	0.38
Methionine + cystine	0.62	0.61	0.61	0.61
Choline (mg/kg)	1,598	1,714	2,461	3,203

ME, metabolizable energy.
Control = Corn and soybean meal diet; Alg = Control plus with 1% MAL; Ch0.1 = Alg plus 0.1% choline chloride; Ch0.2 = Alg plus 0.2% choline chloride.
[1]Provided per kilogram of diet: vitamin A, 19,200 IU; vitamin D, 4,850 IU; vitamin E, 13 IU; thiamine, 3.2 mg; riboflavin, 8.8 mg; niacin, 66 mg; pantothenic acid, 11 mg; choline, 551 mg; pyridoxine, 2.0 mg; folacin, 0.50 mg; vitamin B_{12}, 0.018 mg; calcium, 745 mg; chloride, 1.2 mg; magnesium, 9.4 mg; sulfur, 112 mg; copper, 10 mg; iodine, 5.7 mg; iron, 97 mg; manganese, 125 mg; selenium, 0.60 mg; zinc, 101 mg.

Table 9.3. Choline containing ingredients and their calculated contributions of choline in the diet.

Ingredient	Choline in diet (mg/kg)			
	Control	Alg	Ch0.1	Ch0.2
Choline Chloride (60%)	0	0	748	1,491
Premix	551	551	551	551
Microalgae Supplement	0	124	124	124
Soybean meal	677	673	675	676
Corn	371	366	364	362
Corn oil	0.05	0.05	0.05	0.05
Total	1,598	1,714	2,461	3,203

Control = Corn and soybean meal diet; Alg = Control plus 1% MAL; Ch0.1 = Alg plus 0.1% choline chloride; Ch0.2 = Alg plus 0.2% choline chloride.

Table 9.4. Analyzed nutrient composition of experimental diets.

Item, %	Dietary Treatment			
	Control	Alg	Ch0.1	Ch0.2
GE (kcal/kg)[1]	3,563	3,678	3,627	3,615
Crude protein[1]	16.3	17.0	16.8	16.3
Ether extract	4.24	4.44	5.15	4.78
Ash[1]	11.89	11.88	13.29	14.21
Choline (mg/kg)	1,080	919	1,540	1,937
PE (mg/g)	0.29	0.29	0.30	0.32
PC (mg/g)	2.18	2.51	2.54	2.62
α-tocopherol (mg/kg)	12.34	18.227	16.67	14.93
γ-tocopherol (mg/kg)	25.14	28.90	33.92	35.62
Fatty Acids (g/kg)				
C22:6 n-3	0.00	1.03	0.93	1.05
Total SFA	8.31	8.76	9.81	9.35
Total MUFA	14.36	13.11	15.26	14.27
Total n-6 PUFA	27.46	26.12	29.81	28.07
Total n-3 PUFA	1.01	1.97	1.97	2.03
n-6:n-3	27.79	13.43	15.20	14.44

GE, gross energy; PE phosphatidylethanolamine; PC, phosphatidylcholine; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.
Control = Corn and soybean meal diet; Alg = Control plus 1% MAL; Ch0.1 = Alg plus 0.1% choline chloride; Ch0.2 = Alg plus 0.2% choline chloride.
[1]gross energy, crude protein, and ash measured at the Center of Excellence for Poultry Science Central Analytical laboratory of the University of Arkansas.

Table 9.5. Nutrient analysis of microalgae supplement used in experimental diets.

Nutrient (mg/g)	MAL[1]
Ether extract (%)	42.9
α-tocopherol (mg/kg)	5.96
γ-tocopherol (mg/kg)	ND
Choline	12.37
PE	0.40
PC	22.60
Fatty Acids	
C14:0	54.13
C15:0	2.45
C14:1	0.72
C16:0	129.49
C16:1	0.97
C18:0	3.30
C18:1	5.20
C18:2 n-6	3.30
C20:0	1.00
C18:3 n-3	0.73
C20:3 n-6	1.59
C20:4 n-6	2.28
C20:5 n-3	5.96
C22:4 n-6	0.74
C22:5 n-6	52.93
C22:5 n-3	1.66
C22:6 n-3	149.95
Total SFA	190.37
Total MUFA	6.89
Total n-3 PUFA	158.30
Total n-6 PUFA	60.85
n-6:n-3	0.38

MAL, microalgae supplement, PE, phosphatidylethanolamine; PC, phosphatidylcholine; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

9.3.2 Egg production performance

Dietary MAL did not affect hen production performance compared to Control (Table 9.6). Compared to Alg, Ch0.1 increased hen day egg production by an impressive 7.8% on average (p=0.03), daily egg mass by 4.6 g/hen/day (p=0.02), and improved FCR by 0.19 grams of feed per gram of egg laid (p=0.05). Ch0.1 also tended to increase body weight gain during the 16 week feeding period (p=0.08). Ch0.2, however, caused no changes in production.

Table 9.6. Effect of dietary microalgae and choline chloride supplementation on egg production performance during 16 week feeding trial.

Item	Dietary Treatment				SEM	P-value		
	Control	Alg	Ch0.1	Ch0.2		Alg vs. Control	Ch0.1 vs. Alg	Ch0.2 vs. Alg
EP (%)	81.0	79.5	87.3**	83.4	3.32	0.66	0.03	0.25
EW (g)	54.8	53.6	54.2	52.6	1.09	0.31	0.64	0.37
EM (g/h/d)	44.2	42.5	47.2**	43.8	1.81	0.38	0.02	0.50
FI (g/h/d)	103.5	101.1	103.8	100.7	2.41	0.27	0.23	0.29
FCR	2.34	2.39	2.20**	2.30	0.11	0.48	0.05	0.17
BW, initial (kg)	1.60	1.65	1.62	1.58	0.06	0.39	0.62	0.28
BW, gain (kg)	0.14	0.09	0.22	0.16	0.07	0.46	0.08	0.33

EP, hen day egg production; EW, egg weight; EM, daily egg mass; FI, feed intake; FCR, feed conversion ratio; BW, body weight; SEM, standard error of the mean.
Control = Corn and soybean meal diet; Alg = Control plus 1% MAL; Ch0.1 = Alg plus 0.1% choline chloride; Ch0.2 = Alg plus 0.2% choline chloride.
"**" denotes significant difference from Alg ($p \leq 0.05$).

9.3.3 Physical egg quality

Alg did not change any physical egg quality characteristics compared to Control (Table 9.7). Ch0.1 increased albumen thickness (p=0.03) and Haugh unit (p=0.04) compared to Alg, but Ch0.2 did not. Ch0.2 tended to increase shell percentage of the egg (p=0.07).

Table 9.7. Effect of dietary microalgae and choline chloride supplementation on physical egg quality characteristics during 16 week feeding trial.

Egg Component	Dietary Treatment				SEM	P-value		
	Control	Alg	Ch0.1	Ch0.2		Alg vs. Control	Ch0.1 vs. Alg	Ch0.2 vs. Alg
Egg wt. (g)	55.4	53.7	54.5	53.1	1.06	0.14	0.46	0.57
Yolk wt. (g)	15.2	14.9	14.8	14.7	0.34	0.39	0.90	0.61
Shell wt .(g)	5.23	5.07	5.07	5.19	0.11	0.18	0.93	0.32
Albumen wt. (g)	35.0	33.8	34.6	33.2	0.80	0.16	0.31	0.49
Yolk:albumen	0.435	0.441	0.429	0.443	0.01	0.54	0.27	0.88
Shell thickness (mm)	0.326	0.316	0.304	0.326	0.008	0.19	0.15	0.21
Yolk height (mm)	17.86	17.61	17.93	17.31	0.22	0.26	0.16	0.19
Yolk width (mm)	39.6	39.0	39.2	39.0	0.34	0.11	0.57	0.97
Yolk index	0.452	0.452	0.458	0.445	0.006	0.99	0.30	0.25
Yolk (%)	27.4	27.7	27.2	27.7	0.45	0.58	0.33	0.99
Shell (%)	9.46	9.44	9.31	9.78	0.17	0.96	0.42	0.07
Albumen (%)	63.1	62.9	63.5	62.6	0.50	0.57	0.22	0.59
Haugh Unit	86.5	85.1	88.6**	86.4	1.66	0.40	0.04	0.45
Albumen thickness (mm)	7.37	7.03	7.65**	7.20	0.27	0.23	0.03	0.55
Yolk color	7.95	8.13	8.02	7.96	0.13	0.18	0.42	0.23

SEM, standard error of the mean.
Control = Corn and soybean meal diet; Alg = Control plus 1% MAL; Ch0.1 = Alg plus 0.1% choline chloride; Ch0.2 = Alg plus 0.2% choline chloride.
"**" denotes significant difference from Alg (p≤0.05).

9.3.4 Egg nutritional composition

Alg increased DHA content in whole egg yolks by 68 mg on average compared to Control ($p<0.0001$) (Table 9.8). It also increased ALA ($p=0.0008$) and total n-3 PUFA ($p=<0.0001$) content, while decreasing ARA (<0.0001) and C16:1 ($p=0.04$). The only change caused by Ch0.1 was increased total n-6 PUFA content ($p=0.04$). Ch0.2 decreased total FA content ($p=0.03$) and concentration ($p=0.004$). The different FA decreased included SFA, MUFA, and n-3 and n-6 PUFA, including a 14.7 mg reduction of DHA ($p=0.0001$).

Ch0.1 increased the total lipid concentration in egg yolks by an average of 1.24% ($p=0.04$) compared to Alg (Table 9.9). It did not, however, significantly increase the total lipid content after adjustment for yolk weight. Ch0.2 did not change lipid concentration despite the decrease in total FA it caused. None of the diets affected egg yolk TBARS ($p>0.05$).

Egg yolk concentrations of PE ($p=0.02$) and PC ($p<0.0001$) were increased by Alg (Table 9.10). After adjusting for yolk weights, increases in PC ($p=0.002$), combined PL ($p=0.003$) content, and PC as a percentage of total lipids ($p=0.0002$) were significant. Alg did tend to increase PE content as well ($p=0.06$). Compared to Alg, dietary choline only effected PL composition by a tendency of Ch0.1 to decrease PC as a percentage of total lipids compared to Alg ($p=0.08$). Although Alg enriched egg yolks with PC, total choline content did not increase. Strangely, Ch0.1 decreased total choline content of yolks compared to Alg.

Table 9.8. Effect of dietary microalgae and choline chloride supplementation on egg yolk fatty acid content during 16 week feeding trial.

Fatty Acid (mg/yolk)	Dietary Treatment					P-value		
	Control	Alg	Ch0.1	Ch0.2	SEM	Alg vs. Control	Ch0.1 vs. Alg	Ch0.2 vs. Alg
C14:0	14.3	15.4	17.2	14.85	1.10	0.32	0.11	0.65
C16:0	1169	1172	1243	1073**	49.00	0.95	0.16	0.05
C16:1	93	81*	91	77	5.67	0.04	0.10	0.41
C18:0	473	448	478	412**	15.44	0.11	0.06	0.03
C18:1	1886	1963	2025	1744**	103.63	0.46	0.56	0.05
C18:2 n-6	668	678	751	594**	37.52	0.79	0.06	0.04
C18:3 n-3	11.4	16.0*	15.7	11.9**	1.21	0.0008	0.81	0.003
C18:4 n-3	8.9	8.7	9.4	6.9**	0.84	0.85	0.43	0.04
C20:2 n-6	18	8	9	26**	5.77	0.08	0.80	0.005
C20:4 n-6	102	77*	83	70	3.94	<0.0001	0.13	0.11
C20:5 n-3	1.3	1.4	1.1	2.6	0.69	0.95	0.65	0.10
C22:5 n-6	17.7	7.9*	7.7	5.2	0.99	<0.0001	0.87	0.06
C22:6 n-3	21	89*	86	74**	3.23	<0.0001	0.35	0.0001
Total SFA	1,661	1,641	1,745	1,505**	62.98	0.75	0.11	0.04
Total MUFA	1,979	2,045	2,116	1,821**	107.8	0.55	0.52	0.05
Total n-3 PUFA	43	115*	112	96**	3.74	<0.0001	0.43	<0.0001
Total n-6 PUFA	816	774	857**	701	37.40	0.27	0.04	0.07
n-6:n-3	19.9	6.7	7.6	7.4	0.48	<0.0001	0.08	0.19
sum	4,499	4,574	4,830	4,123**	201.7	0.71	0.22	0.03

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SEM, standard error of the mean.
Control = Corn and soybean meal diet; Alg = Control plus 1% MAL; Ch0.1 = Alg plus 0.1% choline chloride; Ch0.2 = Alg plus 0.2% choline chloride.
"*" denotes significant difference from Control ($p \leq 0.05$).
"**" denotes significant difference from Alg ($p \leq 0.05$).

Table 9.9. Effect of dietary microalgae and choline chloride supplementation on total lipid content and peroxidation products in egg yolks during 16 week feeding trial.

Substance	Dietary Treatment				SEM	P-value		
	Control	Alg	Ch0.1	Ch0.2		Alg vs. Control	Ch0.1 vs. Alg	Ch0.2 vs. Alg
Total lipids (g)	4.38	4.25	4.46	4.18	0.15	0.43	0.19	0.63
Total lipids (%)[1]	28.8	28.7	29.9**	28.5	0.58	0.82	0.04	0.79
TBARS (mg/g)	34	33	33	29	2.95	0.63	0.95	0.19

TBARS, thiobarbituric acid reactive substances; SEM, standard error of the mean. Control = Corn and soybean meal diet; Alg = Control plus 1% MAL; Ch0.1 = Alg plus 0.1% choline chloride; Ch0.2 = Alg plus 0.2% choline chloride.
"**" denotes significant difference from Alg ($p \leq 0.05$).
[1]Percentage of fresh, raw egg yolks accounted for by extractable lipids.

Table 9.10. Effect of dietary microalgae and choline chloride supplementation on phospholipid classes and total choline in egg yolks.

Substance	Dietary Treatment				SEM	P-value		
	Control	Alg	Ch0.1	Ch0.2		Alg vs. Control	Ch0.1 vs. Alg	Ch0.2 vs. Alg
PE (mg/g)	16.5	19.0*	19.6	18.4	0.95	0.02	0.50	0.58
PC (mg/g)	70.5	86.3*	86.5	88.2	2.99	<0.0001	0.96	0.54
PE+PC (mg/g)	87	105*	106	107	3.61	<0.0001	0.83	0.72
PC:PE	4.27	4.56	4.45	4.79	0.17	0.11	0.56	0.20
PE (mg/yolk)	272	304	300	299	11.53	0.06	0.81	0.75
PC (mg/yolk)	1,160	1,384*	1,324	1,427	65.94	0.002	0.38	0.51
PE+PC (mg/yolk)	1,432	1,688*	1,624	1,726	78.31	0.003	0.43	0.63
PC (% total lipids)	25.2	32.0*	29.1	33.8	1.56	0.0002	0.08	0.27
Choline (mg/g)	10.51	10.85	10.79	11.23	0.23	0.14	0.80	0.11
Choline (mg/yolk)	173	174	159**	182	4.88	0.83	0.005	0.13

PE, phosphatidylethanolamine; PC, phosphatidylcholine; SEM, standard error of the mean.
Control = Corn and soybean meal diet; Alg = Control plus 1% MAL; Ch0.1 = Alg plus 0.1% choline chloride; Ch0.2 = Alg plus 0.2% choline chloride.
"*" denotes significant difference from Control ($p \leq 0.05$).
"**" denotes significant difference from Alg ($p \leq 0.05$).

9.4 Discussion

The results of this experiment support that dietary MAL is effective for enriching eggs with DHA without affecting egg production performance or egg quality, and suggest that supplemental choline chloride can improve egg production of hens fed MAL. It also provided evidence that there is a threshold level of choline chloride in diets containing MAL, above which hen performance will decline.

When working with choline supplements, it is important to note the difference between choline and choline chloride. Choline is the nutrient, whereas choline chloride is the supplement. Choline chloride is heavier than choline because it also contains chloride. The experimental diets are named after the amount of choline chloride added compared to the control diet. Table 9.5 is included to help clarify how much total choline is in the diets.

The enrichment of egg yolks with DHA by feeding MAL in the current experiment is consistent with previous studies. It is also typical for MAL not to effect hen performance or egg quality (Neijat et al., 2016). The unexpected result of the current experiment was that dietary MAL increased PC and PE in egg yolks. Dietary choline has been shown to increase PC and decrease PE in egg yolk (Tsiagbe et al., 1988; Rajalekshmy, 2010), but it caused no change in egg PC or PE when compared to Alg in the current study. To the best of the author's knowledge, PL concentration of egg yolk in response to MAL or other n-3 PUFA supplements has not been previously quantified. The molecular mechanisms of this result remain unclear, and possibilities are discussed further in Chapter 10.

The improvements in EP, EM, and FCR caused by supplementing diets containing MAL with 0.1% choline chloride are consistent with some of the previous research on choline, but the magnitude is largely unprecedented. There have been very few experiments conducted assessing the effect of choline in the presence of MAL. However, in studies on choline in the absence of MAL, choline did not influence FI (Danicke et al, 2006; Rajalekshmy, 2010; Zhai et al., 2013). Danicke et al. (2006) found that adding very high levels of choline (4,000 mg/kg) improved FCR of laying hens, but levels similar to the current study had no effect, as was the case in Rajalekshmy (2010) and Zhai et al. (2013). Broiler breeders, however, fed diets supplemented with comparable amounts of choline, had improved energy and protein efficiency on a nutrient per egg basis (Rao et al., 2001). Prior evidence of dietary choline supplementation increasing EP and EM in the absence of methionine, cysteine, or B vitamin deficiency, is lacking. Rajalekshmy (2010), Zhai et al. (2013) and Wang et al. (2017) found EP to be unaffected by choline supplementation in adequate basal diets, whereas Griffith et al. (1969) found it to increase in low methionine diets and Schexnailder and Griffith (1972) found that EP increased when choline and vitamin B12 were supplemented to low methionine diets.

Few changes in egg quality are reported in choline feeding trials. Tsiagbe et al. (1988) reported increased egg weight and yolk weight from supplementing 1,000 mg/kg of choline, which was not the case in the current study. Adding 1,000 mg/kg of choline chloride increased the Haugh unit of eggs in the current study. Zhai et al. (2013) observed

a similar response in older hens (59-68 wks. of age), but not in young hens as those in the current study.

Another interesting result of the current experiment was the differential responses of laying hens to choline level in diets containing MAL. A decline in laying performance when diets were supplemented with more than 1,700 mg/kg of choline chloride was reported by Zhai et al. (2013), but the author did not discuss potential mechanisms. Possibilities include toxic effects of choline or chloride. Chloride has an acidogenic effect on the acid/base balance of the diet, and consequently lowers blood pH of chickens (Hamilton and Thompson, 1980; Hurwitz et al., 1973). Very low sodium plus potassium to chloride ratio (0.40) has decreased FI, EP, and egg shell strength (Hamilton and Thompson, 1980). The narrow range of ion ratios in the current experiment (2.59-3.03, Table 9.2), however, were not found to alter performance by Hamilton and Thompson (1980). Further research is required to elucidate the mechanism of choline chloride threshold in laying hens.

Supplementing 0.1 or 0.2% of choline chloride to diets caused varying responses in the outcomes measured in this trial. In diets containing 1% Alg, adding 0.1% choline chloride generally improved egg production performance and albumen thickness, but not chemical characteristics of the egg yolk. Supplementation of 0.2% choline chloride, on the other hand, did not affect production performance or egg quality, but broadly reduced FA deposition in egg yolks. The contrast in effectiveness between the two choline levels is further emphasized by direct comparison between Ch0.1 and Ch0.2. Ch0.2 reduced feed intake by 3.15 g/hen/day compared to Ch0.1 ($p=0.03$), a likely cause of further

differences between the two treatment groups. Eggs laid by hens fed Ch0.2 had lower yolk index ($p=0.04$) and thicker shells ($p=0.01$) that accounted for a higher percentage of egg weight ($p=0.01$), and a tendency towards lower albumen percentage ($p=0.08$) than those laid by hens fed Ch0.1. Compared to Ch0.1, Ch0.2 decreased every FA measured ($p=0.0002$ to 0.08), with the exception of EPA and C20:2 n-6, which it increased. The concomitant increase in EPA and decrease in DHA suggests that Ch0.2 either decreased DHA synthesis or increased its retroconversion to EPA. A decrease in DHA synthesis is unlikely given that all diets were already low in ALA and EPA, so the latter is more likely. Ch0.2 also decreased total lipid concentration ($p=0.02$) compared to Ch0.1, and tended to decrease total yolk lipids by an average of 280 mg ($p=0.08$). Ch0.2 did increase PC percentage of total lipids ($p=0.007$) and total choline content ($p=0.0001$) in egg yolks, and tended to increase PC to PE ratio ($p=0.07$) compared to Ch0.1.

The results of this experiment support MAL as an effective alternative to fish oil for enriching eggs with DHA without affecting egg production performance or egg quality. It also provided strong evidence that the optimal level of choline in laying hen diets containing MAL is close to 2,460 mg/kg. Because choline supplementation has not increased egg production of hens fed standard diets in previous studies, it is possible that its effect in diets containing MAL in the current study was the result of a nutrient interaction. Therefore, it would be beneficial to include a diet with no MAL and 0.1% choline chloride, when replicating this experiment, in order to quantify the effect of choline supplementation alone and its interaction with MAL.

10 EXPERIMENT 2. EFFECT OF CHOLINE IN DIETS CONTAINING MICROALGAE ON LIPID METABOLISM OF LAYING HENS

10.1 Introduction

The hen liver is a very important organ for lipid metabolism. The liver serves as the primary site of lipogenesis in chickens (Griffin and Hermier, 1988). Most dietary lipids are delivered to the liver as portomicrons via the hepatic portal vein after absorption in the small intestine. FA in the liver exert their metabolism regulating effects in hepatocytes, undergo β-oxidation, are synthesized, elongated, desaturated, and esterified to PL and TAG. Processing of fat in the liver allows the hen's body some control over the FA composition of tissues, including egg yolks. The apolipoproteins required for VLDL are synthesized in the liver, and hepatic lipids are packaged in VLDL and exported to peripheral tissues (Walzem, 2012).

Dietary DHA and choline are both substrates for PL synthesis in laying hens. Incorporation of LC PUFA by both PC and PE contributes to their roles as functional lipids used in membrane biogenesis throughout the body. PC and PE are the two most abundant PL found in VLDLy and egg yolk (Cherian, 2005). Choline affects FA metabolism through several pathways. Oxidation of choline to betaine allows the compound to serve as a methyl donor in the synthesis of PC from existing PE via the PEMT pathway, potentially increasing PC synthesis by both PEMT and the CDP-choline pathways (Caudill et al., 2013). Additionally, the role of choline in one carbon metabolism interconnects with the antioxidant cycle. Choline therefore has the potential to ameliorate oxidative stress and prevent downstream negative health effects. As choline

is also integral to the synthesis of lipoproteins, choline supplementation may also reduce hepatic lipidosis.

The objective of this experiment was to determine if choline dosage influences hepatic lipid metabolism in laying hens fed MAL. It was hypothesized that choline would alter PL synthesis, hepatic fat accumulation, and oxidative status.

10.2 Materials and Methods

All animal work was approved by the Oregon State University Institutional Animal Care and Use Committee (ACUP #4913). Animals were housed indoors at the Oregon State University Harrison Poultry Farm in Corvallis, Oregon for the duration of this work.

10.2.1 Birds, diet and management

A total of 56 white leghorn hens (24 weeks age) were kept in individual cages (46 cm x 53 cm x 58 cm (l·w·h)) and randomly assigned to one of the four treatments. Hens were fed experimental diets during a two week adaptation period prior to physiological measurements beginning at 26 weeks of age. Each treatment had 14 hens kept in seven replicate groups with two hens per each group (n=7 per treatment). The experimental trial period lasted 18 weeks. Feed and water were provided ad libitum. At the end of the feeding trial, one hen from each replicate (n=7 per treatment, n=28 total) was euthanized for tissue collection.

The basal diet (Control) was a commercial type corn and soybean meal mash. Diets Alg, Ch0.1, and Ch0.2 were supplemented with 1% MAL. Ch0.1 and Ch0.2 were also supplemented with 0.1 and 0.2% choline chloride, respectively (Table 10.1). The diets are described in more detail in Chapter 9.

Table 10.1. Summary of supplements added to experimental diets.

Diet	Control	Alg	Ch0.1	Ch0.2
Microalgae Supplement (%)	0	1	1	1
Choline Chloride (%)	0	0	0.1	0.2

Control = Corn and soybean meal diet; Alg = Control plus 1% MAL; Ch0.1 = Alg plus 0.1% choline chloride; Ch0.2 = Alg plus 0.2% choline chloride.

10.2.2 Tissue collection

One bird from each replicate cage was euthanized for tissue collection at the end of the study, for a total of 28 birds. Birds were euthanized at 18 weeks from the start of the trial, by CO_2 gas asphyxiation. Bodies were weighed, decapitated, and dissected for collection of livers, hearts, and blood.

Blood was collected by cardiac puncture in glass tubes with no additives and centrifuged at 1500 × g for 20 min. to separate plasma. Blood plasma was kept frozen at -20°C until analysis. Hearts were trimmed of their major vessels, rinsed, weighed, and discarded. Livers were patted dry and weighed before slicing out a section about three millimeters wide for histology. These sections were placed in buffered formalin solution and brought to the Oregon Veterinary Diagnostic Laboratory at Oregon State University

for histology preparation. The rest of the liver was frozen kept at -20°C for further analysis.

10.2.3 Tocopherol analysis

Egg yolk samples collected at week 14 of the feeding trial, and liver and plasma from week 18, were subject to vitamin E analysis. αT and γT were quantified using a Shimadzu LC-2010 HT high performance liquid chromatograph (HPLC) with a LC2010 AHT High Speed Autosampler and a Shimadzu RF-535 fluorescence detector (Shimadzu, Columbia, MD). The column used was a Supelcosil LC-18, 250 × 4.6 mm, and Superguard LC-18 guard column (Sigma-Aldrich Corp., St. Louis, MO). The mobile phase was 97.5% methanol and 2.5% ultra-pure water. The oven temperature was 40°C, the flow rate one mL/min., and the wavelength 295 nm. The procedure was based on that described in Podda et al. (1996). Samples were prepared by first homogenizing 25 and 20 mg of liver or egg yolk, or 100 μL of plasma, in water. The homogenate was saponified by incubating in a hot water bath at 70°C with ascorbic acid (AA) and KOH. The AA and saturated KOH reagents were doubled from two and 0.3 milliliters, respectively, to two and 0.6 milliliters in liver and yolk samples, because of their high fat content. The samples were then extracted with butylated hydroxytoluene (BHT), AA, and two milliliters of hexane. After mixing and allowing the layers to separate, 1.5 mL of the hexane layer was removed and dried under nitrogen gas. The sample was then resuspended in 1.5 mL or 0.2 mL of 1:1 ethanol:methanol mixture for liver and plasma, or egg yolk, respectively, and passed through a 0.45 μm nylon syringe filter. A 20 μL of

eluted sample was injected into the HPLC for analysis. Tocopherol concentrations were calculated by comparing their elution peak areas to standard curves of αT and γT standards analyzed in the same sequence as the samples.

The αT (DL-all-*rac*-α-tocopherol, cat. no. T3251) and γT ((R,R,R)-γ-tocopherol, cat. no. T1782) standards were both ≥96% pure, HPLC grade, purchased from Sigma-Aldrich Corp. (St. Louis, MO). The two standards were diluted together to 1342 μM of αT and 363 μM of γT with 1:1 ethanol:methanol mixture. They were passed through a syringe filter and standard curves were generated by injecting five, 10, 20, 30, and 40 μL from the same vial.

10.2.4 Lipid extraction, fatty acid, and phospholipid analysis

Lipids were extracted from liver tissue and blood plasma by Folch's method as described in the previous chapter, with tissue specific differences (Folch et al., 1957). Lipids were extracted from liver samples weighing two grams. The amount of plasma available for lipid extraction was limited following vitamin E analysis. Consequently, sample volumes of plasma ranged between 0.5 and two milliliters based on availability. This was accounted for by PL concentration calculations. Also, one plasma sample from Control and one from Alg were not available for PL analysis.

Samples for PL analysis were prepared with 25 μL of liver lipid extract. Volumes of plasma lipid extract varied between 50 and 200 μL based on original sample size. After evaporation under nitrogen, liver samples were resuspended in 600 μL of methanol and plasma samples in 800 μL before filtration and HPLC analysis.

Liver lipid extract was also subjected to gravimetric measurement of total lipid content, and FA composition analysis using the procedures for egg yolk described in Chapter 9.

10.2.5 Total choline and thiobarbituric acid reactive substances in liver

Total choline content of livers was measured using the enzymatic, colorimetric assay described in Chapter 9. Lipid peroxidation products in the liver were evaluated by measuring TBARS in two gram samples as described in Chapter 9. Values represent milligrams of MDA equivalents per gram of liver tissue.

10.2.6 Liver histology

Microscopic images of liver samples were prepared for analysis of fat deposits by the Oregon Veterinary Diagnostics Laboratory at Oregon State University. The samples were fixed in 10% neutral buffered formalin, routinely processed for embedding in paraffin, sectioned at five to seven microns, and stained with hematoxylin and eosin for light microscopy. The microscope was a Nikon Eclipse 50i, and images were taken with a 40X objective, 10X ocular on a Nikon DS-Fi1 camera and NIS-Elements software provided by Nikon. The brightness levels on most images were adjusted using Adobe Photoshop. Lipid droplets were measured and counted maunually on full sized printouts, and categorized as small (three to five millimeters), large (five to seven millimeters), and extra-large (over seven millimeters) by diameter. Sufficient information was not available to calculate diameter to scale.

10.2.7 Statistical Analysis

The effect of dietary treatment on all response variables was analyzed with a general linear mixed model using the Mixed procedure of SAS software (version 9.4) (SAS Institute). The experimental unit was replicate group in all models (n=7 per treatment). Because a dosage effect of choline was observed between the two dietary levels of choline, comparisons between treatment means were made by orthogonal contrast. For all response variables, the effect of Alg vs Control was calculated, and Ch0.1 and Ch0.2 were compared separately to Alg to quantify the effect of adding choline chloride to diets containing MAL. P-values were considered significant at ≤ 0.05 and trending at $0.05 \leq p \leq 0.10$. Least square means and pooled standard error of the means (SEM) are reported.

10.3 Results

10.3.1 The liver: Lipid content, TBARS, and FA composition

Diet had no significant effect on organ weights or lipid content (Tables 10.2 and 10.3). Alg increased hepatic TBARS ($p=0.01$) compared to Control. Ch0.1, however, reduced TBARS compared to Alg ($p=0.005$), and Ch0.2 tended to reduce them as well ($p=0.09$).

Alg increased hepatic concentrations of DHA by an average of 1.27 mg/g ($p<0.0001$) and total n-3 PUFA by 1.65 mg/g ($p<0.0001$) compared to Control (Table 10.4). It also tended to increase ALA ($p=0.06$) and C14:0 ($p=0.07$). Compared to Alg, Ch0.1 had no effect on FA composition, and Ch0.2 tended to decrease total n-3 PUFA ($p=0.08$). EPA was undetectable in all liver samples.

Table 10.2. Effect of dietary microalgae and choline chloride supplementation on hen organ weights.

Organ	Dietary Treatment				SEM	P-value		
	Control	Alg	Ch0.1	Ch0.2		Alg vs Control	Ch0.1 vs Alg	Ch0.2 vs Alg
Liver (g)	56	64	61	57	7.49	0.27	0.63	0.31
Carcass (kg)	1.78	1.83	1.90	1.70	0.11	0.66	0.54	0.24
Liver (% of body)	3.1	3.5	3.2	3.3	0.30	0.23	0.33	0.48
Heart (g)	7.0	7.7	7.7	7.0	0.65	0.30	0.96	0.30
Heart (% of body)	0.39	0.42	0.40	0.41	0.03	0.26	0.48	0.65

SEM, standard error of the mean.
Control = Corn and soybean meal diet; Alg = Control plus 1% MAL; Ch0.1 = Alg plus 0.1% choline chloride; Ch0.2 = Alg plus 0.2% choline chloride.

Table 10.3. Effect of dietary microalgae and choline chloride supplementation on lipid content and lipid peroxidation products in hen liver.

Substance	Dietary Treatment				SEM	P-value		
	Control	Alg	Ch0.1	Ch0.2		Alg vs. Control	Ch0.1 vs. Alg	Ch0.2 vs. Alg
Total lipids (%)	13.6	15.6	15.4	14.8	2.99	0.45	0.86	0.72
Total lipids (g/liver)	8.0	10.8	9.4	8.8	2.64	0.31	0.61	0.47
TBARS (mg/g)	5.3	8.7*	4.7**	6.4	1.31	0.01	0.005	0.09

TBARS, thiobarbituric acid reactive substances; SEM, standard error of the mean. Control = Corn and soybean meal diet; Alg = Control plus 1% MAL; Ch0.1 = Alg plus 0.1% choline chloride; Ch0.2 = Alg plus 0.2% choline chloride.
"*" denotes significant difference from Control ($p \leq 0.05$).
"**" denotes significant difference from Alg ($p \leq 0.05$).

Table 10.4. Effect of dietary microalgae and choline chloride supplementation on fatty acid concentrations in hen liver.

Fatty Acid (mg/g liver)	Dietary Treatment					P-value		
	Control	Alg	Ch0.1	Ch0.2	SEM	Alg vs. Control	Ch0.1 vs. Alg	Ch0.2 vs. Alg
C14:0	0.63	1.06	0.91	0.85	0.23	0.07	0.52	0.37
C16:0	47	54	50	48	11.07	0.54	0.72	0.64
C16:1	4.1	4.3	4.3	3.8	1.04	0.80	0.98	0.60
C18:0	20	24	21	22	4.20	0.37	0.53	0.59
C18:1	90	103	96	93	23.34	0.60	0.79	0.67
C18:2 n-6	18	22	24	20	3.96	0.28	0.67	0.51
C18:3 n-3	ND	0.32	0.30	0.06	0.16	0.06	0.88	0.13
C20:4 n-6	2.38	1.88*	1.82	1.86	0.14	0.002	0.66	0.86
C20:5 n-3	ND	ND	ND	ND	-	-	-	-
C22:6 n-3	0.10	1.37*	1.32	1.34	0.11	<0.0001	0.62	0.77
Total SFA	68	79	72	71	15.48	0.48	0.66	0.62
Total MUFA	94	107	101	96	24.34	0.61	0.80	0.66
Total n-3 PUFA	0.10	1.75*	1.61	1.40	0.19	<0.0001	0.47	0.08
Total n-6 PUFA	20	24	26	21	3.91	0.33	0.67	0.50
n-6:n-3	-	13.8	15.7	15.5	2.43	-	0.43	0.48
sum	182	212	200	190	42.99	0.50	0.79	0.62

ND, not detected; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SEM, standard error of the mean.
Control = Corn and soybean meal diet; Alg = Control plus 1% MAL; Ch0.1 = Alg plus 0.1% choline chloride; Ch0.2 = Alg plus 0.2% choline chloride.
"*" denotes significant difference from Control ($p \leq 0.05$).

10.3.2 Phospholipids and choline

Alg increased PC (p=0.0003) and PE (p=0.0003) concentrations significantly in the liver, and insignificantly in blood plasma (p>0.05) (Tables 10.5 and 10.6). Increased hepatic PC and PE also resulted in their increase in egg yolks, as presented in Chapter 9 (p<0.0001; p=0.02) (Table 9.10). Supplemental choline did not change hepatic or plasma PL in comparison to Alg. Total choline content in the liver was not significantly affected by diet.

Table 10.5. Effect of dietary microalgae and choline chloride supplementation on phospholipid and total choline content of hen liver.

Substance	Dietary Treatment				SEM	P-value		
	Control	Alg	Ch0.1	Ch0.2		Alg vs. Control	Ch0.1 vs. Alg	Ch0.2 vs. Alg
PE (mg/g)	6.2	7.8*	7.1	7.6	0.49	0.003	0.16	0.61
PC (mg/g)	9.1	11.6*	11.3	12.3	0.75	0.003	0.69	0.38
PC+PE (mg/g)	15.3	19.5*	18.5	19.9	1.07	0.0008	0.36	0.70
PC:PE	1.46	1.50	1.60	1.65	0.10	0.69	0.37	0.15
PE (mg/liver)	347	493*	432	420	47.26	0.005	0.21	0.14
PC (mg/liver)	504	745*	682	704	91.86	0.01	0.50	0.66
PC+PE (mg/liver)	850	1,237*	1,113	1,123	135.1	0.009	0.37	0.41
PC (% total lipids)	8.3	8.4	8.7	9.2	2.41	0.97	0.91	0.74
Choline (mg/g)	1.38	1.37	1.49	1.42	0.14	0.93	0.42	0.75
Choline (mg/liver)	77	86	90	84	12.34	0.47	0.72	0.90

PE, phosphatidylethanolamine; PC, phosphatidylcholine; SEM, standard error of the mean.
Control = Corn and soybean meal diet; Alg = Control plus 1% MAL; Ch0.1 = Alg plus 0.1% choline chloride; Ch0.2 = Alg plus 0.2% choline chloride.
"*" denotes significant difference from Control (p≤0.05).

Table 10.6. Effect of dietary microalgae and choline chloride supplementation on phospholipid concentrations of hen blood plasma.

Phospholipid	Dietary Treatment				SEM	P-value		
	Control	Alg	Ch0.1	Ch0.2		Alg vs. Control	Ch0.1 vs. Alg	Ch0.2 vs. Alg
PE (mg/mL)	2.1	2.4	2.4	2.4	0.38	0.52	0.91	0.98
PC (mg/mL)	8.8	10.0	9.9	10.5	1.73	0.52	0.98	0.74
PC:PE	4.17	4.21	4.07	4.41	0.19	0.86	0.46	0.27
PE+PC (mg/mL)	10.9	12.3	12.3	12.9	2.09	0.51	1.00	0.78

PE, phosphatidylethanolamine; PC, phosphatidylcholine; SEM, standard error of the mean.
Control = Corn and soybean meal diet; Alg = Control plus 1% MAL; Ch0.1 = Alg plus 0.1% choline chloride; Ch0.2 = Alg plus 0.2% choline chloride.

10.3.3 Vitamin E

Alg did not change levels of vitamin E in the liver or blood plasma ($p>0.05$) (Tables 10.7 and 10.8). Compared to Alg, Ch0.1 increased hepatic concentrations of γT and αT ($p=0.03$, $p=0.005$), as did Ch0.2 ($p=0.0002$, $p=0.001$). Plasma tocopherols, however, were not affected by diet. In the egg yolk, Ch0.1 increased γT concentration in comparison to Alg ($p=0.05$) (Table 10.9).

Table 10.7. Effect of dietary microalgae and choline chloride supplementation on tocopherol content of hen livers.

Tocopherol	Dietary Treatment				SEM	P-value		
	Control	Alg	Ch0.1	Ch0.2		Alg vs. Control	Ch0.1 vs. Alg	Ch0.2 vs. Alg
γ-tocopherol (µg/g)	2.83	2.41	3.03**	3.65**	0.28	0.15	0.03	0.0002
α-tocopherol (µg/g)	5.7	4.8	6.9**	7.5**	0.71	0.25	0.005	0.001
Total tocopherol (µg/g)	8.5	7.2	10.0**	11.2**	0.95	0.19	0.007	0.0004
γ-tocopherol (µg/liver)	153	155	185	215	31.53	0.93	0.34	0.07
α-tocopherol (µg/liver)	308	309	425	439	68.85	0.98	0.09	0.07
Total tocopherol (µg/liver)	460	465	610	654	98.57	0.97	0.14	0.07

SEM, standard error of the mean.
Control = Corn and soybean meal diet; Alg = Control plus 1% MAL; Ch0.1 = Alg plus 0.1% choline chloride; Ch0.2 = Alg plus 0.2% choline chloride.
"**" denotes significant difference from Alg ($p\leq0.05$).

Table 10.8. Effect of dietary microalgae and choline chloride supplementation on tocopherol concentrations of hen blood plasma.

Tocopherol	Dietary Treatment				SEM	P-value		
	Control	Alg	Ch0.1	Ch0.2		Alg vs. Control	Ch0.1 vs. Alg	Ch0.2 vs. Alg
γ-tocopherol (μg/mL)	1.3	1.4	1.6	1.8	0.34	0.91	0.61	0.21
α-tocopherol (μg/mL)	2.9	2.5	2.4	3.1	0.56	0.55	0.73	0.30
Total tocopherols (μg/mL)	4.2	3.9	3.9	5.0	0.90	0.74	0.98	0.26

Control = Corn and soybean meal diet; Alg = Control plus 1% MAL; Ch0.1 = Alg plus 0.1% choline chloride; Ch0.2 = Alg plus 0.2% choline chloride.

Table 10.9. Effect of dietary microalgae and choline chloride supplementation on tocopherol content of egg yolks during 16 week feeding trial.

Tocopherol	Dietary Treatment					P-value		
	Control	Alg	Ch0.1	Ch0.2	SEM	Alg vs. Control	Ch0.1 vs. Alg	Ch0.2 vs. Alg
γ-tocopherol (µg/g)	15.0	13.5	16.4**	12.6	1.43	0.29	0.05	0.55
α-tocopherol (µg/g)	30.0	28.0	27.2	26.1	2.44	0.44	0.74	0.42
Total tocopherol (µg/g)	45	41	44	39	3.75	0.36	0.56	0.45
γ-tocopherol (µg/yolk)	242	216	264	207	25.44	0.31	0.06	0.72
α-tocopherol (µg/yolk)	483	449	441	426	44.64	0.46	0.85	0.59
Total tocopherol (µg/yolk)	725	665	704	633	68.77	0.39	0.56	0.63

SEM, standard error of the mean.
Control = Corn and soybean meal diet; Alg = Control plus 1% MAL; Ch0.1 = Alg plus 0.1% choline chloride; Ch0.2 = Alg plus 0.2% choline chloride.
"**" denotes significant difference from Alg ($p \leq 0.05$).

10.3.4 Liver histology

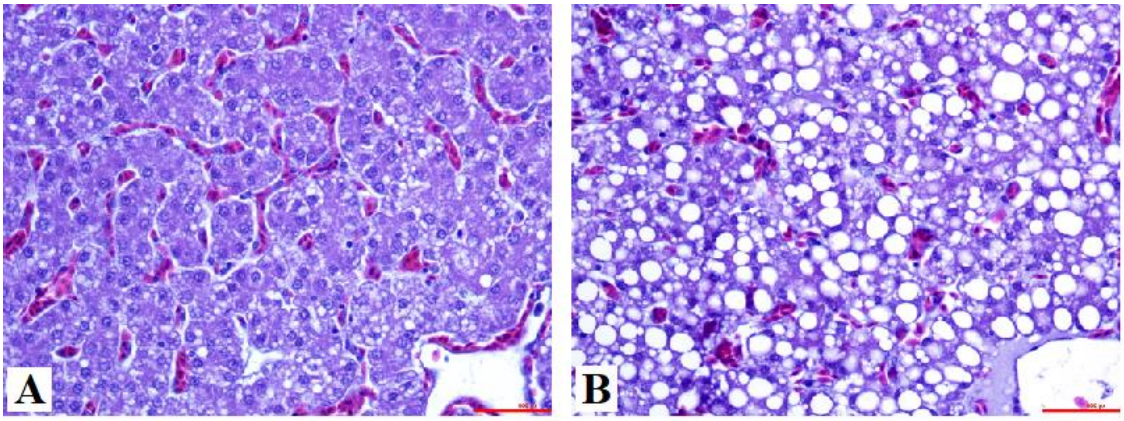
The number and size of lipid droplets in hen livers were highly variable. Every dietary treatment included livers ranging from almost no, to huge amounts of fat. The only effect of diet was a tendency for Ch0.1 to decrease the number of extra-large lipid droplets compared to Alg (p=0.10) (Table 10.10). This is consistent with the trend of Ch0.1 to reduce liver total lipids and liver weights compared to Alg, although not statistically significant. Figure 10.1 demonstrates the range of fat accumulation in hen livers.

Table 10.10. Effect of dietary microalgae and choline chloride supplementation on the average number of hepatocyte lipid droplets per microscopic frame.

Droplet size[1]	Dietary Treatment				SEM	P-value		
	Control	Alg	Ch0.1	Ch0.2		Alg vs. Control	Ch0.1 vs. Alg	Ch0.2 vs. Alg
Small	53	59	65	47	16.54	0.69	0.75	0.46
Large	25	37	24	34	17.28	0.49	0.45	0.84
Extra-large	4	10	1	8	5.20	0.27	0.10	0.64
Large + Extra Large	30	47	25	42	21.64	0.42	0.32	0.79
Total	82	107	90	89	32.76	0.46	0.61	0.58

SEM, standard error of the mean.
Control = Corn and soybean meal diet; Alg = Control plus 1% MAL; Ch0.1 = Alg plus 0.1% choline chloride; Ch0.2 = Alg plus 0.2% choline chloride.
[1]Lipid droplets were measured and counted by hand on original sized printouts, and categorized as by diameter. Small, 3-5 mm; large, 5-7 mm; and extra-large, >7 mm.

Figure 10.1. Histology of hen livers. White lipid droplets can be seen in “B”. Hens in every dietary treatment had livers with lipid droplets ranging from the relatively healthy liver in “A” to the extremely fatty liver in “B”. “A” happens to be from Control, and “B” from Ch0.1.



10.4 Discussion

Contrary to previous research (Depner et al., 2013; Griffeth et al., 1969; Ruiz et al, 1982), fat accumulation in hen livers was not significantly affected by diet in the current experiment. The insignificance may be due to the high level of fattiness and variation for the small sample size in the current experiment, or the young age of the hens. The FA composition of liver tissue primarily reflected that of the diet, consistent with previous literature (Cherian and Hayat, 2009). Dietary choline did not increase hepatic PUFA despite ameliorating oxidative damage, similar to the findings of Cherian and Hayat (2009), who supplemented αT to laying hen diets with flaxseed.

Hens in the current experiment were kept in a hygienic environment, with minimal stressors, and experimental diets were sufficient in all antioxidant ingredients including vitamin E. The increase in hepatic lipid peroxidation products by Alg could be explained by higher PUFA intake of hens fed MAL, as has been previously documented (Cherian and Hayat, 2009). Alternatively, DHA can have antioxidant properties as it upregulates antioxidant enzymes (Long et al., 2018). In Long et al. (2018), dietary MAL increased total antioxidant capacity and reduced MDA in breast and thigh muscle of broiler chickens. The decrease in hepatic TBARS caused by dietary choline compared to Alg in the current experiment could be caused by the overlapping of one carbon metabolism and the antioxidant cycle (Li et al., 2014), because hepatic vitamin E was also increased by choline.

Tocopherol concentration in the liver is another marker of oxidative status. Dietary DHA has been shown to decreased hepatic αT (Depner et al., 2013). Feeding

hens MAL in the current study did not significantly decrease tocopherols in the liver, but supplemental choline significantly increased them. Tocopherols in the blood followed a similar pattern, but without statistical significance, indicating that overall tocopherol status was not improved. In the egg yolk, γT concentration remained increased by Ch0.1, but eggs were not enriched with αT.

Dietary MAL increased hepatic PC and PE concentrations, and a similar trend was seen in blood plasma, though plasma changes were statistically insignificant. The increase in PC and PE was also reflected in egg yolk lipids of hens fed MAL ($p<0.0001$ and $p=0.02$, respectively). The increase in PL synthesis due to dietary MAL, and not choline, was unexpected. Previous research has shown that dietary choline can increase PC in egg yolk, but it tends to decrease PE (Tsiagbe et al., 1988; Rajalekshmy, 2010). Although the effect of dietary MAL on FA composition of PC and PE have been previously reported (Gladkowski et al., 2014), the absolute content of PC and PE themselves has not been previously quantified. One possible mechanism for the increase in PC and PE in response to MAL is that DHA availability in the liver stimulates PC and PE synthesized via the Kennedy pathway, due to the stimulatory effect of DAG on the CT and ET enzymes (Caudill et al., 2013). Enzymes involved in PE synthesis may be more likely to utilize DHA, as PE incorporates more DHA than PC does (Neijat et al., 2017). In that case, the increase in hepatic PC in the current experiment could be a result of methylating PE via the PEMT pathway, as PE-DHA is the preferred substrate of the PEMT enzyme (Ridgway and Vance, 1992). DHA may also increase the density and PL

content of VLDL secreted from the liver (Berge et al., 2015), offering another possible explanation for the observed increase in PL.

To the best of the author's knowledge, the current study was the first to demonstrate that dietary MAL increased PC and PE in the laying hen liver and egg yolks. This finding suggests that choline is not the limiting substrate in PC synthesis, and supplementing choline to balanced laying hen diets containing MAL does not increase DHA enrichment of egg yolks by the mechanism proposed by Wang et al. (2017).

11 CONCLUSION

This thesis provides evidence that eggs can be a good source of DHA when hen diets are supplemented with MAL. Consumption of two eggs produced by hens fed Alg would provide 181 mg of LC n-3 PUFA, which is 36% of the daily intake recommended by the AHA, or 72% of that recommended by the Office of Disease Prevention and Health Promotion.

Dietary MAL did not affect physical egg quality or production performance, but the performance of hens fed MAL was improved when dietary choline was 2,460 mg/kg in the diet as fed, suggesting that corn and soybean meal diets containing MAL need to be supplemented with about 1,300 mg/kg of choline (1,740 mg/kg of choline chloride as fed). Because this greatly exceeds current recommendations for standard diets, further research with different ages and strains of hens is needed to confirm whether an interaction exists between MAL and choline, and whether other sources of dietary n-3 PUFA react similarly. Like most nutrients, however, there is a threshold level of choline chloride consumption beyond which undesirable affects may be observed. Results of the current study suggest that this value lies beyond 1,740 and is closer to 2,740 mg/kg of choline chloride in the laying hen diet. The most current NRC nutrient recommendations for laying hens was published in 1994, and choline requirements were based on studies measuring its ability to improve egg production by compensating for amino acid deficient diets (Parsons and Leeper, 1984; Keshavarz and Austic, 1985; Miles et al., 1986). Studies like this thesis may provide useful evidence upon which to base choline requirements for practical diets in future editions.

The current study also demonstrated that dietary MAL may increase hepatic synthesis of PE and PC, and that this change is reflected in the lipid composition of egg yolks. This suggests that choline content was adequate in all diets for the purpose of PL synthesis, and contradicts the hypothesized mechanism by which choline could alter lipid metabolism in favor of DHA enrichment of egg yolk. Although there was no evidence that MAL or choline prevented hepatic fat accumulation in the current study, dietary choline significantly ameliorated oxidative damage in the liver of hens fed MAL. This could have contributed to the ability of hens fed Ch0.1 to produce more eggs than those fed Alg. Laying hens in commercial facilities experience much higher environmental stress, so controlling oxidative damage is important for normal metabolism in support of production. Dietary choline also demonstrated a possible sparing effect on vitamin E in the current study, warranting further investigation.

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