OMEGA-3 FATTY ACID ENRICHMENT OF CHICKEN EGGS:

REGULATION OF LONG CHAIN POLYUNSATURATED FATTY ACID

METABOLISM IN LAYING HENS

A thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

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in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Animal Science

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ACKNOWLEDGMENTS

I would like to thank my advisor, Dr. James D. House, Professor and Head of Department, Human Nutritional Sciences, University of Manitoba, for the supervision and support throughout the entire period of my doctorate program. His thoughts and ideas in this research and guidance throughout the program has made me reach this far. His mentorship is admirable and creates a great foundation in my career. I would also like to thank my committee advisory members: Dr. M. Suh, Dr. P. Eck, and Dr. W.K. Kim. Valuable inputs and guidance in their field of expertise obtained from Dr. Suh and Dr. Eck during the course of this study is highly appreciated. In addition, the willingness to use their lab is also gratefully acknowledged. Dr. Kim′s comment and ideas shared during committee meetings is also gratefully acknowledged. I would also like to thank my external examiner, Dr. R. A. Elkin, for his helpful comments and for sharing invaluable wealth of knowledge in his field of expertise during the examination of this thesis.

I would like to acknowledge financial support provided by Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grants Program (Winnipeg, MB, Canada); Canadian Hemp Trade Alliance (CHTA); Canadian Agricultural Adaptation Program (CAAP); and Province of Manitoba Science and Technology International Collaboration Fund. I also highly appreciate the generous donation of algal DHA product from DSM Nutritional Products North America (Parsippany NJ, USA) that was used in my study.

I would like to extend my sincere appreciation for my peer lab members Dr. Shyam Mayengbam and Dr. Erin Goldberg for being a motivating company. I would also like to acknowledge technical assistance from Dr. Alemu Regessa, Dennis Labossiere, Dennis Peter, Jason Neufeld, Naveen Gakhar, Jerry Levandoski, Aurel Chartier and Harry Muc provided at

different stages of my program. The University of Manitoba and in particular the Department of

Animal Science academic, technical and research staff members and fellow graduate students are

all highly thanked for providing their support during the course of my program. Finally, my

appreciation goes to my family members for giving me the strength in the years of my study.

Above all, I would like to thank the Almighty God (Allah) for guiding me and making

this accomplishment possible.

DEDICATION

This thesis is dedicated in memory of my late father, Mohamed Said Ibrahim, who installed in

me all the values of education and hard work.

FOREWORD

This thesis was prepared following a manuscript format. There are five manuscripts. The authors and the publishing journals for each manuscript are as indicated in section "publications arising from this thesis". For this thesis, all manuscripts are formatted to one version, according to the guidelines of Poultry Science. Different sections of each manuscript have been presented at national and international conferences/symposia/workshops. Manuscripts I and II had been presented as posters at the Banff Egg Forum, March 28-30, 2012, Banff, AB; 33rd Western Nutrition Conference, September 19-20, 2012, Winnipeg, MB; Poultry Science Association (PSA) Annual Meeting, July 22-25, 2013, San Diego, CA; and Canadian Nutrition Society (CNS) Annual Conference, May 28-30, 2015, Winnipeg, MB. Oral presentations were conducted for manuscript III at the CNS Post-conference Lipid Workshop, May 31-June 1, 2015, Winnipeg, MB; 35th Western Nutrition Conference, September 29-30, 2015, Winnipeg, MB; manuscripts IV and V at Northern Great Plains Lipid Conference, June 11-12, 2016, Grand Forks, ND; PSA Annual Meeting, July 11-14, 2016, New Orleans, LA; and at the International Egg Symposium, October 4-6, 2016, Banff, AB.

PUBLICATIONS ARISING FROM THIS THESIS

1- Neijat, M., N. Gakhar, J. Neufeld, and J.D. House. 2014. Performance, egg quality, and blood plasma chemistry of laying hens fed hempseed and hempseed oil. Poult. Sci. 93:2827–2840.

2- Neijat, M., M. Suh, J. Neufeld, and J.D. House. 2016. Hempseed products fed to hens effectively increased n-3 polyunsaturated fatty acids in total lipids, triacylglycerol and phospholipid of egg yolk. Lipids. 51(5):601-614.

3- Neijat, M., M. Suh, J. Neufeld, and J.D. House. 2016. Increasing levels of dietary hempseed products leads to differential responses in the fatty acid profiles of egg yolk, liver and plasma of laying hens. Lipids. 51(5):615-633.

4- Neijat, M., O. Ojekudo, and J.D. House. 2016. Effect of flaxseed oil and microalgae DHA on the production performance, fatty acids and total lipids of egg yolk and plasma in laying hens. Prostaglandins Leukot. Essent. Fat. Acids. 115:77-88.

5- Neijat, M., P. Eck, and J.D. House. Impact of dietary precursor ALA versus preformed DHA on fatty acid profiles of eggs, liver and adipose tissue and expression of genes associated with hepatic lipid metabolism in laying hens. *(Submitted for publication on 31st Nov., 2016 to Prostaglandins Leukot. Essent. Fat. Acids).*

6- Neijat, M., and J.D. House. Fate of dietary essential fatty acids (ALA) in laying hens – A review. (*Review, in progress*).

LIST OF ABBREVIATIONS

ACSL	Long chain acyl-CoA synthetases
ADF	Acid detergent fibre
ALA (18:3n-3)	Alpha-linolenic acid
AME	Apparent metabolisable energy
ANOVA	Analysis of variance
ApoVLDL-II	Very-low-density apolipoprotein II
ARA (20:4n-6)	Arachidonic acid
CE	Cholesteryl ester
CPT1A	Carnitine palmitoyl transferase 1A
DAG (AT)	Diacylglycerol (acyltransferase)
DHA (22:6n-3)	Docosahexaenoic acid
DPA (22:5n-3)	Docosapentaenoic acid
EFA	Essential fatty acids
EPA (20:5n-3)	Eicosapentaenoic acid
ER	Endoplasmic reticulum
FA	Fatty acid
FC	Free cholesterol
FFA	Free (unesterified) fatty acid
G-3-P	Glycerol-3-phosphate
GLA (18:3n-6)	Gamma-linolenic acid
GPAT	Glycerol 3-phosphate acyltransferase
h	Hour(s)
HDL	High density lipoprotein
HO	Hempseed oil
HS	Hempseed
LA (18:2n-6)	Linoleic acid
LCPUFA	Long chain polyunsaturated fatty acid(s)
LPL	Lipoprotein lipase
MAG (AT)	Monoacylglycerol (acyltransferase)

min	Minute(s)
mol	Amount of substance
MUFA	Monounsaturated fatty acid(s)
NDF	Neutral detergent fiber
PC or PtdCho	Phosphatidylcholine
PE or PtdEtn	Phosphatidylethanolamine
PLA2	Phospholipase A2
PUFA	Polyunsaturated fatty acid(s)
SDA (18:4n-3)	Stearidonic acid
SFA	Saturated fatty acid(s)
TAG	Triacylglycerol
TLC	Thin layer chromatography
VLDL	Very low density lipoprotein
wk	Week(s)
wt	Weight

ABSTRACT

Background: Eggs enriched with omega-3 polyunsaturated fatty acids (PUFA), particularly the longer chain PUFA (LCPUFA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) can boost human consumption of these fatty acids implicated in human health. Alpha-linolenic acid (ALA) from plant seeds/oils, primarily serve as the source of omega-3 PUFA for hens, however, the scarcity of ALA-rich plants and the limited conversion of ALA to LCPUFA are challenges for egg enrichment.

Objective: To determine potential factors regulating egg enrichment of omega-3 LCPUFA based on detailed assessment of PUFA profiles in different lipid pools of hen tissues.

Design: Two major experiments were conducted. In experiment 1, supplementation of graded levels of hempseed products, provided ~ 0.1 to 1.3% of ALA in the diets. Experiment 2, investigated dietary supplementation of flaxseed oil (ALA-rich) and algal DHA (preformed LCPUFA), each providing similar graded levels of total omega-3 PUFA.

Results: Both ALA-containing models demonstrated a plateau in DHA enrichment of eggs at higher ALA intakes. ALA-containing diets led to high concentrations of ALA in the triacylglycerol (TAG) fraction of eggs and plasma, and the adipose tissue of flaxseed oil-fed hens. In total phospholipid (PL), particularly the phosphatidylethanolamine (PE), the levels of EPA and ALA in the yolk were linearly associated with those in the liver. In all tissues, DHA dominated the PE pool, exhibiting a plateau with a strong inverse correlation to the ratio of ALA to EPA in the liver, suggesting limited ALA availability for egg DHA enrichment. The use of algal DHA should therefore permit further accumulation of DHA in the total PL and TAG fractions of yolk. However, enrichment via preformed DHA (at 3.36% algal product) was also limited by hepatic PL resulting in more DHA and EPA being shunted to the adipose TAG,

concurrent with elevated hepatic acyl-CoA synthetase (ACSL1) expression. As a function of

total omega-3 PUFA intakes (regardless of source), similar levels of stearidonic acid (SDA) and

particularly EPA accumulated in liver PE.

Conclusion: Hepatic PL regulation, possibly aimed at maintaining EPA level, may potentially be

limiting the amount of ALA accumulation in the same pool; hence, limiting the endogenous

synthesis of DHA and subsequent enrichment in eggs.

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CHAPTER 1 GENERAL INTRODUCTION

Specialty eggs, including omega-3 enriched eggs, were first introduced in Canada, in part, to improve the image of eggs in view of the contentious nature of egg cholesterol composition and its relationship to heart disease (Kennedy, 2000). Over the past few years, recommendations for intakes of omega-3 polyunsaturated fatty acids (PUFA) have been of interest as measures that help prevent cardiovascular disease, the most documented bioactive role (Yashodhara et al., 2009). Such recommendations include Adequate Intake (AI) for alpha-linolenic acid (ALA, 18:3n-3) in the range of 1.1 to 1.6 g/day for adult population and 10% of the AI for ALA being provided by eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) (IOM, 2005). Estimated dietary intakes of 0.25 to 0.50 g/day of EPA plus DHA have also been established in order to decrease the risk of coronary heart disease (Harris et al., 2009). Hence, the concept of "specialty eggs" has now positioned the chicken egg, as a value added product, with a potential alternative (non-fish food) and sustainable source of omega-3 fatty acids for human consumption with numerous health benefits (Fraeye et al., 2012).

Eggs naturally contain ~ 0.06 g/egg of total omega-3 PUFA (USDA, 2015), however, the hen has the ability to endogenously convert precursor ALA, an essential fatty acid, into the longer chain PUFA (LCPUFA) as in humans through a similar pathway utilizing the desaturation and elongation enzymatic steps (Leveille et al., 1975). The incorporation of omega-3 PUFA into chicken eggs through dietary manipulation has been extensively studied by utilizing varied plant seed/oil-based ingredients (Cherian and Sim, 1991; Baucells et al., 2000; Grobas et al., 2001; Gakhar et al., 2012; Goldberg et al., 2013), fish/marine oils (Van Elswyk et al., 1995; Cachaldora et al., 2005; Lawlor et al., 2010) and microalgae (Sefer et al., 2011; Lemahieu et al., 2013; Ao et

al., 2015). The latter two sources provide preformed (exogenous) forms of LCPUFA that can directly be incorporated into the egg.

Traditionally, omega-3 PUFA supplementation in hens' diet is based on ALA-rich plant seeds/oils, however, this is a scarce resource for inclusion in poultry diets. Potential feed ingredient, such as products of hemp (*Cannabis sativa* L.), hempseed (HS) and hempseed oil (HO), have been regarded valuable for poultry and other livestock diets (Mustafa et al., 1999; Callaway, 2004; Silversides and Lefrançois, 2005), however, to date it has not been certified for use in feeding these animals due to limited information regarding their safety and efficacy. Hempseed oil is an excellent source of PUFA (80%) with linoleic acid (LA, 18:2n-6) to ALA ratio of approximately 3 to 1 (Parker et al., 2003). It has also been demonstrated in our lab (Gakhar et al., 2012; Goldberg et al., 2012) that HS or HO can be used in diets of laying hens up to levels of 20 and 12%, respectively to enrich eggs with omega-3 PUFA without influencing sensory quality of eggs. However, higher levels, in particular the intact seeds, have not been evaluated.

Furthermore, the efficiency of conversion of dietary ALA into DHA is known to be limited in poultry (Cherian and Sim, 1991; Scheideler and Froning, 1996; Baucells et al., 2000; Gakhar et al., 2012) as it is in humans (Salem et al., 1999; Plourde and Cunnane, 2007). Previous studies have shown non-proportional increases and attenuation of plateau in the accumulation of DHA in eggs total lipid (Grobas et al., 2001; Gakhar et al., 2012), liver and plasma PL in rats (Tu et al., 2010) as well as in cell cultures using human liver carcinoma cells (HepG2) in membrane PL (Portolesi et al., 2007) as a function of ALA intake/substrate concentration. The most documented factor considered to be responsible for the low incorporation of omega-3 LCPUFA into egg yolk is the rate limiting effect of the desaturase and

elongase enzymes, in particular due to the competition between the omega-6 and omega-3 fatty acid series for these enzymes in the metabolic pathway of the LCPUFA (Burdge, 2004; Nakamura and Nara, 2004; Brenna et al., 2009; Gregory et al., 2011). Apparently, the regulation of the endogenous synthesis of omega-3 LCPUFA (DHA) from precursor ALA is not fully explained by changes in the expression of enzymes or related transcription factors involved in fatty acid metabolism (Cheng et al., 2006; Tu et al., 2010), hence, complicating mechanistic/biological views to understand endogenous synthesis of omega-3 LCPUFA from precursor ALA, and subsequently egg enrichment with these fatty acids. Therefore, the current project aims to provide detailed characterization of fatty acid profiles (amounts and distribution) of different tissues and eggs of hens in the identification of potential factor(s) regulating the metabolism of omega-3 fatty acids in laying hens. This would provide an insight and direction for potential in improving the enrichment of omega-3 LCPUFA in chicken eggs.

In this thesis, Chapter 2 summarizes the current literature, subsequently leading to the hypothesis and objectives of this research in Chapter 3. Chapter 4 evaluates higher inclusion of hempseed in diets for hens. Chapter 5 provides further characterisation of hempseed product-derived omega-3 fatty acids in eggs. Chapter 6 utilizing precursor ALA derived from hempseed products relates lipid pools of different tissues to understand the mechanistic view of omega-3 fatty acid egg enrichment. In Chapter 7, a comparison, based on performance indices between precursor ALA- and preformed DHA-fed hens is presented. Chapter 8 presents further detailed assessment of lipid pools of different tissues to elucidate mechanistic views of omega-3 fatty acid enrichment in eggs based on a strong model of precursor ALA (flaxseed oil) along with a preformed DHA supplementation. Finally, Chapters 9, 10 and 11 provide a general discussion, overall conclusion of the work and considerations for future research directions, respectively.

CHAPTER 2 LITERATURE REVIEW

2.1 Omega-3 Fatty Acids

2.1.1 Nomenclature and biology

Lipids consist of glycerol esters and long chain aliphatic acids (fatty acids), which are either saturated or unsaturated. The number of double bonds between the carbon atoms in the backbone of the fatty acid molecule determines the saturation. Fatty acids can have no double bond (saturated fatty acids, SFA), one double (monounsaturated fatty acids, MUFA) or more than one double bond (polyunsaturated fatty acids, PUFA). Fatty acids are hydrocarbon chains with a methyl group at one end of the molecule (designated omega, ω) and a carboxyl group at the opposite end. In the latter, the carbon atom next to the carboxyl group designated alpha, α. Polyunsaturated fatty acids can be divided into families characterized by the position of first double bond from the methyl end of the carbon chain. As such, with the first double bond at the third carbon atom, the chain is called omega-3 (or n-3), while a first double bond at the sixth carbon atom, the fatty acid belongs to the omega-6 (or n-6) family.

The principal fatty acids of these families, alpha-linolenic acid (ALA, 18:3n-3) and linoleic acid (LA, 18:2n-6) for omega-3 and omega-6 families, respectively, cannot be synthesized in birds (Hulbert et al., 2002) or mammals (Goyens et al., 2006; Glaser et al., 2010) because they lack the enzymes (Δ12- and Δ15-desaturase) for *de novo* synthesis responsible for converting oleic acid (18:1n-9) into LA and ALA. The lack of these enzymes in higher animals, unlike the situation in plants, hinders the ability to create a double bond between the existing omega-9 double bond and the methyl end of oleic acid (Demandre et al., 1986). Hence, LA and ALA are essential fatty acids (EFA) and must be provided by the diet. Subsequently, these EFA,

are converted to the long chain PUFA (LCPUFA). This review will mainly focus on the omega-3 fatty acids.

2.1.2 Health benefits and recommendations in humans

The physiological importance of omega-3 LCPUFA: eicosapentaenoic acid (EPA, 20:5n-3), docosapentaenoic acid (DPA, 22:5 n-3) and docosahexaenoic acid (DHA, 22:6n-3) can generally be ascribed to their structural, metabolic and functional roles. These biological roles, primarily relate to the ability of the LCPUFA to incorporate into cellular membrane phospholipids (PL) and exert their influence through the formation and functioning of the cell membranes (Clandinin et al., 1994). However, several other mechanisms are involved and form the basis for the mode by which omega-3 LCPUFA exert their actions. These include the regulation of cell metabolism through the control of gene expression, modification of cell signalling, and the formation of eicosanoids (Jump et al., 2008; Adkins and Kelley, 2010). Omega-3 PUFAs have been shown to be therapeutic and contribute to the prevention and treatment of coronary artery disease, hypertension, diabetes, cancer and some inflammatory disorders (Simopoulos, 1991; Griffiths and Morse, 2006). In addition, as a component of the membrane PL, omega-3 LCPUFA (predominantly, DHA) has also shown to be important for pregnant women (for the fetus) and infants for brain and visual development, as well as cognitive function (Clandinin et al., 1994; Suh et al., 2000).

The American Heart Association has recommended the consumption of no more than 30% of total energy in the diets of adult population as fat (Harris et al., 2009). Polyunsaturated fatty acids contribute to ~ 7% of total energy intake (Harris et al., 2009). Of this proportion, LA, the major PUFA, comprises ~86% of the total PUFA energy intake and ALA contributes 9–11% (~10 times less that of LA) of the total PUFA energy intake, i.e. ~ 0.7 % of total energy (Kris-

Etherton et al., 2000). The Adequate Intake value for ALA for adults has been recommended at 1.1 and 1.6 g/day for females and males, respectively (IOM, 2005). Eicosapentaenoic acid and DHA can replace up to 10% of the requirement for ALA, indicating a daily intake in the range of 250 to 500 mg in order to decrease the risk of coronary heart disease (Harris et al., 2009). Additionally, according to the American Heart Association, patients with documented chronic heart disease are recommended a daily intake of 1 g DHA and EPA, currently, achievable from fish oils (Kris-Etherton et al., 2002). However, sustainable and alternative sources of omega-3 fatty acids, in the form of regularly consumable food sources such as the chicken egg fortified with omega-3 PUFA, have been positioned as potential dietary sources of omega-3 PUFA.

2.2 The Chicken Egg

2.2.1   Overall composition

Structurally composed of ~ 9.5% to 11% eggshell (including shell membrane), 58% to 63% egg white, and 27.5% to 31% yolk (Stadelman, 1995), the chicken egg mainly consists of water (50%), protein (15%), lipids (30 - 35%), and carbohydrates ($< 1\%$) as well as other nutrients in minor percentage including minerals, vitamins and carotenoids (Anton and Gandemer, 1997; Leskanich and Noble, 1997). Overall, the egg contains the necessary nutrients for the chicken embryo, provided in a form that can be well metabolized by the developing chick and essential for its growth and development (Griminger, 1986; Etches, 1996). Furthermore, the chicken egg is of particular interest because it is relatively rich in fatty acids, and the associated fat soluble compounds. Egg lipids found in the yolk exist mainly as a lipoprotein complex (Shen et al., 1993). The lipid fraction of the egg yolk is the main focal point in this review.

2.2.2   Yolk lipid composition

Egg yolk lipid represents 7 to 8 % of the weight of whole eggs and, being particularly confined to the egg yolk (33% by wt of yolk), is a rich source of a variety of biochemically important compounds differing in chemical composition. The lipids of the egg yolk are mainly associated with lipoprotein assemblies (Shen et al., 1993). Of the 33% proportion of total yolk weight (about 65% of the dry matter of egg yolk component; Noble, 1987), the lipids of the egg yolk are composed of triacylglycerols (TAG, ~65%), phospholipids (PL, 25 - 29%; of which 86% are phosphatidylcholine (PC) and 14% phosphatidylethanolamine (PE)), free cholesterol (5%), in addition to cholesteryl ester (CE, 1%) and traces of free fatty acids (FFA) (Anton and Gandemer, 1997). Minor PL classes found in egg yolk lipids are phosphatidylserine, phosphatidylinositol and the lyso-compounds of PC and PE as well as plasmalogens (a glycerol-ether of PL) (Noble and Moore, 1965).

Most lipids contain at least one fatty acid of varying chain lengths, degrees of saturation and configurations. Based on a standardized diet, the fatty acid composition of egg lipids is about 30–35% (1.5 g/egg) SFA, 40–45% (1.9 g/egg) MUFA, and 20–25% (0.7 g/ egg) PUFA (Song and Kerver, 2000; Anton, 2007). Each fatty acid group is represented in abundance by palmitic acid (16:0, 20–25%), oleic acid (40–45%) and LA (15–20%), respectively (Kuksis, 1992), depending on the fatty acid concentration in the birds' diet (Anton and Gandemer, 1997). Although the composition of PUFA in egg lipid is greatly influenced by the diet (Cherian and Sim, 1991; Baucells et al., 2000; Gakhar et al., 2012), SFA and MUFA levels tend not to be (Caston and Leeson, 1990; Scheideler and Froning, 1996; Meluzzi et al., 2001). In addition to fatty acids, the egg yolk lipid also includes steroids (such as cholesterol), fat-soluble vitamins

and carotenoids (Anton, 2007). Cholesterol, contained at a level of ~200 mg/egg (Griffin, 1992),

is a result, partly, from the hens' feed and that from synthesis in the liver.

2.2.3   Human consumption of chicken eggs

In human nutrition, the egg is a valuable source of nutrients. In addition to its excellent

protein quality (biological rating of 100) positioning the egg as a standard reference in

comparison against the quality of other food proteins (Shrimpton, 1987), the chicken egg is well

established as an excellent source of most essential nutrients for human consumption. Balanced

for most nutrients in relation to its low calorie content (Hu et al., 2001), eggs are also known to

provide diverse biological functions beyond basic nutrition in humans (Kovacs-Nolan et al.,

2005), particularly with reference to omega-3 fatty acids, the basis of this research. Following a

lowest level decline (in the middle 1990s), due to changing dietary habits, in part, related to high

cholesterol content in eggs, egg consumption in Canada has increased in the last decade by 20%

(Statistics Canada, 2013; Figure 2.1). This may possibly relate to an increased public awareness

of the role of eggs and their repositioning as a healthy food, due to the growing understanding

that there is a lack of correlation between dietary cholesterol, which is found in eggs, and serum

total or low-density lipoprotein cholesterol levels (Clarke et al., 1997). The latter finding is true

from an epidemiological/population standpoint but is not from an individual standpoint, in that

within a population, 15 to 30% of individuals exhibit a hyper-response to dietary cholesterol, as

reviewed by Elkin (2006). Eggs and egg products occur widely in people's diets. Per capita egg

consumption (eggs per year) currently sits at 258 in Canada (Statistics Canada, 2013, Figure 2.1)

and 252 in the US (USDA, 2011; USPoultry, 2013). Currently in Canada, the market for omega-

3 fatty acids enriched eggs contributes to ~15% of the total shell egg market (Flax Council of

Canada, 2016). Based on the current positive trend in egg consumption, the incorporation of
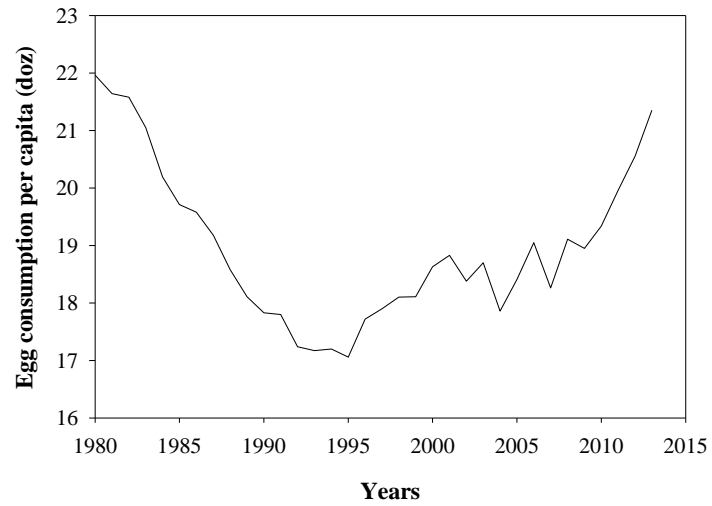
Figure 2.1. Egg consumption per capita, per annum (doz eggs) in Canada from 1980 to 2013. (Statistics Canada, 2013)

omega-3 PUFA in eggs may represent an additional and viable way to boost the intake of these fatty acids.

2.3 Omega-3 Enrichment of Eggs

The regular chicken egg is naturally poor in ALA, DPA and DHA and contain little EPA (Yalcyn et al., 2007; Souza et al., 2008; Elkin et al., 2015). This primarily relates to the fact that the commercial poultry feeds, grains and seeds-based, supply mainly omega-6 PUFA, particularly LA, a predominant fatty acid of the lipids stored in grains and seeds and a small amount of omega-3 PUFA, i.e. ALA (Morrison, 1977). However, it has been established that the egg is easily modifiable and is strategic for enrichment of omega-3 PUFA (Cherian and Sim, 1991; Hargis and Van Elswyk, 1993). The latter benefit stems from the fact that chickens are capable of converting ALA, the primary precursor obtained from the diet, to the longer chain metabolites in the liver, with DHA as the end-product (Anderson et al., 1989).

2.3.1   Regular versus omega-3 fatty acids enriched eggs

A comparison of the lipid content of regular eggs versus omega-3 fatty acids enriched eggs derived from hens fed typical ALA-rich ingredients is shown in Table 2.1. The nutrient contents (per egg): energy (80 vs. 74 kcal), protein (7.03 vs. 6.2 g), carbohydrate (0.40g) and total fat (5.33 vs. 4.8 g), respectively, between a regular egg and that of an omega-3 fatty acid-enriched egg are similar (Scheideler and Lewis, 1997). However, egg enrichment indicates an overall increase in the amounts of omega-3 PUFA in the egg yolk, at the expense of the omega-6 PUFA (Table 2.1). In addition, although modified eggs may contain less cholesterol, up to 50% reduction when pharmacological levels of copper, garlic extract or statin drugs were used in laying hen diets (Elkin, 2006, 2007), there are other suggestions that egg yolk cholesterol content

Table 2.1: Comparison of the lipid contents of regular versus omega-3 fatty acids enriched eggs

Lipids (g)	Regular eggs[1]	Omega-3 PUFA enriched eggs[2]
Saturated fatty acids	1.75	1.50
Monounsaturated fatty acids	2.05	2.10
Polyunsaturated fatty acids	1.07	1.30
Alpha-linolenic acid (ALA)	0.020	0.25
Docosahexaenoic acid (DHA)	0.032	0.10
Total n-6 fatty acids[3]	0.98	0.78
Total n-3 fatty acids[4]	0.056	0.35
n-6/n-3 ratio	17.1	2.60
Cholesterol	0.21	0.18

[1]Lipids content of a fresh, whole (56g mass) regular egg obtained from United States Department of Agriculture (USDA, 2015)

[2]Averages in eggs obtained from flaxseed fed hens (Scheideler and Froning, 1996; Morris, 2003)

[3]Total n-6 fatty acids calculated as the sum of linoleic acid (LA), gamma-linolenic acid (GLA) and arachidonic acid (ARA)

[4]Total n-3 fatty acids calculated as the sum of alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA)

is not affected by dietary fatty acid composition (Cobos et al., 1995; Ferrier et al., 1995; Scheideler and Froning, 1996). On average an egg contains 200 mg cholesterol (Hargis, 1988; Van Elswyk et al., 1991; Griffin, 1992).

With reference to the level of enrichment presented in Table 2.1, on average, hens fed diets containing flaxseed up to 15 to 20% by wt (Ferrier et al., 1995; Scheideler and Froning, 1996; Lewis et al., 2000) produce eggs containing about 250 mg/egg of ALA, 10-fold higher than that observed in regular eggs. In addition, one omega-3 enriched egg would contribute to ~25% of the AI value of ALA for the adult population, particularly for women (~ 1.1 g/day, IOM, 2005), while achieving the nutritional recommendations in Canada of lowering the omega-6/omega-3 (n-6/n-3) PUFA ratio within the range of 4 - 5 to 1 (Kris-Etherton et al., 2000; Holub, 2002). However, the n-6/n-3 ratio of PUFA in the regular egg is high (17 to 1 ratio). The Canadian Food Inspection Agency permits a claim for eggs that states "contains omega-3 PUFA" if the total omega-3 PUFA is at least 0.30 g per reference amount (50 g per shell egg, equivalent to serving size of 50 to 100g; CFIA, 2015). Based on this allowance, an enriched egg (56 g mass, Table 2.1) obtained from flaxseed supplementation in laying hen diets, provides 0.35 g/egg total omega-3 PUFA and would thus achieve this claim. A consumption of 2 omega-3 enriched eggs per day (per calculation, Table 2.1), would provide almost half (50%) of the stated daily intake with the range of 250 to 500 mg of EPA and DHA in order to decrease the risk of cardiovascular disease (Harris et al., 2009). Moreover, the incorporation of omega-3 fatty acids into the egg yolk is considered to be a promising way to reduce or mitigate potential cholesterogenic effects of eggs (Song and Kerver, 2000). However, other studies have indicated that the enrichment of omega-3 fatty acids, results in decreased hepatic cholesterol and *de novo* fatty acid synthesis, consequently reducing the incorporation of cholesterol and its esters into

yolk precursors (vitellogenin and VLDL). Very-low-density lipoproteins and vitellogenin account for approximately 64% and 20% of yolk dry matter (Burley et al., 1993), respectively, and 95% and 5% of yolk cholesterol (Griffin, 1992), respectively.

Other documented benefits of the egg yolk lipids for humans can be attributed to the egg yolk PL (estimated to be more than 3-fold higher than natural soy PL). When consumed, egg yolk PL has been shown to increase plasma and brain choline levels and accelerate neuronal acetylcholine synthesis (Kovacs-Nolan et al., 2005). The latter is known to be important for pregnant and nursing women to ensure healthy fetal brain development and potentially alleviate some of the symptoms of Alzheimer disease (Zeisel, 2006). Generally, LCPUFA serve as a structural component of cell membranes (Ackerman, 1995), as such, the major proportion of these fatty acids (primarily DHA as well as arachidonic acid (ARA, 20:4n-6)), are present as esterified PL. The latter point is biologically and nutritionally significant because PL fatty acids are well absorbed from the intestine (Cansell et al., 2003). In addition, egg yolk PL enriched with LCPUFA is also produced for infant formulas (Trautwein, 2001). Although the majority of the health benefits of the omega-3 LCPUFA relate to EPA and DHA (Fraeye et al., 2012), ALA (the major proportion of the omega-3 fatty acid enriched egg) is also of nutritional importance (Barceló-Coblijn and Murphy, 2009; Coorey et al., 2015) due to its involvement in numerous biological roles in humans (Kinsella et al., 1990) as well as in poultry (Bautista-Ortega et al., 2009).

2.3.2   Functional benefits of yolk lipids in hens

2.3.2.1 Principal role of egg yolk lipids for the chicken

Birds are oviparous and deposit eggs (with enveloping shell) that contain a significant portion of nutrients which are essential for embryonic development and growth (Griminger,

1986; Kovacs-Nolan et al., 2005). Hence, considered as a vital cell, the egg must contain every

necessary nutrient to develop a new life. As such, in addition to numerous important

components contained in the egg, e. g. amino acids, vitamins and minerals, the egg lipids have a

high biological and nutritional value for the embryo. The egg lipid, primarily the TAG, serves as

the main source as well as a store of energy for chicken embryo tissue development and growth

(Noble and Cocchi, 1990; Speake et al., 1998b). The oxidation of fatty acids, possibly related to

the limited ability to modify the levels of SFA and MUFA in the egg (Jiang et al., 1991; Baucells

et al., 2000), provides almost 94% of the total energy needs for the embryo during development

(Noble and Cocchi, 1990). Fatty acid oxidation accounts for approximately 189 kJ of energy

which is equivalent to about 75% of the embryo's total energy requirement for hatching

(Walzem et al., 1999). On the other hand, the PL mainly composed of PUFA, are structural

lipids and generally incorporated into cell membranes (Nobel, 1991), providing essential

components for embryo tissue development and functionality (Speake et al., 1998b).

2.3.2.2 Omega-3 fatty acids in laying hen nutrition

In hens the incorporation of omega-3 PUFA through their diets into the egg and,

consequently, into the developing progeny during embryonic development, is primarily involved

in the development of neural tissue (Speake et al., 1998a; b). Most importantly, DHA has been

shown to be important for the development of brain and retina in chicks (Cherian and Sim,

1991). Subsequently, the incorporation of dietary omega-3 fatty acids into the egg and

consequently into the developing progeny, can lead to improvements in chick growth, health and

development (Cherian and Sim, 1991; Cherian et al., 1997; Hall et al., 2007). However, fertility

and hatchability of chicks (in broiler breeders) was not shown to be influenced (Bautista-Ortega

et al., 2009). Nonetheless, there is an indication that chicks hatched from hens fed diets low in

omega-3 fatty acids may be at higher risk of developing cardiovascular complications related to high ARA concentrations in the heart and blood cells during the first week of age (Bautista-Ortega et al., 2009). The authors indicated that modulating egg yolk omega-3 PUFA enhances chick tissue omega-3 PUFA and reduces the production of pro-inflammatory eicosanoids.

Requirements for specific fatty acids have traditionally focused on the need for LA at 1% (NRC, 1994) or 2% (Lohmann LSL-Classic, 2004) of the diet for hens consuming 100 g per day. It is recognized that both the omega-6 and omega-3 series of PUFA play essential roles in embryonic and chick development (Cherian and Sim, 1991; Speake et al., 1998a). However, to date, little evidence exists to support a requirement estimate for omega-3 fatty acids, primarily ALA, in laying hen diets (NRC, 1994). Alterations in the dietary levels of omega-6 and omega-3 fatty acids can result in significant changes in the egg or tissue omega-6 and omega-3 PUFA contents, liver desaturase enzyme activities, PUFA-derived eicosanoid synthesis and immune responses. As such, an understanding of the metabolic fate of the parent omega-3 fatty acid (ALA) is critical, and this is addressed in the next section.

2.4 Fate of Dietary Essential Fatty Acids in Laying Hens

The fate of dietary essential fatty acids, particularly ALA, requires an understanding of the processes involved in the digestion, absorption, transport and hepatic metabolism of this fatty acid, as well as its final deposition into the egg yolk (Figure 2.2). Consumption of specific dietary fatty acids, have differential effects on biological processes. Therefore, a mechanistic framework that links uptake of these fatty acids to biological pathway(s) and the final deposition of egg yolk lipids is important. Although the intestine and the liver are considered the two important organs involved in maintaining lipid homeostasis (Xiao et al., 2011), each stage within
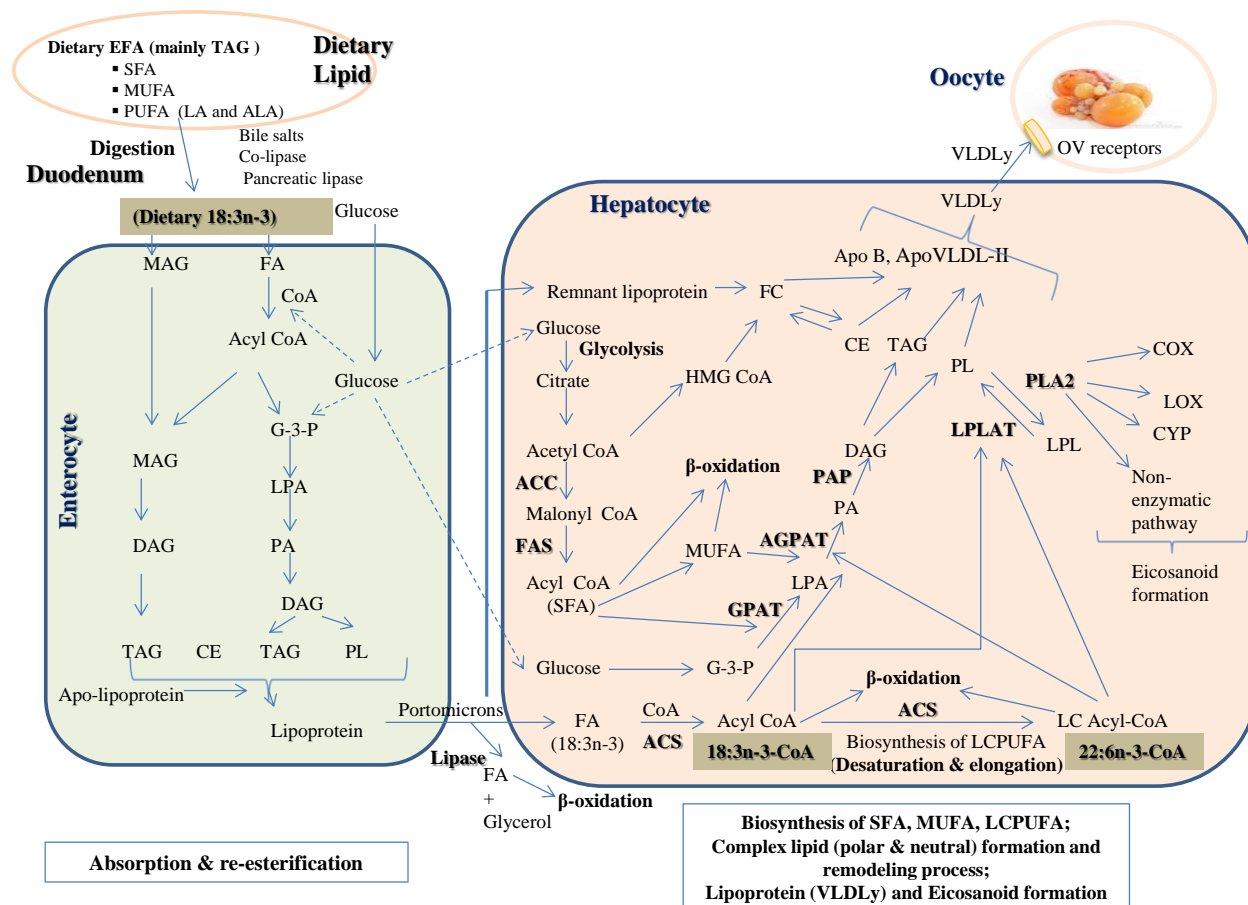
Figure 2.2. Schematic representation of a mechanistic framework linking dietary ALA intake to the deposition of DHA into chicken eggs.

Abbreviation: EFA, essential fatty acids; ALA (18:3n-3), alpha-linolenic acid; FA, fatty acid; MAG, monoacylglycerol; DAG, diacylglycerol; TAG, triacylglycerol; G-3-P, glycerol-3-phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; PL, phospholipid; CE, cholesteryl esters; ACC, acyl CoA carboxylase; FAS, fatty acyl synthase; HMG-CoA, hydroxymethylglutaryl-CoA; FC, free cholesterol; Apo B, apolipoprotein B; VLDL, very-low-density lipoproteins; apoVLDL-II, very-low-density apolipoprotein II; VLDLy, yolk targeted VLDL; OV receptor, oocyte vitellogenin/VLDL receptor; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; ACS, acyl CoA synthase; CoA, coenzyme A; PAP, phosphatidic acid phosphatases; GPAT, glycerophosphate acyltransferase; AGPAT, acylglycerophosphate acyltransferase; LC Acyl-CoA, long chain acyl CoA; DHA (22:6n-3), docosahexaenoic acid; PLA2, phospholipase A2; LPLAT, lyso-phospholipid acyl transferase; LPL, lyso-phospholipid; COX, cyclooxygenases; LOX, lipoxygenases; and CYP, cytochrome P450s.

the framework, herein described, may represent a potential regulatory point in the deposition of LCPUFA in egg yolk endogenously synthesized from dietary ALA.

2.4.1   Digestion, absorption and transport of dietary lipid

The bulk of dietary lipid is principally present as neutral fat (mainly TAG). Hence, dietary EFA (along with SFA and MUFA) are supplied in the hen diet mostly as acyl chains of TAG (90%) and enters the site of digestion, namely the duodenum, in that form (Hermier, 1997; Ramírez et al., 2001). However, PL, cholesterol, CE and fat-soluble vitamins are also contained in dietary lipids, constituting the remaining 10% (McFarland, 2003). In addition to dietary PL, biliary PL (endogenous PL) is also an important source of intestinal EFA (Bregendahl, 2006). Lipid digestion involves the resolution of the lipid fraction into its constituent fatty acid components (Hurwitz et al., 1973). During digestion, bile salts emulsify fat and with the aid of co-lipase, that helps provide more surface area for the digestive enzymes to act upon, micelles are formed which aid absorption. During absorption, pancreatic lipase hydrolyses the emulsified TAG on sn-1 and sn-3 positions to release 2-monoacylglycerols (MAG) and two FFA. Similarly, cholesterol esterase hydrolyses cholesterol-fatty acid esters into cholesterol and free fatty acids, and phospholipase A2 (PLA2) cleaves phospholipids at the sn-2 position to release lyso-phosphatidylcholine and FFA (Christie, 2011; Tornheim and Ruderman, 2011). Absorption of dietary fat from the intestinal lumen to the enterocyte proceeds after the hydrolysis products diffuse into the aqueous phase of the intestinal mucosa which then get translocated to the brush border into the enterocytes where re-esterification of the FFA into TAG takes place (Krogdahl, 1985). In the enterocytes of intestines, up to 75-80% of the TAGs are resynthesized mainly via a MAG pathway. However, glycerol-3-phosphate (G-3-P), produced by the catabolism of glucose (glycolysis), referred to as the alpha-glycerophosphate pathway, is

used in the biosynthesis of phosphatidic acid (PA) for the synthesis of TAG and PL (Coleman

and Lee, 2004; Christie, 2011). The G-3-P, as a source of carbon, is further described in a latter

section of this review (complex lipid formation in the liver). In the MAG pathway, the MAG

acylations are first activated by an acyl coenzyme A: monoacylglycerol acyltransferase (MGAT)

with the formation of sn-1, 2-diacylglycerols (DAG), mainly as the first intermediate in the

process. Additionally, sn-2, 3-diacylglycerols are also produced, and further acylated by acyl

coenzyme A:diacylglycerol acyltransferase (DGAT) leading to TAG formation (Christie, 2011).

In this way, free fatty acids are first re-esterified to TAG before being transported to the liver.

Although up to 75% of the fatty acids absorbed into the enterocyte are re-esterified and

exported to the circulation as TAG, the remaining (~25 to 30%) of the non-esterified fatty acids

(mainly composed of LCPUFA) are transported as such in the circulation (Sklan et al., 1996), but

still rapidly converted to fatty acyl CoA to help keep low intracellular levels of FFA and

transported to intracellular compartments bound to protein for metabolism (Jump et al., 2008).

Besides, the rate of absorption of the ingested dietary fatty acids can also be influenced by the

degree of dietary fat saturation. This may, in turn, translate to differences in the uptake of other

nutrients as the capacity for the absorption of nutrients, including fatty acids, in the intestine

depends on the intestinal morphology, particularly the development of the mucosal surface area

(Ferrer et al., 2003). The composition of dietary lipids can influence the fluidity of brush border

membranes of the intestine (Vázquez et al., 1997) that, in part, could also alter the transportation

and diffusion of certain nutrients (including fatty acids) across the intestinal mucosa, possibly by

modifying the functions of enzymes and nutrient transporters present in the mucosal membrane.

The newly resynthesized TAGs are combined with cholesterol, CE, PL and some specific

proteins (apolipoproteins) into very large lipoprotein particles called portomicrons, which are

analogous to chylomicrons. Unlike mammals, birds have a rudimentary lymphatic system; hence, transport of portomicrons to the liver for further synthesis occurs via the portal venous system (Bensadoun and Rothfeld, 1972; Krogdahl, 1985). Chicken portomicrons are similar in composition to mammalian chylomicrons (Schneider, 2009). In birds, as in mammals, the metabolism of portomicrons is similar with remnant TAG particles being converted to LDL before being removed from the circulation by the liver or extrahepatic tissue (Brindley, 1984).

2.4.2 Hepatic lipid metabolism

In avian species (Leveille et al., 1975), as in humans (Patel et al., 1975), the liver is the principal site of lipid metabolism. In the liver, ingested fatty acids, including ALA, may enter several metabolic fates, including β-oxidation (for cellular energy, ATP), conversion to LCPUFA, esterification into structural lipids (complex lipid formation) and transportation to the extra hepatic tissues including muscle, adipose or ovarian tissues. The latter will receive particular emphasis in this review. The long chain metabolites of ALA may also be used as precursors for eicosanoids synthesis. In this review, each of these metabolic fates of the ingested ALA will be briefly described following the schematic layout in Figure 2.2.

2.4.2.1 Biosynthesis of LCPUFA

In the liver, essential fatty acids can be converted to the longer chain metabolites. This is because birds are capable of synthesizing the longer chain omega-3 and omega-6 PUFA (up to 22 or more carbons), from the principal fatty acids, ALA and LA, respectively (Cherian and Sim, 1991). The biosynthesis of LCPUFA (omega-3 and omega-6 PUFA pathways) in birds is described to be similar as those in mammals, and involves the action of Δ5- and Δ6-desaturase and elongase enzymes (Goyens et al., 2006). In these processes ALA can be converted to EPA (to a limited extent) and later to DHA (as the major end-product), while LA can be converted to

ARA (Bézard et al., 1994; Hastings et al., 2001; Glaser et al., 2010). In hens, the enrichment of

egg yolk can also occur through a direct deposition of preformed LCPUFA from the diets

containing ingredients that provides preformed EPA and/or DHA, such as marine oils (source of

EPA and DHA; Lawlor et al., 2010) and oils from algae (source of DHA; Herber and Van

Elswyk, 1996; Sefer et al., 2011). A general layout in the biosynthesis of the omega-3 fatty acids

is shown in Figure 2.3.

The endogenous synthesis of DHA from dietary ALA undergoes a series of reactions

including desaturation (addition of a double bond/removal of hydrogen) and elongation (addition

of two carbon atoms within microsomes) (Brenner, 1974). Firstly, ALA is converted to

stearidonic acid (SDA, 18:4n-3) by Δ6-desaturase (encoded by FADS2) then elongated to

eicosatetraenoic acid (ETA, 20:4n-3). This is followed by two desaturations (firstly by Δ5-

desaturase, encoded by FADS1; then by Δ6-desaturase) and elongations and a β-oxidation of

tetracohexaenoic acid (THA, 24:6n-3) into DHA in the peroxisomes via a multifunctional

enzyme (Sprecher, 2000). All reactions occur in the endoplasmic reticulum (ER) except the

latter process which is known to occur in the peroxisomes, indicating that the ER does not

contain an acyl-CoA-dependent Δ4-desaturase (Sprecher et al., 1995). Hence, the LCPUFA

(either TPA and THA or the latter only) translocates from the ER to the peroxisomes (referred to

as the Sprecher pathway) where β-oxidation takes place (by removal of 2 carbons) to generate

DHA, after which DHA moves to the ER, where it is esterified into membrane lipids (Luthria et

al., 1996; Sprecher, 2000). This intracellular site for the retro-conversion of THA to DHA is

evident, in part, because patients lacking peroxisomes resulted in DHA deficiency

(Ferdinandusse et al., 2001). Furthermore, a retro-conversion of DHA into EPA is also known to
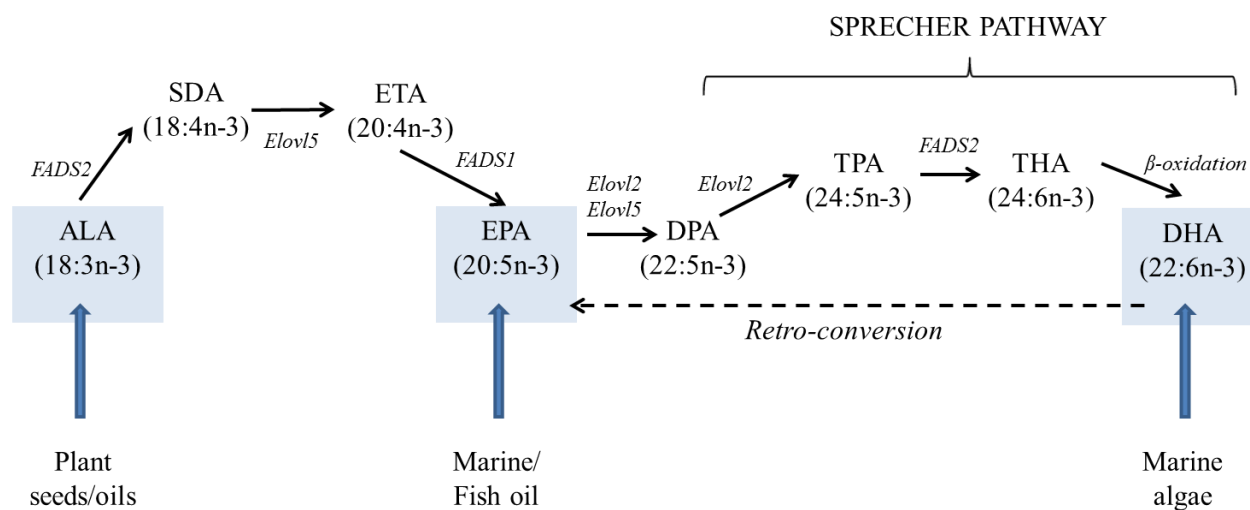
Figure 2.3. General overview of omega-3 PUFA biosynthesis pathway.

Abbreviations: ALA, alpha-linolenic acid; SDA, stearidonic acid; ETA, eicosatetraenoic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; TPA, tetracosapentaenoic acid; THA, tetracosahexaenoic acid; and DHA, docosahexaenoic acid. Adapted (Sprecher, 2000; Ferdinandusse et al., 2001; Brenna et al., 2010; Jump et al., 2013).

take place by partial peroxisomal β-oxidation (Gronn et al., 1991; Sprecher et al., 1995; Willumsen et al., 1996). Likewise, in the omega-6 series, LA can be converted to gamma-linolenic acid (GLA, 18:3n-6) by Δ6-desaturase and then GLA can be elongated to dihomo-gamma-linolenic acid (20:3n-6) and further to ARA, utilizing similar enzymes as those for the omega-3 series. The omega-6 family member, docosapentaenoic acid (22:5n-6), is also a product of peroxisomal β-oxidation generated in the same way (involving the Sprecher pathway) as for DHA.

Arising from this metabolic pathway are several factors that may regulate the metabolism of LCPUFA in the conversion of ALA to DHA. Some of these factors are further discussed in section 2.5. Firstly, given the shared enzymatic steps, competition between the omega-6 and omega-3 series (Brenner et al., 1969) and within the omega-3 series (Tu et al., 2010) of PUFA for desaturation and elongation occurs in the metabolic pathway. Considering that there is no inter-conversion between metabolites (between series) or sparing effect of the fatty acids in either family member (Sprecher, 2000), the balance of essential fatty acids in the diets is very important for physiological functions (Calder, 2002). Secondly, within the same family members, the conversion of ALA to stearidonic acid (SDA, 18:4n-3), encoded by fatty acid desaturase 2 (FADS2, Δ6-desaturase), is known to be a rate-limiting step in LCPUFA biosynthesis (Sprecher, 1981; Brenna, 2002). In addition, both ALA and TPA (24:5n-3) are substrates for the same FADS2 gene (Sprecher et al., 1995), this can have a direct influence on the efficiency of conversion. It has been shows that FADS2 has higher substrate specificity for ALA than TPA (Portolesi et al., 2007). Furthermore, the retro-conversion of DHA to EPA is a possible means of controlling hepatic 22-carbon PUFA levels during hepatic metabolism of LCPUFA and maintaining lipid homeostasis (Jump, 2008). Besides these limitations, the

chicken has the ability to utilize both ELOVL2 and ELOVL5 to efficiently elongate DPA

(Gregory et al., 2013). The latter gene, ELOVL5, is not only functional but also abundant in the

chicken compared with other species (Gregory et al., 2013). However, there is high protein

sequence identity between the chicken and the human FADS1 and FADS2 of 79 and 77%,

respectively (NCBI, 2012).

2.4.2.2 *β-oxidation of fatty acids*

Both omega-3 and omega-6 PUFA have been shown to promote fatty acid oxidation

while decreasing the rates of *de novo* lipid synthesis (Sampath and Ntambi, 2005). As

mentioned earlier in this review, the first step of fatty acid metabolism is the activation of fatty

acids (Digel et al., 2009; He et al., 2014). In the process, long chain acyl-CoA synthetases

(ACSLs) first activate the long-chain fatty acids with coenzyme A (forms the acyl-CoA), which

then get esterified (Digel et al., 2009). Different isoforms of ACSL exist. ACSL1 located to

mitochondria play an major role in activating fatty acids destined for β-oxidation in tissues, such

as heart and adipose (Mashek, 2013). The regulation of long chain acyl-CoA use for β-oxidation

(Coleman and Lee, 2004) reflects the need to control hepatic metabolism of LCPUFA and

maintain lipid homeostasis (Jump, 2008).

Unlike saturated and monounsaturated fatty acids, a diet that provides 2-5% of energy as

20- and 22-carbon PUFA (synthesized or preformed), stimulates hepatic fatty acid oxidation

(Kris-Etherton et al., 2000; Clarke, 2004; Gibson et al., 2013). As described earlier, the final

step in the biosynthetic pathway in the conversion of ALA to LCPUFA (Figure 2.3), involves a

process of peroxisomal degradation of THA (24:6n-3), a prerequisite in the biosynthesis of

DHA, as well as a possible retro-conversion of DHA to EPA (Voss et al., 1991; Sprecher et al.,

1995). Each step (the peroxisomal partial β-oxidations), in itself, is a means to help regulate the

levels of LCPUFA (Sprecher, 2000). On the other hand, mitochondrial β-oxidation is mainly

responsible for the breakdown of fatty acids up to 18-carbons (Cunnane and Anderson, 1997;

Brenna, 2002). A high percentage of dietary ALA, is directed toward mitochondrial β-oxidation,

equivalent to ~85% observed in growing rats (Poumès-Ballihaut et al., 2001). In humans, ~ 25%

of ALA is β-oxidized in the first 24 h, reaching 60% by 7 days (Brenna, 2002). This rate of

oxidation may account for the low conversion of ALA into the LCPUFA. Other dietary derived

PUFA are also β-oxidized in similar percentages (Cunnane and Anderson, 1997).

Fatty acids, particularly LCPUFA, regulate lipid metabolism by modulating the

expression of numerous genes (Sampath and Ntambi, 2006; Adkins and Kelley, 2010). At the

cellular level, both omega-3 and omega-6 PUFA function via peroxisome proliferator-activated

receptor α (PPARα) to upregulate the transcription of genes involved in β-oxidation, such as

acyl-CoA oxidase (ACOX), a rate-limiting enzyme in peroxisomal fatty acid oxidation

(Willumsen et al., 1996) and carnitine palmitoyl transferase-1 (CPT-1) genes, involved in

mitochondrial β-oxidation (Sampath and Ntambi, 2006). Microsomal oxidation targeting

cytochrome P450 (CYP) genes are also activated by PPARα. However, PUFA are differentially

β-oxidized. For example, in a cultured rat hepatocyte study, using ethyl esters of EPA and DHA

as substrates, Willumsen et al. (1996) showed mitochondrial β-oxidation to be stimulated only in

the presence of EPA but not DHA. Although the same authors found that peroxisomal β-

oxidation was stimulated after both EPA and DHA treatments (indicated by increased activity in

the fatty acyl-CoA oxidase, a rate-limiting enzyme in peroxisomal fatty acid oxidation).

Poumès-Ballihaut et al. (2001) provided evidence that DHA was a poor substrate for both

peroxisomal as well as mitochondrial degradations. In addition, while DHA is a weak activator

of PPARα, the retro-conversion mechanism generates EPA, a strong PPARα activator (Jump,

2008). Hence, this may position EPA as an important point in the metabolic pathway of omega-3 PUFA.

2.4.2.3 Complex lipid formation (lipid classes)

Fatty acids are esterified into acceptors to yield neutral lipids and phospholipids where they are used and/or transported to other tissues. The resultant movement of the chain-shortened product (DHA) from the peroxisome back to the ER (microsomes) for esterification is critical for its use in membrane lipid synthesis (Sprecher et al., 1995), rather than serving as a substrate for continued peroxisomal β-oxidation (Sprecher, 2000).

Fatty acids synthesized in the liver are metabolised into various lipid classes such as PL and TAG (Nguyen et al., 2008), mainly through the esterification of G-3-P with the activated form of the fatty acids (acyl-CoAs) (Christie, 2011). In the *de novo* pathway of complex lipid formation, phosphatidic acid (PA) is a key intermediary in the TAG (glycerolipids) and PL (glycerophospholipids) biosynthetic pathways (Christie, 2011, 2014). As an example, considering the biosynthesis of glycerophospholipids, they are first formed by the *de novo* pathway (Kennedy pathway) using acyl-CoAs as donors. Firstly, a fatty acyl-CoA, usually a SFA, is added to G-3-P at the sn-1 position to produce lysophosphatidic acid (LPA). This is catalyzed by glycerol 3-phosphate acyltransferase (GPAT). A second fatty acyl-CoA, often unsaturated (MUFA), is added to LPA at the *sn*-2 position to form PA, catalyzed by acylglycerol-3-acyltransferase (acylglycerophosphate acyltransferase) (AGPAT). The sequential reactions of acyl-CoA:G-3-P acyltransferase (GPAT) and acyl-CoA:1-acyl-G-3-P acyltransferase (AGPAT) or lysophosphatidic acid acyltransferases (LPAAT), are involved in the incorporation of fatty acids into the glycerol backbone of PL. Phosphatidic acid can be dephosphorylated by PA phosphatases (PAP) to DAG to synthesize phosphatidylcholines (PC),

phosphatidylethanolamines (PE), phosphatidylserines (PS) or TAG (Yamashita et al., 2014).

Therefore, the primary physiological role of acyl-CoA:LPAAT is to provide PL having a SFA at

the sn-1 position and an unsaturated fatty acid at the sn-2 position. Subsequently, PUFA are

incorporated into complex lipids mainly through remodelling (Lands et al., 1982) via the

deacylation-reacylation pathway involving phospholipase A (PLA2). The latter releases the fatty

acid at the sn-2 position. An acyl transferase (e. g. lyso-phospholipid acyltransferase; LPLAT)

catalyzes the reacylation (Balsinde et al., 1995) to reform an intact PL. Alternatively, PLA2

enzymes can catalyse the hydrolysis of membrane phospholipids, releasing lyso-phospholipids

and free fatty acids, leading to the formation of eicosanoids. Hence, the major route for the

incorporation of LCPUFA into PLs is mediated by the deacylation/reacylation cycle described as

the Land's cycle/pathway (Lands et al., 1982). This pathway allows the remodeling of cellular

PL leading to the selective distribution of LCPUFA at the sn-2 position as well as regulating the

basal levels of eicosanoid synthesis (Balsinde et al., 1995).

Both SFA and MUFA are primarily obtained from the diet, since *de novo* synthesis of

these fatty acids can be inhibited by the presence of PUFA (Sampath and Ntambi, 2005; Jump,

2008). However, in the presence of low fat diets, SFA and MUFA are synthesized *de novo.* The

de novo synthesis, following the layout in Figure 2.1, involves acetyl-CoA carboxylase

catalysing the conversion of acetyl-CoA to malonyl-CoA and the formation of palmitate from

another molecule of acetyl-CoA and malonyl-CoA by fatty acid synthetase (Annison, 1983).

The acetyl CoA for fatty acid synthesis comes mostly from the glycolytic breakdown of glucose.

Fatty acid synthetase is a multi-enzyme polypeptide and, in vertebrates, the enzyme possesses

seven distinct activities required for the synthesis of long-chain fatty acids from malonyl-CoA

(Annison, 1983). A sequence of six steps increases the fatty acid chain by 2 carbon atoms with

the release of the 16-carbon SFA (palmitate) as the final step. Palmitate can then be modified

through chain elongation and desaturation to give rise to longer chain unsaturated fatty acids,

with one desaturation step leading to the MUFA. Otherwise, omega-3 LCPUFA biosynthesis

inhibits *de novo* fatty acid synthesis. Hence, within the complex lipid classes, the composition of

fatty acids, as well as the positional distribution of fatty acids on the glycerol molecule, can

differ depending on the lipid sources. In addition, competition between LCPUFA for

esterification at the sn-2 position of glycerophospholipid (Yamashita et al., 2014) and selective

activation of LCPUFA by the enzymes is also known to exist. This may result in differences in

preferential incorporation of fatty acids into different pools. For example, EPA and its CoA

derivative, EPA-CoA, are known to be poor substrates for diacylglycerol acyltransferase

(DGAT), an enzyme involved in the esterification of 1,2-diacylglycerol (Dircks and Sul, 1999).

Hence, the distribution pattern in the incorporation of fatty acids into the different lipid classes

may have regulatory as well as functional roles. As such, PLs, predominantly containing

LCPUFA, are major constituents of cell membranes and play an essential role in the

biochemistry and physiology of cells (Kim et al., 2010).

2.4.2.4 Eicosanoid formation

As a subsequent fate of essential fatty acid metabolism (Figure 2.2), following the

esterification (remodeling process) of LCPUFA into various PLs, upon cell activation

(immunological activation/challenge, in animal could be stress induced increase in cytokine

secretion), phospholipases (PLA2) can cleave the esterified fatty acids from membrane PL

(Chilton et al., 2014). Given the shared enzymatic steps involved in the biosynthesis of the

omega-3 and omega-6 PUFA (Sprecher, 2000), the metabolites of the omega-3 LCPUFA (EPA,

DPA and DHA) are also known to compete with those of the omega-6 fatty acid series (ARA).

Upon mobilization from the membrane PL through the action of PLA2, the free fatty acids (of either series) can serve as the metabolic precursors for the synthesis of oxylipins, including eicosanoids (Buczynski et al., 2009). These are oxidized products of LCPUFA. When ARA is released from its storage site in membrane PL, this serves as the first step in the synthesis of eicosanoids (Dennis, 2000). It has thus been suggested that the omega-3 LCPUFA (particularly EPA and DHA) inhibit ARA incorporation into cell membranes and the subsequent ARA metabolism to eicosanoids (Glaser et al., 2010; Chilton et al., 2014). In this instance, the omega-3 LCPUFA would act as the substrate(s) for the synthesis of oxylipins. In either case, the mobilized free fatty acids are then oxidized to distinct classes of oxylipins such as eicosanoids (derived from free ARA or EPA) and docosanoids (derived from free DHA). This occurs via three major enzymatic pathways, including the cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP) pathways (Cherian, 2007; Glaser et al., 2010; Tourdot et al., 2014). The syntheses of these oxidized lipid mediators can significantly influence inflammatory processes (Calder, 2008). Eicosanoids derived from the omega-6 series can act as potent pro-inflammatory mediators, and include such compounds as prostaglandin (PG) E2, thromboxane (TX) A2, and leukotriene (LT) B4. Those derived from the omega-3 series are less potent as pro-inflammatory molecules, with this class of compound including the eicosanoids PGE3, TXA3, and LTB5 (Calder, 2006; Chilton et al., 2014). The fact that both the omega-6 (ARA) and the omega-3 LCPUFA (EPA and DHA) serve as metabolic substrates for the synthesis inflammatory mediators leads to the positioning of the widely held belief that inflammatory diseases are associated with a higher ratio of omega-6 to omega-3 PUFA (Schmitz and Ecker, 2008). In poultry, studies focused on feeding PUFA (omega-6 and omega-3 LCPUFA) on immune tissue fatty acid composition and immunomodulation have traditionally been based on

studies with breeder hens (Hall et al., 2007; Bautista-Ortega et al., 2009), with limited information available for the laying hen. Studies in breeder hens have shown that by modulating the maternal dietary omega-6 and omega-3 PUFA, one can alter leukotriene production in chicks which could lead to less inflammatory-related disorders (Hall et al., 2007).

2.4.3   Transfer of yolk lipid components from liver to egg yolk

The lipid components synthesized in the liver of the laying hens are transported to the ovary by lipoproteins (Shafey et al., 2003), mainly by the very-low-density lipoproteins (VLDL) (Bickerstaffe and Annison, 1969; Chapman, 1980). The latter consist of Apo B (the main structural protein of VLDL), PL, TAG, free cholesterol and cholesteryl ester (CE).

Fatty acids can be esterified with glycerol or cholesterol (Kvilekval et al., 1994). In the egg yolk, >90% of cholesterol is free while <10% is esterified (Bitman and Wood, 1980; Kuksis, 1992). Cholesteryl esters are synthesized in the liver mainly during the esterification of free cholesterol by binding with PUFA (Ng, 2012). This is mediated by lecithin:cholesterol acyltransferase (LCAT) as a key enzyme in maintaining cholesterol homeostasis and regulating its transport in the blood and returning it to the liver (Jonas, 2000; Spector and Haynes, 2007) for biliary elimination (Wu and Cohen, 2005). The chicken LCAT possesses 72.6% protein sequence identity to that of its homologue, the human LCAT (gene ID 396136 and 3931 for *G. gallus* and *H. sapiens*, respectively; NCBI, 2012). In hens, VLDL is by far the predominant cholesterol-carrying lipoprotein class; in roosters, it is HDL (Schneider, 2009). Hence, systemic cholesterol homeostasis in hens is maintained by an ApoB-specific receptor on somatic cells (different from the oocyte receptor), which expresses a similar function as the mammalian LDL receptor, in which a small amount of VLDL becomes converted to LDL (Nimpf and Schneider, 1991).

In the laying hen, TAG-rich lipoproteins serve as precursors of egg yolk lipid (Chapman, 1980). Yet, it has also been shown that dietary sources of omega-3 fatty acids decrease hepatic production of VLDL in laying hens (Vance and Vance, 1990). However, the TAG lipid fraction in the egg yolk constitutes the main source of energy for the developing embryo (Speake et al., 1998b; Sinanoglou et al., 2011). As such, yolk lipid formation depends upon a hen's capacity to initiate and sustain the assembly of VLDL. Hence, at maturity, with the onset of egg production, estrogen shifts hepatocytic lipoprotein production from generic VLDL to VLDLy (yolk targeted; Walzem et al., 1999) which is resistant to the lipolytic activity of LPL. Distinctively, VLDL (yolk targeted) also contain apoVLDL-II on the particle's surface (Schneider, 2009). While ApoB is necessary for binding to the VLDL/vitellogenin receptor, apoVLDL-II is an lipoprotein lipase (LPL) inhibitor (Schneider, 2009), to enable a high concentration of TAG in the yolk (Griffin et al., 1982). The chicken VLDL (destined for yolk, VLDLy) is similar in content to mammalian VLDL, except for the presence of apoVLDL-II (LPL inhibitor) on the surface which allows the VLDLy particles to travel from the liver to the ovary without being broken down to low density lipoprotein (LDL) (Griffin et al., 1982; Schneider, 2009). In addition, a small amount of *bona fide* high density lipoprotein (HDL) are also known to be taken up into the oocyte from the serum of the laying hen and deposited into the yolk (Vieira et al., 1995). The mechanism of yolk formation has been described in detail in an earlier reviews (Speake et al., 1998a; Schneider, 2009).

Finally, during yolk development, the major yolk components, vitellogenin and VLDL are taken up from the blood into the developing follicles (oocytes) in the ovary via receptor mediated endocytosis, and the oocyte vitellogenin/VLDL receptor (OV receptor; Walzem et al., 1999; Schneider, 2007). This receptor (OV receptor) belongs to the LDL receptor superfamily

that shows high sequence identity with the mammalian LDL receptors. It exists in the plasma membrane of the oocyte and is an essential receptor in avian species (Schneider, 2007). Unlike the major yolk precursors (VLDL and vitellogenin), the HDL particles (a *bona fide* HDL) found in yolk is not induced by estrogen, but rather via a receptor-independent mechanism such as bulk-phase uptake via the general endocytotic activity of the oocyte surface (Bujo et al., 1997). The transport and removal of lipoproteins from the blood in the laying hen for deposition in the yolk (the ovary) represents a potential regulatory point for fatty acid deposition. This is because it creates a system in which the massive transport of lipid to one organ (the ovary) coexists with regulatory mechanisms for the control of lipid homeostasis in other tissues.

Taken together, the metabolic fate of dietary EFA, such as ALA, discussed above, highlights several control factors, which in addition to other external factors (such as resource availability), discussed in the next section, can contribute to challenges in the final deposition of omega-3 PUFA in chicken eggs.

2.5 Challenges in Egg Enrichment with Omega-3 PUFA

2.5.1   Resource related factors

In Canada, a plant source that is rich in ALA and currently added to hen diets is flaxseed (Caston and Leeson, 1990). Flaxseed, an oilseed that contains approximately 30% lipid, contains an omega-6/omega-3 fatty acid ratio of 0.3:1, and is a rich source of ALA (53% of total fatty acids; Morris, 2007). Some commercially available flaxseed oils now contain 70% ALA (e.g., HiOmega® 70 Flaxseed Oil; Polar Foods, Fisher Branch, MB, Canada). Flaxseed is considered a minor crop in North America compared to other oilseeds such as canola and soybean, yet the latter two crops contain only 11 and 8% of total fatty acids in the oil as ALA, respectively (Jhala

and Hall, 2010). However, the greater usage for its direct consumption by humans may present a challenge for the future use of the same ingredient for poultry or other animal diets. In addition, its inclusion level in laying hen diets is limited to less than 10% (by wt of diet) due to issues related to negative sensory attributes in eggs at higher levels of inclusion (Scheideler et al., 2003). The applicability of other potential ingredients for inclusion in laying hen diets, such as the products of hemp (*Cannabis sativa* L.), hempseed and hempseed oil (containing 80% PUFA with an LA to ALA ratio of approximately 3 to 1; Parker et al., 2003) have recently been evaluated (Mustafa et al., 1999; Callaway, 2004; Silversides and Lefrançois, 2005; Gakhar et al., 2012; Goldberg et al., 2012). However, the usage of industrial hemp products (seeds and oil) in Canada is not currently legalized for commercial use in poultry diets due to the limited available of data on safety and efficacy.

Alternatively, as a means to enhance the omega-3 LCPUFA content of chicken eggs, the hen diet can be modified via the inclusion of preformed sources of the omega-3 LCPUFA (exogenous incorporation of LCPUFA). Such sources include EPA/DHA-rich fish/marine oils (Lawlor et al., 2010) and microalgae (as a source of EPA and/or DHA; Herber and Van Elswyk, 1996; Lemahieu et al., 2013). Unlike plant-sourced PUFA (rich in precursor ALA), when preformed sources of LCPUFA are supplemented in hen diets, a proportionate increase in egg DHA level in yolk is achieved as a function of intake (Lawlor et al., 2010; Sefer et al., 2011). The upper levels of inclusion are primarily dictated by the generation of undesirable sensory attributes. Marine/fish oil are susceptible to peroxidation and are oxidatively unstable, resulting in fishy flavours and aroma in eggs (Hargis et al., 1991; Leskanich and Noble, 1997; Gonzalez-Esquerra and Leeson, 2000). Strategies to ameliorate these undesirable effects may include limiting the inclusion levels of omega-3 fatty acid sources, the inclusion of high levels of dietary

anti-oxidants such as vitamin E and selenium, and the use of microencapsulation (Lawlor et al., 2010). Other limiting issues in the use of marine/fish oil for feeding hens relate to the concerns about potential pollutants/contaminants associated with fish products, such as methyl-mercury dioxins and polychlorinated biphenyls.

2.5.2 Enrichment of endogenously synthesized LCPUFA in eggs is limited

Although plant ingredients rich in ALA are primarily used to enrich eggs with omega-3 fatty acids, the efficiency of conversion of ALA to EPA and DHA is limited in hens (Cherian and Sim, 1991; Scheideler and Froning, 1996; Baucells et al., 2000; Gakhar et al., 2012), as it is in humans (Burdge 2006; Brenna et al., 2009). In humans, the conversion efficiencies of ALA to DHA range between 0.2–9% of ingested ALA to DHA (Pawlosky et al., 2001; Brenna, 2002; Burdge, 2004; Plourde and Cunnane, 2007).

Cruickshank (1934) was the first to report that the fatty acid profile of eggs is influenced by the content and quality of the dietary fat provided to the hens. In addition, not all dietary omega-3 fatty acid sources are biologically equivalent (Barlow et al., 1990). Hence, utilizing data adapted from previous studies for flaxseed (Scheideler and Froning, 1996; Bean and Leeson, 2003) and hempseed/oil (Gakhar et al., 2012), the levels of ALA and DHA deposited in egg yolk were compared in this review and are represented in Figure 2.4. Both plant sources of ALA, flaxseed oil (50%; Scheideler and Froning, 1996) and hempseed oil and hempseed (18% and 7%, respectively; Parker et al., 2003) showed a similar trend of ALA accumulation in the egg yolk, increasing in a dose dependent manner as a function of levels of ALA in the diets of laying hens. Although the levels of DHA incorporated into the egg yolk was higher when hen diets where supplemented with flaxseed (Scheideler and Froning, 1996; maximum at ~90mg/yolk, at 1.68% ALA obtained from 10% inclusion of ground flaxseed) compared to that
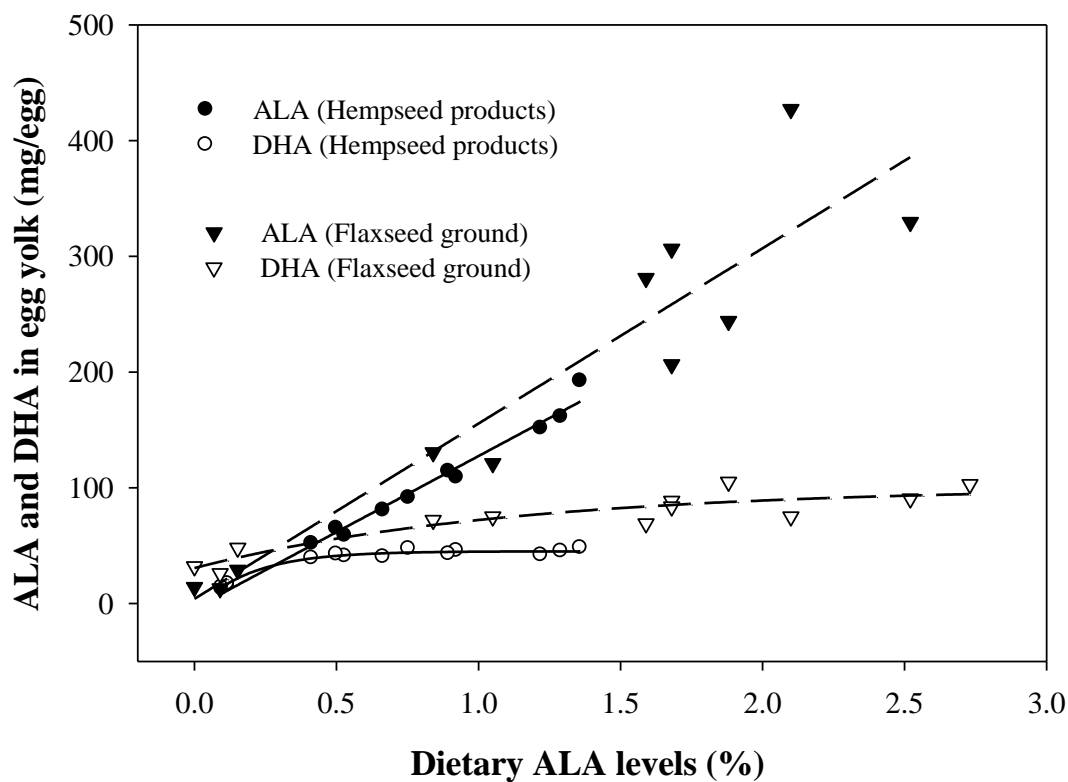
Figure 2.4. Alpha-linolenic acid (ALA) and docosahexaenoic acid (DHA) levels in egg yolk (mg/yolk) as a function of dietary ALA (% of diet).

Modeling of levels of ALA and DHA in egg yolk for laying hens consuming hempseed products (● and ○) and ground flaxseed (▼ and Δ), respectively. Data sources: flaxseed (Cherian and Sim, 1991; Aymond and Van Elswyk, 1995; Scheideler and Froning, 1996; Baucells et al., 2000; Bean and Leeson, 2003) and hempseed products (hempseed and hempseed oil; Gakhar et al., 2012). Data provided as a % of total weight were converted to mg/egg based on 5g of lipid per egg/13g yolk/50g egg.

achieved with hempseed products (Gakhar et al., 2012; maximum at ~48 mg/yolk, at 20% hempseed inclusions equivalent to 0.60% ALA in the diet), a saturation point (plateau) in the enrichment of DHA in the egg yolk was eventually reached in both cases.

In the conversion of ALA to omega-3 LCPUFA, the competition between ALA and LA for the same desaturase enzymes is widely mentioned (Sprecher, 2000; Burdge, 2004; Mennicken et al., 2005). The competition is said to occur at the level of the active sites on the Δ6-desaturase enzyme, with ALA being the preferred substrate (Sprecher, 2002). At a lower ratio of omega-6 to omega-3 PUFA the efficiency of endogenous conversion of ALA to EPA and DHA is increased (and vice versa) (Kris-Etherton et al., 2000; Burdge, 2004). Studies in our lab (Goldberg et al., 2013) used different strategies to reduce competition between ALA and LA. In one of the studies, it was observed that by increasing the ratio of SFA: LA + oleic acid, the levels of all omega-3 PUFA, including DHA were increased. The results indicate that there is a potential for enhancing the enrichment of eggs by reducing competition between dietary ALA and LA.

In other studies, involving hen strain, source and different levels of fat inclusion in laying hen diets, Grobas et al. (2001) observed the attainment of a plateau in DHA accumulation in egg yolk. The authors suggested that when using plant sources for the enrichment of eggs with DHA, only moderate amounts of ALA (less than 1% by wt of diet) in laying hen diet is required. Similar results of a plateau in the accumulation of DHA as a function of ALA intakes was observed in other studies using cell culture of human liver HepG2 cells in PL fraction (Portolesi et al., 2007), mice tissue PL (Broughton et al., 1991) and circulating plasma PL in rat studies (Gibson et al., 2013).

Hence, taken together, the inefficiency in the conversion of ALA to DHA is related to the fact that, regardless of the source of dietary ALA, DHA level in eggs or tissues, reaches a plateau, so further increases in ALA, does not translate into a similar proportional increase in the accumulation of DHA. The observed plateau in the endogenous synthesis of DHA from ALA at a high level of the substrate (precursor ALA) may be regarded as a saturation point of the desaturase activity taking place (Grobas et al., 2001). This may indicate that further conversion to longer chain metabolites at higher levels of ALA may not necessarily occur. However, it may also imply that there is a mechanism other than substrate competition between the two series of PUFA or in combination that is involved in the regulation of the conversion of ALA to DHA.

Comparing endogenous synthesis of LCPUFA versus preformed/exogenous LCPUFA in the enrichment of eggs with LCPUFA, extensive studies have demonstrated that, unlike plant sources rich in ALA the inclusion of preformed sources such as marine/fish oils permits significant enrichment (linearly, as a function of intake; Figure 2.5) of eggs with DHA (Lawlor et al., 2010). However, previous studies have also indicated that although supplementation of laying hen diets with preformed EPA and/or DHA from sources such as fish oil (Van Elswyk et al., 1995; Nitsan et al., 1999; Cachaldora et al., 2008; Lawlor et al., 2010) and microalgae (Sefer et al., 2011; Lemahieu et al., 2013; Ao et al., 2015) may significantly enrich DHA in the egg yolk, EPA is only incorporated to a much lesser extent. In addition, in earlier studies, Abril and Barclay (1998), who used 0.30 and 0.60 g/hen/day DHA from marine microalgae, observed enrichment levels of 172.8 and 243 mg/egg of total omega-3 PUFA, respectively. While in other studies, Herber and Van Elswyk (1996) supplemented hen diets with either 2.4 or 4.8% (equivalent to 2.4 and 4.8 g/hen/day, respectively) of marine microalgae *Schizochytrium sp.*
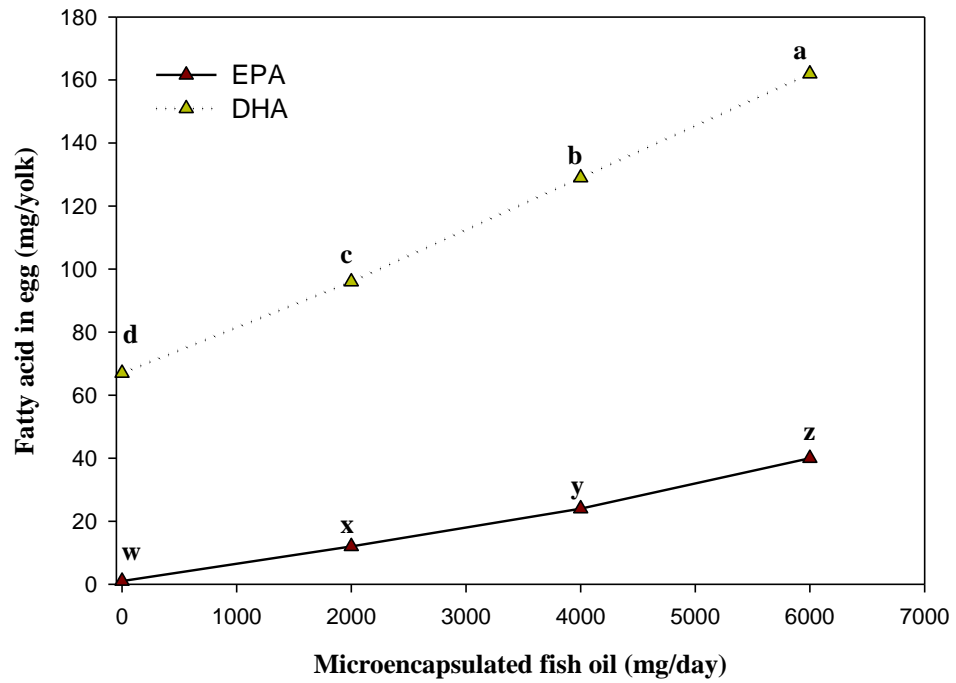
Figure 2.5. Supplementation of microencapsulated fish oil and levels of EPA and DHA enrichment in eggs.
(Data adapted from Lawlor et al., 2010).

resulted in the enrichment levels of 9.5 mg/g (~152 mg/yolk, considering yolk weight of 16 g/egg) and 11.5 mg/g (~184 mg/yolk) of omega-3 PUFA yolk, respectively. The difference between these studies may relate to differences in the composition of the background diet, in which the former contained higher levels of ALA derived from flaxseeds. The impact of background diet composition, particularly in relation to the levels of PUFA, on the accumulation levels of LCPUFA in rat tissues has been demonstrated previously (Gibson et al., 2013).

To date, the mechanisms regulating omega-3 LCPUFA enrichment of chicken eggs is not clearly understood. Extensive research has been conducted on gene regulation to explain factors associated with the rate limiting effect of the desaturase and elongase enzymes in the metabolic pathway of the LCPUFA (Hastings et al., 2001; Nakamura and Nara, 2004; Tu et al., 2010; Gakhar et al., 2012). In addition, previous studies have also indicated the lack of correlation between the levels of tissue fatty profiles and changes in the expression of mRNA of genes and/or regulatory transcription factors in rats (Tu et al., 2010) and laying ducks (Cheng et al., 2006) related to endogenous synthesis of omega-3 LCPUFA from precursor ALA. Based on the metabolic fates of ALA described in earlier section of this review, there is little information available on the distribution of fatty acids in the different lipid pools in eggs and tissues of hens in relation to dietary sources of omega-3 PUFA. Previous work focused on the fatty acid composition of total yolk lipids, without considering the fatty acid composition of the different lipid classes. Hence, detailed information of distribution of the fatty acids in the different lipid pools in eggs in relation to other tissues of the hen may elucidate potential control points in the chicken that relates to the mechanisms regulating the enrichment of omega-3 LCPUFA in chicken eggs. The impetus for this research comes at a time when a new health claim for DHA and EPA on TAG lowering effects has been permitted in Canada with the goal for eggs to reach

the required amount per serving (500 mg) of DHA and EPA, while retaining sensory properties.

Hence, identifying potential factor(s) in the regulation of endogenous synthesis of omega-3

longer chain metabolites derived from precursor ALA and their enrichment in chicken eggs

would be valuable information for the egg industry.

CHAPTER 3 HYPOTHESIS AND OBJECTIVES

Hypotheses:

- Amount and distribution of polyunsaturated fatty acids (PUFA) in the different lipid classes (neutral and polar lipids) of different tissues provide a basis in the regulation of omega-3 long chain PUFA (LCPUFA) metabolism.

- The level of omega-3 LCPUFA (particularly docosahexaenoic acid, DHA) enrichment of eggs is limited by the phospholipid (PL) pool of the egg.

Objectives

The main aim of this research was to identify potential factor(s) that regulate the enrichment of endogenously synthesized omega-3 LCPUFA (particularly DHA) in chicken eggs. In addition, the research aimed to evaluate, concurrently, potential plant (terrestrial) source of omega-3 PUFA (primarily, ALA) for use in laying hen diets. These were achieved through the following objectives:

- To characterize the fatty acid profiles in total and individual lipid classes (neutral and polar lipids) in the egg yolk, liver, plasma and adipose in laying hens supplemented with

  - Novel plant sources of precursor ALA from hempseed and hempseed oil.

  - ALA-rich plant source (flaxseed oil) and preformed LCPUFA (algal DHA), aimed at comparing differences in the enrichment efficiency between the two sources.

- To determine the mRNA expression of key hepatic genes and transcription factor involved in synthesis, β-oxidation and activation of fatty acids to further elucidate differences in the metabolism between endogenous versus exogenous (preformed) omega-3 LCPUFA and enrichment efficiency in eggs.

- To evaluate hen performance indices and plasma chemistry to determine safety and efficacy of utilizing hempseed and oil (dietary sources of ALA) in laying hen diets.

CHAPTER 4 MANUSCRIPT I

Performance, egg quality and blood plasma chemistry of laying hens fed hempseed and

hempseed oil

4.1 ABSTRACT

The aim of this study was to compare the performance of hens (feed intake, rate of lay, egg

weight and body weight gain), egg quality and blood plasma biochemical variables (enzymes,

electrolytes, proteins and other plasma constituents) of laying hens fed diets containing hemp

products. Forty-eight Lohmann LSL-Classic (white-egg layers) (19 weeks of age) were

individually caged and fed one of 6 wheat-barley-soybean-based diets for a period of 12 weeks.

The diets consisted of either hempseed (HS; 10, 20 or 30%) or hempseed oil (HO; 4.5 or 9.0%)

or a control diet (corn oil-based). All diets were formulated to contain similar levels of crude fat

(11%), energy (2800 kcal/kg) and crude protein (17%). Data were analyzed as a Completely

Randomized Design using the repeated measure analysis of the Proc Mixed procedure of SAS.

The results indicated that the inclusion of up to 30 and 9.0% HS and HO, respectively, to diets of

laying hens had no significant effects on hen performance, egg quality or plasma level of

metabolites (proteins, glucose, uric acid and cholesterol) and electrolytes (Na, K, Cl, P and Ca).

Overall plasma enzyme concentrations, particularly gamma-glutamyl transferase, was

significantly ($P < 0.01$) lowest at the 10 and 20% levels of HS inclusion, or at the 4.5% of HO

levels of inclusion of the hempseed products in laying hen diets compared to the higher levels or

control fed hens. Similar effects were also observed for plasma aspartate aminotransferase levels

but with the HS enriched diets only ($P < 0.05$), particularly being lowest at the inclusion levels of

10 and 20% HS compared to the control. The results may imply a possible protective effect of

HS and HO containing diets, particularly at 10% HS, 20% HS and 4.5% HO levels, on liver

damage/injury. In summary, both HO and HS appear to be well tolerated by laying hens,

without affecting hen performance and liver function (health) as judged by markers of plasma

clinical chemistry, supporting the safety and efficacy of hemp products for use in laying hen rations.

Key words: laying hen, hemp product, performance, plasma chemistry

4.2 INTRODUCTION

Since 1998, the commercial production of industrial hemp (*Cannabis sativa L.*) has proceeded in Canada under strict licensing requirements (Health Canada, 2012). Despite their current, albeit limited, availability, hemp products, including whole hempseed, hempseed oil, and hempseed meal/cake are not approved for use in commercial feeds for any class of livestock in Canada. Evidence for the safety and efficacy of these products are required by regulatory agencies in order to approve their use. Industrial hemp is bred to contain low levels ($< 0.3\%$) of the psychoactive substance delta-9-tetrahydrocannabinol (THC), and this fact has renewed interest in the quality of the seed and seed products as dietary sources of protein and energy for animals, including poultry.

With respect to its nutritive value, whole hempseed contains 24% crude protein and 30% crude fat (House et al., 2010). The fecal digestibility of the protein in hempseed, dehulled hempseed, and hempseed meal, as measured in a rat bioassay, was determined to be between 85 and 96%, with the neutral detergent fiber (hull) fraction having a suppressive effect on digestibility values. With respect to protein quality, lysine is the first limiting amino acid in hemp proteins for most animals (House et al., 2010). The formulation of laying hen rations with hempseed meal, at up to 20% of the diet, provided evidence that the protein and energy associated with the meal could support production performance of laying hens over a 4-week study period (Silversides and Lefrançois, 2005). More recently, we have documented the efficacy of whole hempseed and hempseed oil in providing protein/ energy in support of egg production in Bovan White laying hens after 12 weeks of feeding (Gakhar et al., 2012). Hempseed oil is relatively rich in the n-3 fatty acid, ALA (17% of total fatty acids; Gakhar et al., 2012). Given the high ALA content of hemp oil, we documented increases in both ALA and the

long chain omega 3 fatty acid DHA in eggs, as a function of increased hempseed and hempseed oil inclusion (Gakhar et al., 2012), without impacts on the overall sensory qualities of the eggs (Goldberg et al., 2012). While data exists to support the efficacy of hemp products in maintaining egg production, additional data is required in support of the safety of these products for the hen particularly at higher inclusion levels.

In chickens, the liver is the principal site of lipid metabolism (Hermier, 1997), and due to portomicron absorption (Bensadoun and Rothfeld, 1972), the liver is exposed to fat that reflects dietary origin. Hemp products contain high content of unsaturated lipids, making them susceptible to oxidation (Leskanich and Noble, 1997). Lipid peroxidation of hepatocyte and organelle membranes can result in oxidative damage, increasing the susceptibility of laying hens to hepatic diseases, including fatty liver haemorrhagic syndrome (Squires and Leeson, 1988; Schumann et al., 2003). Given the key role of the liver in lipid metabolism, it is therefore vital to include assessments of hepatic and metabolic health in the face of consuming novel dietary lipids. Concentrations of biochemical variables measured in either the serum or plasma, including aspartate aminotransferase (AST) and gamma glutamyl transferase (GGT), are often used for assessing liver function in chickens (Fernandez et al., 1994; Diaz et al., 1999), and may be associated with liver damage in birds (Hochleithner, 1994). Although, the amount and type of fat in diets of hens is known to improve the digestibility of other dietary components (Mateos and Sell, 1980), changes in the content of plasma nitrogenous compounds correlate closely with the metabolic changes in the body, particularly before peak egg production (Gyenis et al., 2006). In addition, changes in hepatic function can influence the metabolism of minerals, including calcium (Ca) and phosphorus (P) necessary for skeletal integrity and eggshell quality (Jiang et al., 2013). The novelty of this paper lies in the following aspects i) assessing the impact

of higher levels of inclusion of hemp-derived products, particularly intact hempseed, in laying

hen diets and ii) evaluating indices of blood chemistry in relation to overall production

performance and egg quality measures; these aspects have not been reported in earlier studies.

Hence, the results from this study will provide data to help support or refute efficacy and safety

claims for hemp products as feed ingredients in laying hen diets. However, possible presence of

THC or its metabolite residues in eggs and other poultry products may also require further

investigation, to determine the safety for human consumption.

4.3 MATERIALS AND METHODS

4.3.1 *Birds and housing*

A total of forty-eight Lohmann LSL-Classic (white-egg layers) (19 weeks of age) were

individually placed in conventional-type cages that provided a floor space of 1,032 cm^2 per hen

and a perch. Hens were housed under semi-controlled environmental conditions whereby, the

heating/ventilating control system depended on the temperature of the inflow air and recorded

based on average temperature. During the period of study (winter climates), the temperature and

relative humidity in the barn were on average $21.6 \pm 1.06^{o}C$ and $23.3 \pm 1.53\%$, respectively. In

addition, the hens were exposed to a 16-h photoperiod for a period of 12 weeks. The hens were

allowed a period of 2 weeks to adapt to their individual cages. During the first week of

adaptation, hens were fed a commercial layer diet, and then transitioned to a 50:50 blend of the

commercial and test diets in the second week. Feed and water were available to permit *ad*

libitum consumption. All management procedures used during this study were in accordance

with recommendations established by the Canadian Council on Animal Care (1993) which were

reviewed and approved by the University of Manitoba's Animal Care Protocol Management and

Review Committee.

4.3.2 *Diets and experimental approach*

Six wheat-barley-soybean-based diets were formulated to meet all (except the level of essential fatty acids) nutrient specifications for Lohmann LSL-Classic hens within Phase 1 of the production cycle (50% egg lay to 45 weeks of age) consuming 105 to 115 g/hen/day feed, as indicated in strains' layer management guide. Although the recommended daily hen requirement level of LA for Lohmann LSL-Classic laying hens in Phase 1 is 2.0% of the diet (Layer Management Guide, LOHMANN LSL-CLASSIC), the levels in the diets of the current study were higher because the diets were formulated to accommodate the highest inclusion level of HS (30%) and HO (9.0%) being tested which consequently contain higher levels of total fat (11% of the diet). However, the LA content in all diets was formulated to be similar (in the range of 3.87 $\pm$ 0.15%). Information on the diet formulations and treatment regimens are presented in Table 4.1. The HS and HO used in the formulation of the diets were sourced through Hemp Oil Canada, St. Agathe, MB, Canada. Although the chemical composition of the HO (processes oil) used in this study was similar to those previously used in the same lab (Gakhar et al., 2012), the chemical profile of the HS for this study is indicated in Table 4.2. For a homogeneous mix in the diets, the hempseeds (whole intact seeds) were first mixed with wheat grains (coarse particle size) in a ratio of 50:50 (based on the highest inclusion level of HS to wheat ratio), and then milled using a small hammer-mill before the diets were formulated. The diets consisted of a control (corn oil-based), hempseed (HS; 10, 20 or 30% by weight) or hempseed oil (HO; 4.5 or 9.0% by weight), with diets formulated to be isocaloric (AME$_n$), isonitrogenous and isolipidic (energy, 2800 kcal/kg; CP, 17% and crude fat, 11% by weight). Due to the high PUFA content of the diets, additional vitamin E (total of 150 IU/kg diet) was included in the diet, along with synthetic antioxidants (Table 4.1). Diets were mixed immediately before the commencement of

Table 4.1: Formulation and nutrient composition of diets containing hempseed (HS) and hempseed oil (HO) compared to the control diet

	Control	10% HS	20% HS	30% HS	4.5% HO	9.0%HO
Ingredients (%)						
Wheat	34.99	33.43	31.87	30.31	34.99	34.99
Barley	15.00	15.00	15.00	15.00	15.00	15.00
Soybean Meal	21.53	16.90	12.24	7.59	21.53	21.53
Hemp Seed	0.00	10.00	20.00	30.00	0.00	0.00
Hemp Oil	0.00	0.00	0.00	0.00	4.50	9.00
Corn Oil	9.68	6.48	3.28	0.07	5.18	0.68
Vitamin-mineral premix[1]	2.50	2.50	2.50	2.50	2.50	2.50
Limestone	13.86	13.53	12.96	12.39	13.86	13.86
Dicalcium Phosphate	1.88	1.60	1.56	1.52	1.88	1.88
Salt	0.34	0.35	0.35	0.35	0.34	0.34
DL-Methionine	0.116	0.095	0.073	0.051	0.116	0.116
L-Lysine-HCl	0.083	0.128	0.173	0.218	0.083	0.083
L-Threonine	0.005	0.000	0.000	0.000	0.005	0.005
Calculated nutrient contents						
AMEn (Kcal/kg)	2800	2800	2800	2800	2800	2800
CP (%)	17	17	17	17	17	17
Crude fat (%)	11.00	11.00	11.00	11.00	11.00	11.00
Calcium (%)	5.48	5.30	5.09	4.87	5.48	5.48
Total Phosphorus (%)	0.70	0.72	0.80	0.87	0.70	0.70
Available Phosphorus (%)	0.50	0.45	0.45	0.45	0.50	0.50
Sodium (%)	0.16	0.16	0.16	0.16	0.16	0.16
Chloride (%)	0.26	0.26	0.25	0.25	0.26	0.26
Methionine	0.36	0.36	0.36	0.36	0.36	0.36
Total Lysine (%)	0.85	0.85	0.85	0.85	0.85	0.85
Threonine	0.60	0.60	0.61	0.62	0.60	0.60
Analyzed nutrient contents[2]						
DM (%)	91.9 ± 0.21	91.8 ± 0.25	91.9 ± 0.21	91.9 ± 0.30	92.1 ±0.36	91.90± 0.30
Energy (GE, kcal/kg)	3805 ± 19.9	3848 ± 22.8	3848 ± 14.2	3873± 32.1	3806 ± 13.9	3797 ± 2.55
CP (%)	16.9 ± 0.58	17.8 ± 0.35	17.3 ± 0.37	16.8 ± 1.67	17.0 ± 0.72	17.7 ± 0.50
Crude fat (%)	11.6 ± 0.62	12.7 ± 0.90	12.5 ± 0.02	12.5 ± 0.44	12.0 ± 0.01	11.3 ± 0.28
Calcium (%)	5.65 ± 0.43	5.64 ± 0.41	5.29 ± 0.38	5.93 ± 1.04	5.50 ± 0.81	5.73 ± 0.15
Total Phosphorus (%)	0.75 ± 0.03	0.75 ± 0.07	0.80 ± 0.01	0.86 ± 0.04	0.70 ± 0.04	0.75 ± 0.01
Potassium (%)	0.30 ± 0.02	0.30 ± 0.02	0.27 ± 0.01	0.24 ± 0.02	0.30 ± 0.01	0.30 ± 0.03
Sodium (%)	0.35 ± 0.00	0.37 ± 0.07	0.37 ± 0.00	0.37 ± 0.06	0.30 ± 0.09	0.36 ± 0.04
ADF (%)	2.91	5.45	7.38	8.83	2.84	3.27
NDF (%)	14.25	16.80	18.23	21.63	11.83	13.32
Linoleic acid (LA, %)	3.81	3.92	3.86	3.63	3.90	4.09
Alpha-linolenic acid (ALA, %)	0.10	0.50	0.90	1.22	0.66	1.28
Ratio (LA to ALA)	39.8	7.83	4.32	2.99	5.87	3.18

[1]Provided per kilogram of diet, vitamin-mineral premix contained: 11,000 IU of vitamin A; 3,000 IU of vitamin D_3, 150 IU of vitamin E, 3 mg of vitamin K_3 (as menadione), 0.02 mg of vitamin B_{12}, 0.2 mg of biotin, 6.5 mg of riboflavin, 4 mg of folic acid, 10 mg of calcium pantothenate, 39.9 mg of niacin, 2.2 mg of thiamine, 4.5 mg of pyridoxine, 1000 mg of choline chloride, 125mg antioxidant (ethoxyquin), 66 mg of manganese oxide, 70 mg of zinc oxide, 80 mg of ferrous sulfate, 10 mg of copper sulfate, 0.3 mg of sodium selenite, 0.4 mg of calcium iodate, 0.67 mg of sodium chloride (salt).
[2] Mean values ± standard deviation (SD) for duplicate analysis per sample

Table 4.2: Chemical composition of the hempseed used during the study

Composition	Hempseed
Provided by supplier[1](dry matter basis)	
DM (%)	93.8
AMEn (poultry; kcal/kg)	2521
CP (%)	37.8
Crude fibre (%)	29.2
Ash (%)	6.24
ADF[2] (%)	35.8
NDF[2] (%)	46.0
Analyzed[2](as-is basis)	
DM (%)	92.5 ± 0.03
GE (kcal/kg)	5780 ± 20.1
Crude fat (%)	32.8 ± 1.05
CP (%)	23.5 ± 0.31

[1]Supplier provided information, as per certificate of analysis.
[2]ADF = acid detergent fiber; NDF = neutral detergent fiber.
[3]Analyzed values ± SD for duplicate analysis per sample.

the study, and stored in a cool, dry storage room. Sub-samples of each diet (150g) were ground

and analyzed for dry matter (DM) following the method 925.09 (AOAC, 1990), gross energy

(GE) using a Parr adiabatic oxygen bomb calorimeter (Parr Instrument Co., Moline, IL), crude

fat (CF) following method 920.39 (AOAC, 1990), and nitrogen (N) using a Leco analyzer (NS-

2000, Corp., St. Joseph, MI). Dietary mineral levels were determined by inductively coupled

plasma mass spectrometry (Varian Inc., Palo Alto, CA) after samples were ashed for 12 h and

digested according to AOAC (1990) procedures (method 990.08).

4.3.3 *Hen performance*

At the end of each week, total feed consumption was determined as the difference

between feed offered and residual feed remaining in feeders. Egg weight and egg production

were recorded on a daily basis, and hen body weights were measured weekly. Hen performance

variables were expressed as averages over the corresponding study week and average body

weight gain was calculated as the difference between consecutive weekly weight measurements.

4.3.4 *Egg quality measurements*

Egg yolk weight and eggshell measures (eggshell weight and thickness) were recorded on

week 4, 8 and 12 (from eggs collected for 3 consecutive days within each period). Eggs were

broken to determine yolk weight using a digital scale. Thereafter, the shells of the same eggs

were washed with the eggshell membrane intact and allowed to dry at room temperature for 2

days before final weight and thickness measurements. Egg shell weights obtained during periods

8 and 12 were expressed per unit of surface area to obtain the shell index as described by

Sauveur (1988). Shell thickness was determined using a thickness gauge micrometer (B. C.

AMES Co., Waltham, MA) on 3 replicate shell chips obtained from areas along the equator of

the egg. The obtained thickness values (in thousandths of an inch) were converted into

micrometers by multiplying by 25.4. Egg specific gravity and Haugh unit scores (an index of albumen quality) were determined as previously described (Samli et al., 2005).

4.3.5 Plasma clinical chemistry

On week 6 and 12, blood samples were obtained from the wing vein using heparin-treated syringes then immediately transferred into lithium heparinized vacutainers. The samples were kept in an ice box and transferred to the Manitoba Veterinary Services Laboratory (Winnipeg, Manitoba, Canada) for further assays and analysis using an automated analyzer (Cell-Dyn 3500 System; Abbot Laboratories Abot Park, IL). The plasma chemistry analysis included enzymology tests for creatine kinase (CK), aspartate amino-transferase (AST) and gamma glutamyl-transferase (GGT); electrolytes (sodium, Na; potassium, K; chloride, Cl; phosphorus, P; and calcium, Ca); and plasma proteins (total protein, albumin and globulin) and other plasma constituents (uric acid, glucose and cholesterol).

4.3.6 Statistical analysis

All data were analyzed as a Completely Randomized Design with the individual hen as the experimental unit. The HS (control (0), 10, 20 and 30% levels) and HO (control (0), 4.5 and 9.0% levels) treatments were analyzed as 2 separate experiments/trials, since the biological efficiency in the utilization of the seeds vs. the oils by the hens would be expected to differ. The same data for the control diet was applied in either treatment group. Apart from the data for cholesterol, all other data were used in the repeated measure analysis of Proc Mixed procedure of SAS according to the following model:

$$Y_{ijk} = \mu + d_i + h_{ij} + w_k + dw_{ik} + e_{ijk};$$

where μ = overall mean, d_i = fixed effect of diet (i = 1 to 3 for HS containing diets and i = 1 to 2 for HO containing diets), h_{ij} = random effect of hen within diet (treatment) (j = 1 to 8, number of

hens per treatment), w_k = fixed effect of week (k = 1 to 12, for production performance; k = 1 to 3, for egg quality measures; k = 1 to 2, for plasma chemistry) and dw_{jk} = interaction between diet and week (treatment × period), e_{ijk} = random error variation (residual error). Treatment (diet) by week interactions were considered as fixed effects. The data for cholesterol was based on week 12 only and was analyzed using the Proc GLM procedure of SAS. In all analyses, least square means (LSM) were compared using Tukey's procedure after analysis of variance and the significance levels were based on $P < 0.05$. Feed intake, the average of week 6 and 12, was used as a covariate in the analysis of all plasma chemistry, except for cholesterol (feed intake of week 12 only applied). Data points with studentized residuals below or above 3.0 were considered outliers and excluded from the analysis.

4.4 RESULTS

4.4.1 Hen performance

The inclusion of HS or HO in laying hen diets did not significantly affect hen performance variables. In both treatment groups, there was a significant ($P < 0.0001$) week effect for feed consumption, increasing at week 1 and leveling off after week 6 (Table 4.3; individual week data shown only for week 1, 4, 6, 8 and 12). An increase in body weight gain (BWG) was noted in week 1, but decreased in subsequent weeks, reflective of the trend in feed intake, possibly due to hens adapting to diets. However, positive weight gains were achieved earlier for the hens consuming the HS diets than those on the HO treatments and maintained to the end of the experiment. The rate of egg lay increased to more than 90% of production by week 6 to 7, and remained relatively constant through to the end of the study. Egg weight increased substantially from week 1 to 4, steadily increased to week 6 (for HS) and week 8 (for HO)

Table 4.3: Overall performance of Lohmann LSL-Classic hens (21 to 33 weeks of age) fed diets containing hempseed (HS) or hempseed oil (HO)[1]

	Feed intake (g/hen/day)	BWG (g/week)	Rate of egg lay (%)	Egg weight (g/egg)
HS diets				
Effect of diet				
Control	96.1	14.9	97.3	57.9
30% HS	99.9	9.83	97.2	57.0
20% HS	98.0	14.0	98.5	57.0
10% HS	96.1	9.79	97.5	54.2
SEM	1.69	1.79	0.79	1.02
Effect of week (wk)				
wk 1	92.8^b	18.2^a	93.7^b	50.6^c
wk 4	96.2^{ab}	-9.69^b	98.2^{ab}	55.9^b
wk 6	97.6^a	4.69^{ab}	98.7^a	56.4^b
wk 8	98.5^a	10.0^{ab}	97.8^{ab}	58.0^a
wk 12	97.7^a	26.6^a	96.0^{ab}	59.5^a
SEM	1.13	5.60	1.07	0.60
P-values[2]				
Diet	0.29	0.10	0.61	0.082
week	< 0.0001	< 0.0001	< 0.01	< 0.0001
Diet x week	0.079	0.70	0.52	< 0.001
HO diets				
Effect of diet				
Control	96.1	14.9	97.3	57.9
9.0% HO	93.0	16.6	97.4	56.2
4.5% HO	95.4	12.4	98.4	55.8
SEM	1.41	1.59	0.92	1.12
Effect of week (wk)				
wk 1	92.6^b	23.3^a	94.7	50.5^c
wk 4	93.3^b	-5.42^b	96.4	55.8^b
wk 6	96.1^a	-5.42^b	99.4	57.1^{ab}
wk 8	96.8^a	8.75^{ab}	99.8	58.3^a
wk 12	96.2^a	27.0^a	96.4	58.8^a
SEM	1.23	5.96	1.29	0.72
P-values[2]				
Diet	0.29	0.20	0.66	0.37
week	< 0.0001	< 0.0001	0.17	< 0.0001
Diet x week	< 0.01	0.60	0.31	0.84

[a-c]Means with different superscripts within each variable, for either HS or HO treatments, are significantly different at $P < 0.05$ (n = 8 per treatment).
[1]Data are presented as least square means and their SEM.

treatments and remained constant thereafter. Hens consuming the 30% HS had a significantly (P < 0.001; Figure 4.1a) lower egg weight compared to those consuming the control or the lower levels of HS (10 or 20%) between week 3 and 8; after which, egg weights were similar between treatment. On the other hand, a significant (P < 0.01) diet by week interaction in feed intake between treatments was observed in weeks 2 and 3, in which hens consuming the 4.5% (P < 0.0001) and 9.0% (P < 0.05) level of HO had lower intakes relative to those fed the control diet (Figure 4.1b). However, by week 3 there was a substantial increase in feed intake by both groups of hens, similar to those in the control, with no marked differences in feed intake between the three groups of hens to the end of the experiment.

The length of the assay period, in the current study (week 21 to 33), included periods of increasing egg production and rising feed intakes (Layer Management Guide, Lohmann LSL-Classic). Although Lohmann LSL-Classic hens are expected to start a phase-feeding program at 28 weeks, all experimental hens were already in lay and hence started on the experimental layer diets (Phase 1 diets, Table 4.1), depending on the level of production rather than by age. Hence, the observed interactions (feed intake and egg weight) and the age effects on the performance parameters tested, particularly, in the early stages of the production cycle may be related to the degree of development of the digestive tract in relation to nutrient supply.

4.4.2 *Egg yolk weight, Haugh unit score and eggshell quality measurements*

Egg quality measurements including egg yolk weights, Haugh unit scores and eggshell (specific gravity, eggshell weight and eggshell thickness) were not influenced by the inclusion of either HS or HO to diets of the laying hens (data not shown in Tables). For the HS treatment groups (control, 10, 20 or 30% HS), the corresponding variables measured were, egg yolk weight: 14.8, 14.8, 14.1, 14.2 ± 0.33 g; Haugh unit: 91.9, 93.4, 94.0, 94.8 ±1.31; specific gravity
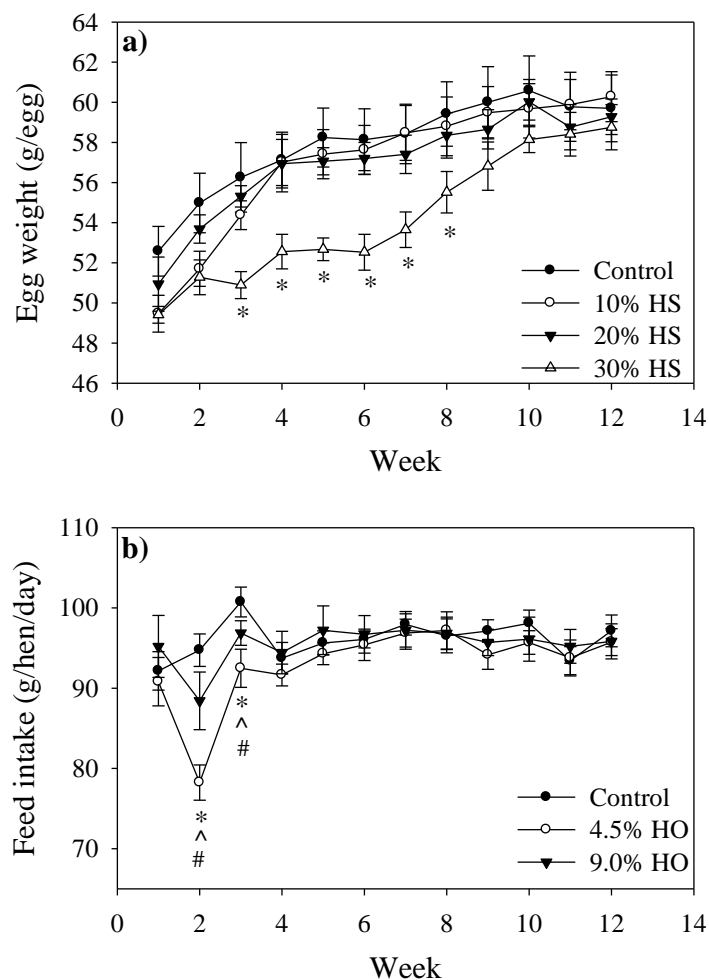
Figure 4.1. Diet by week interaction effects of a) egg weight (g/egg) and b) feed intake (g/hen/day) of Lohmann LSL-Classic hens (21 to 33 weeks of age) consuming diets containing hempseed (HS) and hempseed oil (HO), respectively.

Data points are group means ± standard error (SE) (n = 8 per treatment). *, ^ and # denote significant ($P < 0.05$) differences for either 4.1a) the 30% HS from its counterpart treatments or 4.1b) 4.5% HO from the control diet; 9.0% HO from the control diet; and differences between the two HO treatments, respectively.

(absolute): 1.089, 1.088, 1.089, 1.089 ± 0.001; shell index: 8.16, 7.93, 7.99, 7.83 ± 0.11

g/100cm^2 ; and eggshell thickness: 394, 388, 394, 386 ± 4.95 microns; respectively. Similarly,

for the HO groups (control, 4.5 or 9.0% HO), the measurements were egg yolk weight: 14.8,

14.1, 14.6 ± 0.25 g; Haugh unit: 91.9, 91.6, 92.3 ±1.51; specific gravity (absolute): 1.0890,

1.0883, 1.0885 ± 0.0007; shell index: 8.16, 8.01, 7.95 ± 0.13 g/100cm^2; and eggshell thickness:

394, 393, 387 ±5.57 microns, respectively. Although there were no significant diet by week

interactions, the significant main effects of week for egg yolk weights ($P < 0.0001$), Haugh unit

scores ($P < 0.0001$), shell index ($P < 0.0001$) and eggshell thickness ($P < 0.01$) indicated a

change over time along a pattern that would be expected as hens age. In both treatment groups,

as the egg yolk weight increased with age, Haugh unit, shell index and eggshell thickness

decreased; however, the latter was only evident in the last period (significantly higher in week 4

or 8 compared week 12 (i.e. HS: 393 and 393 vs. 385 ± 2.90; $P < 0.01$) and HO: 397 and 393 vs.

384 ± 3.46; $P < 0.0001$), respectively.

4.4.3   Plasma enzymes and electrolytes

Analyzed plasma enzyme profiles are presented in Table 4.4 (HS) and Table 4.5 (HO).

Overall, the addition of either HS or HO to the diets of the laying hens had no effect on plasma

levels of CK, although in both cases, significantly higher ($P < 0.0001$) plasma CK activity was

observed in week 12 versus week 6. The inclusion of HS in diets of the laying hens had a

significant quadratic effect in the levels of AST ($P < 0.01$ and $P < 0.05$) and GGT ($P < 0.001$ and

$P < 0.0904$; a tendency in the latter) in both week 6 and 12, respectively, with lower levels being

observed for hens consuming diet with the 10 and 20% HS levels versus the control or the 30%

level. A linear effect ($P < 0.01$) due to HS inclusion was also evident for AST levels in the

plasma in week 12, whereby lower levels of the enzyme were noted in the HS fed hens compared

Table 4.4: Plasma enzymes (creatine kinase, CK; aspartate aminotransferase, AST; gamma glutamyl-transferase, GGT) and electrolytes (Na, K, Cl, P and Ca) of laying hens fed diets containing hempseed (HS)[1]

Variables	Week (wk)	Main effect of diet					Main effect of week			P-values[2,3]		
		Control	10% HS	20% HS	30% HS	SE	wk6	wk12	SE	Diet	Week	Diet x week
Enzymes (U/L)												
CK	6	378	385	372	343							
	12	506	446	425	425							
	overall	442	415	399	384	34.4	370	450	19.2	0.68	< 0.0001	0.42
AST	6	177	159	165	190[*]							
	12	184	164	161	163[#]							
	overall	180[a]	162[b]	163[b]	176[ab]	5.10	173	168	2.97	< 0.05	0.15	< 0.01
GGT	6	39.1	36.6	33.9	41.7[*]							
	12	40.0	36.6	36.5	38.5[#]							
	overall	39.6[ab]	36.6[bc]	35.2[c]	40.1[a]	1.05	37.8	37.9	0.63	< 0.01	0.92	< 0.05
Electrolytes (mmol/L)												
Na	6	151	149	149	151							
	12	156	152	153	152							
	overall	154	150	151	151	1.17	150	153	0.66	0.22	< 0.0001	0.21
K	6	4.89	4.79	4.73	4.62							
	12	4.83	4.78	4.73	4.67							
	overall	4.86	4.78	4.73	4.64	0.06	4.76	4.75	0.04	0.13	0.91	0.92
Cl	6	123	121	122	122							
	12	122	121	121	121							
	overall	122	121	122	122	0.92	121.9	121.2	0.48	0.71	< 0.05	0.15
P	6	1.85	1.81	1.92	1.68							
	12	2.14	2.18	2.04	2.01							
	overall	1.99	1.99	1.98	1.85	0.12	1.81	2.09	0.07	0.79	< 0.001	0.60
Ca	6	6.51	6.23	6.69	5.15[#]							
	12	6.97	6.71	6.90	6.80[*]							
	overall	6.74	6.47	6.79	5.97	0.29	6.14	6.84	0.17	0.21	< 0.001	< 0.05

[1]Data are presented as least square means (LSM) ± standard errors of means (SE) (n = 8 per treatment).
[2]Means with different superscript letters within each variable are significantly different at $P < 0.05$.
[3]Means with different superscript symbols [#], [*] denote significant differences ($P < 0.05$) in each treatment group (within a column), comparison of diet by week interaction.

Table 4.5: Plasma enzymes (creatine kinase, CK; aspartate aminotransferase, AST; gamma glutamyl-transferase, GGT) and electrolytes (Na, K, Cl, P and Ca) of laying hens fed diets containing hempseed oil (HO)[1]

Variables	Week (wk)	Main effect of diets				Main effect of week			P-values[2]		
		Control	4.5% HO	9.0% HO	SE	wk6	wk12	SE	Diet	Week	Diet x week
Enzymes (U/L)											
CK	6	369	404	355							
	12	496	540	477							
	overall	432	472	416	35.1	376	504	23.3	0.53	< 0.0001	0.97
AST	6	176	169	166							
	12	184	169	167							
	overall	180	169	167	5.12	170	173	3.33	0.17	0.3677	0.58
GGT	6	39.4	33.0	38.0							
	12	40.3	32.8	37.7							
	overall	39.8[a]	32.9[b]	37.9[a]	1.30	36.8	37.0	0.81	< 0.01	0.80	0.71
Electrolytes (mmol/L)											
Na	6	151	149	151							
	12	156	151	153							
	overall	153	150	152	1.42	150	153	0.91	0.30	< 0.01	0.30
K	6	4.89	4.76	4.9							
	12	4.83	4.48	4.85							
	overall	4.86[a]	4.62[b]	4.88[a]	0.07	4.85	4.72	0.05	< 0.05	0.067	0.33
Cl	6	123	121	123							
	12	122	121	122							
	overall	122	121	122	1.03	122	121	0.64	0.58	0.097	0.87
P	6	1.83	1.90	1.93							
	12	2.13	2.07	2.21							
	overall	1.98	1.98	2.07	0.10	1.89	2.13	0.06	0.78	< 0.001	0.70
Ca	6	6.50	6.22	6.60							
	12	6.96	6.68	6.98							
	overall	6.73	6.45	6.79	0.23	6.44	6.87	0.15	0.55	< 0.01	0.97

[1]Data are presented as least square means (LSM) ± standard errors of means (SE) (n = 8 per treatment).
[2]Means with different superscript letters within each variable are significantly different at $P < 0.05$.

to the control group. Overall, significantly lower ($P < 0.05$) AST and ($P < 0.01$) GGT plasma

levels for the hens consuming diets containing 10 or 20% HS relative to control or the 30% HS

treatment groups (Table 4.4) were observed. Significant diet by week interactions were noted for

plasma AST ($P < 0.01$) and GGT ($P < 0.05$) in hens consuming the HS diets, reflecting a

decrease in the levels of these enzymes in week 12 compared to that in week 6 (163 vs. 190 and

38.5 vs. 41.7 U/L; for AST and GGT, respectively). The interaction effects were mainly due to

the inclusion of the 30% HS that resulted in a significant reduction in the levels of both AST (P

< 0.01) and GGT ($P < 0.05$) in week 12 versus week 6 compared to the control or the 10 and

20% HS levels. The inclusion of HO in the laying hen diets did not affect the plasma AST

levels, however plasma GGT levels of hens consuming the 4.5% HO diet were significantly

lower ($P < 0.01$) compared to levels found in hens consuming either the control or the 9.0% HO

diets (Table 4.5). This was supported by the significant ($P < 0.01$) quadratic responses observed

in both week 6 and 12 in the levels of plasma GGT due to the inclusion of HO. Feed intake was

used as a covariate in the analysis of the plasma chemistry variables of the laying hens, and

significant effects ($P < 0.05$; data not presented in Tables) were observed only for the plasma

GGT levels in hens consuming the HS, but not the HO diet.

There was no significant main effect of the inclusion of HS in the laying hen diet on the

plasma levels of Na, K, Cl, P and Ca (Table 4.4). However, a significant week effect on plasma

ions levels was noted for all analyzed ions except K. While plasma levels of Na increased, the

levels of Cl ions decreased in week 12 than in week 6. In addition, both macro-minerals (Ca and

P), were observed to increase with age. A significant ($P < 0.05$) week by diet interaction in

plasma Ca levels is explained by the increase over time of plasma Ca levels due to the inclusion

of the 30% level of HS ($P < 0.01$) in the laying hen diets; 5.15 vs. 6.80 mmol/L for week 6 and

12, respectively. Similar to the results with the HS diets, plasma levels of Na, Cl, P and Ca ions were not influenced by the inclusion of HO in the diets of the laying hens (Table 4.5). However, the level of plasma K showed a significant ($P < 0.01$) quadratic response in week 12 due to the inclusion of HO, which overall, was significantly lower ($P < 0.05$) in hens consuming the 4.5 % HO diet, compared to those consuming the 9.0% HO diet or the control. In addition, significant effects of week were noted ($P < 0.01$) for plasma levels of Na, P and Ca, with values being higher at week 12 compared to week 6; however, the interaction with diet was not significant.

4.4.4   *Plasma proteins, glucose, uric acid and cholesterol*

Across the levels of HS inclusion, there were no significant differences in the concentrations of plasma metabolites, including plasma proteins, uric acid, glucose and cholesterol (Table 4.6). However, significant week effects ($P < 0.05$) were noted for plasma levels of total protein (51.5 vs. 55.0 ± 1.26 g/L), globulin (32.8 vs. 35.8 ± 1.02 g/L), A/G ratio (0.58 vs. 0.54 ± 0.01) and uric acid (212.6 vs. 248.5 ± 9.94 μmol/L) for week 6 versus week 12, respectively. The plasma levels of albumin (18.7 and 19.3 ± 0.30 g/L) and glucose (13.2 to 13.6 ± 0.19 mmol/L), did not differ over time (Table 4.6). The level of plasma albumin was significantly lower ($P < 0.05$) for hens consuming diets containing 4.5% HO versus those consuming either the control or the 9.0% HO diets (Table 4.5), this was supported by a quadratic response ($P < 0.01$, week 12) due to the inclusion of HO in the diets of the laying hens. While there were no significant main effects of diet on the A/G ratio in the plasma, the ratio was significantly lower ($P < 0.05$) in week 12 versus week 6 in both treatment modalities. Overall, there were no significant differences in the concentrations of plasma cholesterol for hens consuming diets containing either HS (Table 4.6) or HO (Table 4.7), when compared to control conditions.

Table 4.6: Plasma proteins and other constituents of laying hens fed diets containing hempseed (HS)[1]

Variables	Week (wk)	Main effect of diet					Main effect of week			P-values[2]		
		Control	10% HS	20% HS	30% HS	SE	wk6	wk12	SE	Diet	Week	Diet x week
Proteins												
Total protein (g/L)	6	53.4	52.9	51.8	47.9							
	12	55.2	57.3	54.1	53.6							
	overall	54.3	55.1	53.0	50.7	1.95	51.5	55.0	1.26	0.44	< 0.05	0.82
Albumin (A, g/L)	6	19.5	18.6	19.0	17.5							
	12	20.0	19.0	19.3	18.9							
	overall	19.7	18.8	19.2	18.2	0.50	18.7	19.3	0.3	0.21	0.090	0.68
Globulin (G, g/L)	6	34.0	34.1	32.6	30.5							
	12	35.7	38.1	35.0	34.6							
	overall	34.8	36.1	33.8	32.5	1.54	32.8	35.8	1.02	0.42	< 0.05	0.90
A/G ratio	6	0.58	0.55	0.59	0.58							
	12	0.55	0.53	0.55	0.52							
	overall	0.57	0.54	0.57	0.55	0.02	0.58	0.54	0.01	0.60	< 0.05	0.77
Other constituents												
Uric acid (μmol/L)	6	211	252	216	172							
	12	226	261	256	250							
	overall	219	257	236	211	16.5	213	249	9.94	0.24	< 0.01	0.14
Glucose (mmol/L)	6	13.2	12.9	13.1	13.8							
	12	13.2	13.0	13.0	13.3							
	overall	13.2	12.9	13.0	13.5	0.18	13.2	13.1	0.10	0.12	0.29	0.17
Cholesterol (mmol/L)	12	2.87	2.49	2.87	2.72	0.24				0.70	-	-

[1]Data are presented as least square means (LSM) ± standard errors of means (SE) (n = 8 per treatment).
[2]Means with different superscript letters within each variable are significantly different at $P < 0.05$.

Table 4.7: Plasma proteins and other constituents of laying hens fed diets containing hempseed oil (HO)[1]

Variables	Week (wk)	Control	4.5% HO	9.0% HO	SE	wk6	wk12	SE	Diet	Week	Diet x week
		Main effect of diets				Main effect of week			P-values[2]		
Proteins											
Total protein (g/L)	6	53.4	51.3	54.6							
	12	55.1	52.8	54.6							
	overall	54.2	52.0	54.6	1.04	53.1	54.2	0.73	0.19	0.21	0.66
Albumin (A, g/L)	6	19.4	18.5	20.0							
	12	19.9	18.5	19.5							
	overall	19.6a	18.5b	19.7a	0.32	19.3	19.3	0.23	< 0.05	1.00	0.37
Globulin (G, g/L)	6	34.0	32.6	34.6							
	12	35.7	34.3	34.9							
	overall	34.9	33.4	34.7	0.76	33.8	35.0	0.54	0.36	0.071	0.57
A/G ratio	6	0.57	0.58	0.60							
	12	0.55	0.54	0.56							
	overall	0.56	0.56	0.58	0.01	0.58	0.55	0.01	0.37	< 0.05	0.89
Other constituents											
Uric acid (μmol/L)	6	206	223	239							
	12	221	245	279							
	overall	214	234	259	20.9	223	249	14.1	0.33	0.098	0.79
Glucose (mmol/L)	6	13.1	13.2	13.3							
	12	13.2	13.0	13.4							
	overall	13.2	13.1	13.4	0.23	13.2	13.2	0.14	0.60	0.89	0.58
Cholesterol (mmol/L)	12	2.84	2.73	2.45	0.21	-	-		0.43	-	-

[1]Data are presented as least square means (LSM) ± standard errors of means (SE) (n = 8 per treatment).
[2]Means with different superscript letters within each variable are significantly different at $P < 0.05$.

4.5 DISCUSSION

Data exists to support the nutritional value of hemp (*Cannabis sativa* L.) seed and oil for laying hens, in relation to their uses as sources of dietary energy and essential fatty acids (Parker et al., 2003; Callaway, 2004), and for the enrichment of eggs with the long chain n-3 PUFAs (Gakhar et al., 2012; Goldberg et al., 2012). However, little is known about the effect of the inclusion of these products on plasma chemistry values when fed to laying hens. The latter data can serve to provide additional evidence as to the safety and efficacy of these potential feed ingredients for laying hens.

In the current study, the overall hen performance (feed intake, rate of lay, egg weight and body weight gain) was not influenced by the inclusion of graded levels of either HS (up to 30%) or HO (up to 9.0%) in the diet, when compared to hens fed the control diet. These results are consistent with previous studies examining the utilization of hemp products by laying hens (Silversides and Lefrançois, 2005; Gakhar et al., 2012). Consistent with changes in indices of performance with advancing age of hens, the main effect of time was significant for feed intake, body weight gain, rate of egg lay and egg weight. Although overall feed intakes were lower than expected for the breed type (105 – 115 g/hen/day for Lohmann LSL-Classic hens in Phase 1), in part, due to high fat content in the diets (Grobas et al., 2001), peak egg lay was achieved at approximately 6 to 7 weeks of the experiment (approximately, 27 to 28 weeks of age) in accordance with breed standards (Lohmann LSL Classic).

A significant diet by week effect in egg weights for hens consuming the 30% HS, was presumably due to an extended adaptation time by the hens to the feed (between week 3 and 6) without affecting total egg production or egg quality measures as compared with the other groups. Similarly, a depression in feed intake in week 2 by hens consuming the HO diets was

also indicative of the hens adapting to the higher oil diets because after week 3, the hens were able to maintain intakes similar to the control-diet fed hens. Generally, an early adaptation of birds to hemp-derived products during the rearing period may be necessary, particularly when higher level are used (30% HS, and 4.5% or above for HO).

An increase in egg weight is related to the increase in yolk weight with age at the expense of the other egg components (Johnston and Gous, 2007). The interior egg quality, particularly egg yolk weight, can be influenced by the dietary level of lipids (Ayerza and Coates, 1999); although, all diets used in this study contained the same level of crude fat (isolipidic), there is evidence of increased egg weights in laying hens consuming high fat diet (Grobas et al., 2001) because of greater availability of fatty acids for yolk deposition (Whitehead, 1995). The current data agree with earlier studies that provided limited evidence of an effect of different fat sources/levels on eggshell quality (Da Silva Filardi et al., 2005). Overall, the current data provides additional support for the use of HS and HO up to 30% and 9.0% HO in laying hen diets without affecting either hen performance or the quality of their eggs. While further assessment on the levels of THC in poultry tissues are underway in our lab (unpublished data), to determine their safety for human consumption, this study supports the hypothesis that hempseed products are safe for use in laying hen diets.

While data on the efficacy of novel ingredients to support performance and quality measures is important, additional measures are necessary to support their safety for laying hens and satisfy regulatory agencies. To this end, the current study examined clinical markers of metabolic and hepatic health in hens consuming hemp-based products. The levels of plasma enzymes, AST and GGT, neither of which are solely indicative of liver damage in birds (Hochleithner, 1994), are often used for assessing liver function in laying hens (Fernandez et al.,

1994), although the CK activity was also included for the same purpose, the latter is not specific to liver, but rather associated with damage to muscle membranes (Diaz et al., 1999). In the current study, while changes in enzymes were observed, there were no significant increases when hemp product containing diets were fed to the laying hens compared with the control group, hence supporting the safety of using these hemp-derived products in laying hen diets.

Overall, the mean plasma CK activities for both treatments (HS and HO) were observed to increase with the age of the laying hens, possibly reflecting a change in muscle activity, as was observed in our study, both groups of hens lost weight between week 1 and 4 and gained by week 6 (HS) and week 7 (HO). Although the overall plasma CK activities for both treatments (HS and HO) were observed to increase with the age of the laying hens, there was no association between BWG over the two periods (week 6 and 12) and the CK values ($P = 0.59$ and 0.22 for HS and HO treatments, respectively). The observed increases, however, are within the limits of previously (Diaz et al., 1999) documented values of 432 to 1645 U/L indicated for hens at 33 weeks of age. With respect to plasma AST and GGT levels, there were significant reductions in the levels of AST measured for hens consuming the 10 and 20% HS; GGT in hens consuming the 20% HS and 4.5% HO; and AST and GGT (a result of diet by week interaction, 14% and 6% lower in week 12 than 6; respectively) for the 30% HS containing diet in comparison the control diet. As hemp oil is rich in polyunsaturated fatty acids, high inclusion levels may challenge hepatic capacity for metabolism, leading to atherosclerosis (Fraser et al., 1986) and fatty liver disease and associated hemorrhage (Hansen and Walzem, 1993; Hermier et al., 1994). The latter are known to be more prevalent in hens consuming higher fat diets than other high energy-diets/high carbohydrate diets (Zhang et al., 2008). The absence of hemp effects on markers of hepatic and muscular damage provides clinical evidence of the safety of their respective

inclusion in laying hen diets. In the current study, a reduction in the levels of GGT, at intermediary inclusion levels, is suggestive of reduced liver trauma with hemp product-containing diets in laying hen production, possibly due to its pharmacological activity (antioxidant property) given high γ-tocopherol levels (Leizer et al., 2000). However, based on the magnitude of changes observed, while significant, they may not be clinically relevant.

The electrolytes Na, K, and Cl (Nobakht et al., 2006) are important for key physiological processes, including acid-base and osmotic balance (Borges et al., 2004). In laying hens, these minerals are critical for optimal egg production, shell quality, feed efficiency, water intake and body weight gain (Hughes, 1988; Murakami et al., 2001; Pavlík et al., 2009). Although a significant reduction of K level in the plasma was observed for laying hens consuming the 4.5% HO enriched diet, a higher level of HO (9.0%) did not result in a similar or further decline in K plasma level, raising questions as to clinical significance of this treatment effect. Additionally, no week effect or interaction with treatment effect was observed, providing evidence that the inclusion of the HO did not alter the temporal patterns in changes in the level of the K in the plasma.

With respect to other minerals, plasma Ca and P levels in laying hens are also inter-related (Chandramoni et al., 1998; Kebreab et al., 2009). The dietary Ca and P content have an impact on the body's effort to regulate the pH of the blood with a consequence on the acid-base balance (Keshavarz, 1994). In laying hens, maintaining an optimal acid-base balance is necessary for bicarbonate homeostasis and eggshell formation (Pelicia et al., 2009). Differences in eggshell quality have been attributed to changes in blood plasma mineral profiles in hens (Pavlík et al., 2009). In the current study, for either diet types (HS or HO), the significant week effect in blood Ca and P levels, likely reflects changes in the demand for both minerals with age.

For example, the requirement for Ca is highest during peak egg production and can be reflected by serum/plasma calcium concentrations (Gyenis et al., 2006). The increases with time, irrespective of diet, may reflect a relative oversupply of both Ca and P, in relation to metabolic needs, as the hen progress from peak production. A significant treatment by week interaction on blood Ca levels of hens fed diets containing HS (30% level) but not HO was primarily impacted by results from week 6, but the effect was lost later in the study, however the explanation for this observation is not readily apparent.

In laying hens, published ranges of plasma protein levels include 35 to 55 g/L for total protein and 13 to 28 g/L for albumin (Gyenis et al., 2006). In the current study, the plasma protein values for all groups of hens (Tables 4.4 and 4.5) were within a similar range. While typically associated with muscle tissue hypertrophic growth (Gyenis et al., 2006), the absence of significant main effects or interactions in these markers of nitrogen metabolism support the ability of the hemp products to provide both dietary energy (HS and HO) and amino acids (HS) to meet metabolic demands. Hempseed protein, while somewhat limiting in lysine, has been shown to be highly digestible in rodent studies (House et al., 2010). Similarly, a primary end-product of protein metabolism, uric acid, measured in this study, was not affected by the treatment effects. However, plasma uric acid levels of this metabolite increased over time (week 6 versus week 12), likely, reflect a temporal shift in nitrogen metabolism relative to intake as the birds mature. Plasma glucose and cholesterol levels of all groups of hens were not significantly different. Although glucose is necessary as a source of energy in most cells, avian erythrocytes depend primarily on fatty acids and not on glucose, as their energy source (Jones, 1999); nevertheless, the dietary treatments were balanced for energy and total fat content. In addition, in chickens as it is for humans, a key enzyme, lecithin:cholesterol acyltransferase is involved in

maintaining cholesterol homeostasis (Hengstschläger-Ottnad et al., 1995) and regulating its transport in blood as it is in the human (Spector and Haynes, 2007) and returned to the liver for biliary elimination (Jonas, 2000). Hence, this mechanism benefits the hen in controlling its blood cholesterol levels. However, considering that most of the cholesterol in laying hen plasma is carried in VLDL, with only ~25% carried in HDL (Schneider, 2009), oocytic uptake of circulating VLDL plays a much greater role in maintaining the hen's plasma cholesterol levels than LCAT. These findings are supported by Elkin et al. (2012) indicating that when the VLDL receptor is dysfunctional, as in hens of a mutant strain referred to as "restricted ovulator", plasma cholesterol and TAG levels increased on average by 4- to 5-fold compared with normal layers. In addition, a potential benefit of including hempseed products in animal diets may indirectly related to cholesterol homeostasis, through the provision of the fatty acid gamma-linolenic acid which has been reported to normalize cholesterol-induced platelet aggregation (Prociuk et al., 2008). Whether additional benefits of increasing this fatty acid in poultry diets are realized, is yet to be determined.

In summary, data from the current study provides valuable information as to metabolic responses of laying hens consuming diets containing HS and HO, thus supporting their potential for use in laying hen diets. The study provides evidence that up to 30% or 9.0% inclusion of HS or HO, respectively, in diets for laying hens could be provided without affecting hen performance and plasma chemistry. Whereas a decrease in egg weight with the 30% HS during the first 8 weeks was observed, egg weights achieved a similar trend to those observed in hens consuming lower levels of hemp products. The latter data may indicate that higher levels of HS (at 30% levels) can be well tolerated by the hens, but an early adaptation of birds to hemp-

derived products during the rearing period may be required, particularly when used at higher

levels.

CHAPTER 5 MANUSCRIPT II

Hempseed products fed to hens effectively increased n-3 polyunsaturated fatty acids in total

lipids, triacylglycerol and phospholipid of egg yolk

5.1 ABSTRACT

Hempseed products represent potential alternative feed ingredients for poultry. However, their usage is not currently approved due to a lack of data to support their safety and efficacy. In this regard, the current study was conducted to assess the impact of dietary concentration of hempseed (HS) products and duration of their feeding to hens on the polyunsaturated fatty acid (PUFA) composition of egg yolk lipids were studied. In the current study, 48 Lohmann LSL-Classic hens were individually housed in metabolism cages, in a completely randomized design, and provided 1 of 6 diets (wheat-barley-soybean-based) containing either HS (10, 20 and 30%), hempseed oil (HO; 4.5 and 9.0%) or no hempseed product (control) over 12 weeks. Increasing alpha-linolenic acid (ALA) intake via increasing dietary hempseed product inclusion, significantly ($P < 0.0001$) increased the n-3 PUFA contents of yolk total lipid. The values of ALA increased by 12 fold (152 ± 3.56 and 156 ± 2.42 mg/yolk) and docosahexaenoic acid (DHA) by 2 to 3 fold (41.3 ± 1.57 and 43.6 ± 1.61 mg/yolk) over the control, for highest levels of HS and HO inclusion, respectively. Increasing levels of hemp products in laying hen diets proved effective in manipulating the fatty acid profile of the total lipid, triacylglycerol (TAG) and total phospholipid (PL) fractions of yolks, enhancing the n-3 fatty acids and reducing the n-6/n-3 ratio. The latter benefit was achieved within 4 weeks of feeding hens either HS- or HO-containing diets.

Key words: egg yolk; hempseed products; fatty acid profile

5.2 INTRODUCTION

Traditionally, North American laying hen diets are formulated on cereal grains and fat or oil sources that provide substantial levels of n-6 polyunsaturated fatty acids (PUFA), predominantly linoleic acid (LA, 18:2n-6), and only a small amount of n-3 PUFA (Morrison, 1977; Maldjian et al., 1996; Simopoulos, 2000). As a result, eggs typically contain low levels of the n-3 PUFA, namely alpha-linolenic acid (ALA, 18:3n-3), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA), and are virtually devoid of eicosapentaenoic acid (EPA) (Yalcyn et al., 2007; Souza et al., 2008). As such, classic eggs make only minor contributions to recommended daily intakes (Institute of Medicine, 2005) of these fatty acids in the general population. The fatty acid profiles of egg yolk lipids have been shown to be altered by adding natural sources of n-3 PUFA to the hens' feed (Caston and Leeson, 1990; Cherian and Sim, 1991; Baucells et al., 2000). Flaxseed, an oilseed that contains approximately 30% lipid, is a rich source of ALA (53% of total fatty acids in the oil) (Calder, 1998; Surai and Sparks, 2001), and is commonly used to enrich eggs with n-3 fatty acids (Caston and Leeson, 1990; González-Esquerra and Leeson, 2001; Hayat et al., 2009). Flaxseed is considered a minor crop in North America compared to other oilseeds such as canola and soybean (Jhala and Hall, 2010). However, given substantial interest for its direct consumption by humans, flaxseed has a high growth potential for use in functional foods for humans (Morris, 2007), thus potentially creating challenges for its utilization in animal feed. Flaxseed also contains anti-nutritive factors, including linamarin (Raes et al., 2004) and linatine (Mayengbam et al., 2014). These latter factors may lead to limitations in flaxseed use in hen diets in order to generate n-3 fatty acid-enriched eggs, with maximum inclusions levels of 15-20% observed to influence hen body weight, egg size and production (Caston et al., 1994; Aymond and Van Elswyk, 1995; Scheideler

and Froning, 1996). In addition, it has been reported that as little as 5% inclusion of flaxseed in hen diets can cause negative organoleptic qualities, such as a "fishy taint" in eggs (Scheideler et al., 1997; Leeson et al., 1998). Owing to these limitations, opportunity exists to evaluate alternative sources of n-3 fatty acids for laying hen diets.

The seed and oil of industrial hemp (*Cannabis sativa* L.) are rich sources of ALA, with this fatty acid comprising 19-22% of the total fatty acid profile (Callaway, 2004; Gakhar et al., 2012). Additionally, hempseed (HS) can be a valuable feed source for poultry given its protein (24%) and total fat (30%) contents (House et al., 2010; Gakhar et al., 2012; Goldberg et al., 2012). Hempseed oil (HO) is a rich source of PUFA (78% of total fatty acids), with a linoleic acid (LA, 18:2n-6) to ALA ratio of approximately 3 to 1 (Parker et al., 2003). This ratio is often cited as being ideal and well suited for human nutrition (Kris-Etherton et al., 2000; Simopoulos, 2002). Additionally, hemp oil contains approximately 4% gamma-linolenic acid (GLA) (Callaway, 2004), which may confer additional health benefits (Leizer et al., 2000). Despite these positive traits, the usage of industrial hemp products in Canada is not currently approved, owing to the need for safety and efficacy data on these components. The latter requirements stem from the potential for hemp to contain small amounts of tetrahydrocannabinol (THC), a psychoactive agent linked to cannabis varieties. Industrial hemp is grown under license from federal regulatory authorities (Health Canada, 2012), and these regulations are aimed at producing appropriate cultivars containing less than 0.3% (by wt) THC. Hemp products are not currently registered as approved feed ingredients in North America, and approval requires submission of data in support of the safety and efficacy of hempseed products for use in livestock and poultry diets.

Previous research has shown that hempseed products can be used to generate eggs enriched with n-3 PUFA (Silversides and Lefrançois, 2005; Gakhar et al., 2012; Goldberg et al., 2012). This previous work focused on the fatty acid composition of total yolk lipids, without considering the fatty acid composition of the major lipid classes, including the phospholipid (PL) and triacylglycerol (TAG) depots; yet each of these lipid fractions can be a potential food source in food industry. For example, egg phospholipids have been utilized in infant formulas (Makrides et al., 2002). Additionally, egg lipids could serve as a source of TAG molecules (based on their fatty acid composition) in the synthesis of structured lipids (composed of medium chain fatty acids and PUFAs) with improved nutritional benefits for ultimate use in food applications (Osborn and Akoh, 2002). As such, the current study was designed to address the temporal changes in the fatty acid profiles, particularly the n-3 PUFA in chicken eggs as a function of increasing hemp oil, from either the seed or the extracted oil, in laying hen diets over a period of 12 weeks. Furthermore, the distribution of fatty acid profile in the major TAG and PL fractions within egg yolk was investigated.

5.3 MATERIALS AND METHODS

5.3.1 *Animals and diets*

Forty eight Lohmann LSL-Classic (white-egg layers; initial mean weight of 1.41 ± 0.08 kg) at 19 weeks of age, were individually caged (25.4 cm x 40.6 cm dimension, providing floor space of 1032 cm^2 / hen) and maintained under semi-controlled environmental conditions at the University of Manitoba poultry barn. Hens were adapted to the cages and diets over an initial two-week period. During the first week of adaptation, hens were fed a commercial layer diet, and then transitioned to a 50:50 blend of the commercial and test diets in the second week.

Following the adaptation period, the hens were allocated to 1 of 6 wheat-barley-soybean meal-based diets. The experiment included a control diet without hempseed product (corn-oil-based, 9.86% of diet); 3 diets providing 10, 20, or 30% hempseed (HS; containing $32.8 \pm 1.05\%$ lipid); and 2 diets with 4.5 or 9.0% hempseed oil (HO). The layer diets were isocaloric, isonitrogenous and isolipidic and formulated to meet the minimum recommendation of laying hens consuming 105 to 115 g of feed per day in accordance to the strain's management guide (Lohmann LSL-Classic, 2004). The composition and nutrient contents of the experimental diets are shown in Table 5.1. To avoid lipid peroxidation, diets were prepared in two batches and in addition, an antioxidant, vitamin E, was supplemented at levels of 150 IU/kg of diet. The lipid profile of the experimental diets is shown in Table 5.2. Animal usage and care was reviewed and approved by the University of Manitoba Animal Care Protocol Management and Review Committee, and the hens were managed in accordance with the recommendations established by Canadian Council on Animal Care (1993).

5.3.2   *Diet and egg yolk fatty acid extraction*

Diet samples (duplicates per treatment) from both batches and eggs (8 replicates per treatment) collected within the last 3 days of each period (week 4, 8 and 12) of the experiment were used for fatty acid extraction. Diet samples (150 g each) were ground using a commercial grinding mill and stored at $-20°C$ until analyzed. The eggs were broken, the yolks carefully separated from the whites (albumen) using an egg separator, then individually weighed in plastic bags and stored at -20°C until analyzed. Diet (1 g) and egg yolk samples (3 g) were used for the extraction of total lipids using chloroform/methanol (2:1, by vol) containing 0.01% butylated hydroxytoluene (antioxidant) according to Folch et al. (1957). The extracted total lipids were weighed and reconstituted in hexane to a volume of 25 mL. From each extract,

Table 5.1: Composition and calculated nutrients of experimental diets containing hempseed (HS)[1] and hempseed oil (HO)

	Control	10% HS	20% HS	30% HS	4.5% HO	9.0%HO
Ingredients (%)						
Wheat	35.0	33.4	31.9	30.3	35.0	35.0
Barley	15.0	15.0	15.0	15.0	15.0	15.0
Soybean Meal	21.5	16.9	12.2	7.60	21.5	21.5
Hemp Seed	0.00	10.0	20.0	30.0	0.00	0.00
Hemp Oil	0.00	0.00	0.00	0.00	4.50	9.00
Corn Oil	9.68	6.48	3.28	0.07	5.18	0.68
Vitamin-mineral premix[2]	2.50	2.50	2.50	2.50	2.50	2.50
Limestone	13.9	13.5	13.0	12.4	13.9	13.9
Dicalcium Phosphate	1.88	1.60	1.56	1.52	1.88	1.88
Salt	0.34	0.35	0.35	0.35	0.34	0.34
DL-Methionine	0.116	0.095	0.073	0.051	0.116	0.116
L-Lysine-HCl	0.083	0.128	0.173	0.218	0.083	0.083
L-Threonine	0.005	0.000	0.000	0.000	0.005	0.005
Calculated nutrient contents						
AMEn (Kcal/kg)	2800	2800	2800	2800	2800	2800
CP (%)	17.0	17.0	17.0	17.0	17.0	17.0
Crude fat (%)	11.0	11.0	11.0	11.0	11.0	11.0
Calcium (%)	5.48	5.30	5.09	4.87	5.48	5.48
Total Phosphorus (%)	0.70	0.72	0.80	0.87	0.70	0.70
Available Phosphorus (%)	0.50	0.45	0.45	0.45	0.50	0.50
Sodium (%)	0.16	0.16	0.16	0.16	0.16	0.16
Chloride (%)	0.26	0.26	0.25	0.25	0.26	0.26
Methionine	0.36	0.36	0.36	0.36	0.36	0.36
Total Lysine (%)	0.85	0.85	0.85	0.85	0.85	0.85
Threonine	0.60	0.60	0.61	0.62	0.60	0.60
Analyzed nutrient contents[3]						
DM (%)	91.9 ± 0.21	91.8 ± 0.25	91.9 ± 0.21	91.9 ± 0.30	92.1 ± 0.36	91.9 ± 0.30
Energy (gross energy, kcal/kg)	3805 ± 19.9	3848 ± 22.8	3848 ± 14.2	3873 ± 32.1	3806 ± 13.9	3797 ± 2.55
CP (%)	16.9 ± 0.58	17.8 ± 0.35	17.3 ± 0.37	16.8 ± 1.67	17.0 ± 0.72	17.7 ± 0.50
Crude fat (%)	11.6 ± 0.62	12.7 ± 0.90	12.5 ± 0.02	12.5 ± 0.44	12.0 ± 0.01	11.3 ± 0.28
Calcium (%)	5.65 ± 0.43	5.64 ± 0.41	5.29 ± 0.38	5.93 ± 1.04	5.50 ± 0.81	5.73 ± 0.15
Total Phosphorus (%)	0.75 ± 0.03	0.75 ± 0.07	0.80 ± 0.01	0.86 ± 0.04	0.70 ± 0.04	0.75 ± 0.01
ADF (%)	2.91	5.45	7.38	8.83	2.84	3.27
NDF (%)	14.3	16.8	18.2	21.6	11.8	13.3

[1]Contained $32.8 \pm 1.05\%$ fat

[2]Provided per kilogram of diet, vitamin-mineral premix contained: 11,000 IU of vitamin A; 3,000 IU of vitamin D_3, 150 IU of vitamin E, 3 mg of vitamin K_3 (as menadione), 0.02 mg of vitamin B_{12}, 0.2 mg of biotin, 6.5 mg of riboflavin, 4 mg of folic acid, 10 mg of calcium pantothenate, 39.9 mg of niacin, 2.2 mg of thiamine, 4.5 mg of pyridoxine, 1000 mg of choline chloride, 125mg antioxidant (ethoxyquin), 66 mg of manganese oxide, 70 mg of zinc oxide, 80 mg of ferrous sulfate, 10 mg of copper sulfate, 0.3 mg of sodium selenite, 0.4 mg of calcium iodate, 0.67 mg of sodium chloride (salt).

[3]Mean values $\pm$ SD for duplicate analysis per sample.

Table 5.2: Fatty acid profile of experimental diets[1]

Fatty acid[2] (mg/g of diet)	Control	10% HS	20% HS	30% HS	4.5% HO	9.0% HO
SFA						
Myristic (14:0)	0.041	0.041	0.045	0.045	0.040	0.047
Palmitic (16:0)	8.53	7.70	6.35	4.90	7.00	5.60
Stearic (18:0)	1.17	1.35	1.49	1.51	1.38	1.65
Total SFA	9.74	9.09	7.88	6.46	8.42	7.29
MUFA						
Palmitoleic (16:1)	0.074	0.078	0.078	0.074	0.077	0.080
Oleic (18:1)	17.8	14.3	10.4	6.01	12.6	7.62
Total MUFA	17.8	14.4	10.5	6.09	12.7	7.70
PUFA[3]						
Linoleic (LA, 18:2n-6)	38.1	39.2	38.6	36.3	39.0	40.9
Gamma-linolenic (GLA, 18:3n-6)	0.01	0.92	1.83	2.57	1.30	2.70
Arachidonic (ARA, 20:4n-6)	ND	ND	ND	ND	ND	ND
Alpha-linolenic (ALA, 18:3n-3)	0.96	5.00	8.95	12.2	6.64	12.8
Eicosapentaenoic (EPA, 20:5n-3)	ND	ND	ND	ND	ND	ND
Docosapentaenoic (DPA, 22:5n-3)	ND	ND	ND	ND	ND	ND
Docosahexaenoic (DHA, 22:6n-3)	ND	ND	ND	ND	ND	ND
Total PUFA	39.1	45.1	49.4	51.0	46.9	56.5
Ratio LA:ALA	39.8	7.83	4.32	2.98	5.88	3.18

[1]Diets contain no hempseed product (control), hempseed (HS) or hempseed oil (HO)
[2]Saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA)
[3]ND = not detectable

aliquots of a known volume (to contain 40 - 50 mg lipid) were dried under nitrogen, dissolved in

toluene and methylated by heating in the presence of methanol containing 2% (by vol) sulphuric

acid (Burdge et al., 2000). The resulting fatty acid methyl esters (FAMEs) were extracted into

iso-octane for determining the fatty acid profile using gas chromatography (GC). The major

lipid classes (TAG and PL) in the total lipid extracts of egg yolk (obtained in week 12 of the

trial, n = 7 per treatment) were separated by thin layer chromatography (TLC) on silica gel plates

(Silica gel G, Uniplate[TM], Analtech, Inc) according to method described by (Suh et al., 1994),

with slight modification, using petroleum ether/diethyl ether/acetic acid (80:20:1, by vol), as the

developing solvent. Approximately 2.4 mg of yolk lipid extract was applied on the TLC plates.

Lipid classes were visualized with 0.1% (wt/vol) 2, 7-dichlorofluorescein in methanol under UV

light. Prior to methylation, the TAG fraction was saponified according to the method previously

described (Hartman, 1973), and the PL fraction was directly esterified. Lipids were esterified

using borontrifluoride in 14% methanol, and fatty acids quantified by GC using C17:1 (Nu-Chek

Prep Inc., Elysian, MN) as the external standard.

5.3.3   *Fatty acid analysis*

The fatty acid methyl esters (FAME) were analyzed using a Varian 450 GC with flame

ionization detector (FID) and equipped with a DB225MS column (30 m $\times$ 0.25 mm diameter and

0.25 μm film thickness; Agilent Technologies Canada Inc., Mississauga, Ontario). The

temperature program was 70°C for 2 min, then raised to 180°C at 30°C/min, held for 1 min;

raised to 200°C at 10°C/min, for 2 min; raised to 220°C at 2°C/minute and held for 10 minutes

and finally raised to 240°C at 20°C/ min for 5 min. Total run time was 36.67 min, and samples

(1μL injection) were run with a 20:1 split ratio. Hydrogen was used as the carrier gas with a

flow rate of 1.3 mL/min. Each fatty acid was identified by comparing its retention time to

authentic standard samples of known composition (Lipid standards, Nu-Chek Prep, Inc., Elysian, MN, USA). The fatty acid content of egg yolk total lipid was calculated as concentration (mg/yolk) = [(peak area of a given fatty acid × concentration of internal standard (mg/mL)/peak area of internal standard) × dilution factor of extracted lipid] divided by yolk sample weight (g), then multiplied by total yolk weight per egg (g). For lipid classes, TAG and total PL of yolk, the peak area for an individual fatty acid was expressed as a percentage of the total peak area for all identified fatty acids in the lipid samples.

5.3.4 Statistical analysis

The fatty acid content in the egg yolk total lipid and lipid classes were analyzed as a completely randomized design with the individual hen as the experimental unit. The HS (control (0), 10, 20 and 30% levels) and HO (control (0), 4.5 and 9.0% levels) treatments were analyzed as 2 separate experiments/trials, since the biological efficiency in the utilization of the seeds vs. the oils by the hens would be expected to differ. The same data for the control diet was applied in either treatment group. Feed intake was used as a covariate in all analysis, and this may influence the final control data sets between the two treatment groups. Except for lipid classes, the fatty acids in total lipid of egg yolk were run as repeated measure analysis using PROC MIXED procedure of SAS (SAS Institute Inc., Cary, NC) following the model: $Y_{ijk} = \mu + d_i + h_{ij} + w_k + dw_{ik} + e_{ijk}$, where μ = overall mean, d_i = fixed effect of diet (i = 1 to 4 for HS-containing diets or i = 1 to 3 for HO containing diets), h_{ij} = random effect of hen within diet (j = 1 to 8, number of hens per treatment), w_k = fixed effect of week (k = 1 to 3), and dw_{ik} = interaction between diet and week (diet × week), and e_{ijk} = random error variation (residual error). Diet × week interactions were considered as fixed effects. Fatty acids in lipid classes were analyzed with a one-way ANOVA for the HS (control (0), 10, 20 and 30% levels) or HO (control (0), 4.5

and 9.0% levels) treatment groups (n=7 for each diet), using the same statistical package.

Normality of the data distribution was assessed using the Shapiro-Wilk test and data points with

studentized residuals below or above ± 3.0 were considered outliers and excluded from the

analysis. Least squares means (LSM), adjusted using Tukey's significant difference test, were

compared for significant difference ($P < 0.05$).

5.4 RESULTS

5.4.1 *Total egg yolk lipid content*

Total lipid extracts from eggs were not statistically different between treatments for both

treatment groups: HS (31.5, 34.3, 32.9 and 32.3 ± 1.27 (SE) g/100g yolk, $P = 0.51$; for control,

10, 20 and 30% of diet HS inclusion, respectively); and HO (31.5, 34.8 and 34.3 ± 1.37, $P = 0.20$

(SE) g/100g yolk; for control, 4.5 and 9.0 % of diet HO inclusion, respectively).

5.4.2 *Fatty acid composition in the total lipid of egg yolk*

With respect to the total lipid fraction, the composition of egg yolk SFA (myristic (14:0);

palmitic (16:0) and stearic (18:0) acids) and MUFA (palmitoleic (16:1) and oleic (18:1) acids),

as a function of increasing levels of hempseed-derived products, are presented in Tables 5.3 and

5.4 for the HS and HO treatment groups, respectively. There was a significant decrease in the

levels of myristic ($P < 0.05$) and palmitic ($P < 0.001$) acids in eggs from hens receiving the HS

containing diets compared to the control (Table 5.3). With respect to HO treatments (Table 5.4),

the myristic and palmitic acid levels in the egg yolk were significantly ($P < 0.01$ and $P < 0.05$,

respectively) lower for hens fed the 4.5% HO compared to the control or the 9.0% HO.

Additionally, in both the HS (Table 5.3) and HO (Table 5.4) groups, the level of stearic acid was

Table 5.3: Total saturated (SFA) and monounsaturated (MUFA) fatty acids composition in egg yolk (mg/yolk) obtained from hens consuming diets containing increasing levels of hempseed (HS)[1]

Fatty acids[2]	Week (Wk)	Control	10% HS	20% HS	30% HS	SE	Wk 4	Wk 8	Wk 12	SE	Diet	Wk	Diet x Wk
			Effect of diet					Effect of week				P - value	
14:0	4	9.03^A	7.17^B	7.19^B	6.93^B								
	8	8.38^{AB}	7.27^B	7.34^B	6.73^B								
	12	8.00^B	8.29^A	8.12^A	7.83^A								
	Overall	8.47^a	7.58^b	7.55^b	7.16^b	0.23	7.58^b	7.43^b	8.06^a	0.16	< 0.01	< 0.01	< 0.01
16:0	4	757	664	620	608								
	8	795	713	669	636								
	12	759	769	709	703								
	Overall	771^a	716^{ab}	666^{bc}	650^c	17.4	662^c	703^b	735^a	12.5	< 0.001	< 0.0001	0.13
18:0	4	281	277	284	339								
	8	292	296	301	346								
	12	275	316	312	345								
	Overall	282^b	296^b	299^b	344^a	8.63	296^b	309^a	312^a	5.58	< 0.001	< 0.05	0.15
Total SFA		1062	1019	972	1000	22.2	966^b	1019^a	1055^a	16.2	0.053	< 0.001	0.16
16:1	4	34.8	29.6^B	27.7	24.0^B								
	8	34.2	29.0^B	29.7	25.1^B								
	12	32.6	33.2^A	30.5	28.8^A								
	Overall	34.0^a	30.6^{ab}	29.3^{ab}	25.8^b	1.60	29.0^b	29.5^{ab}	31.3^a	0.92	< 0.05	< 0.05	< 0.05
18:1	4	938	800	675	583								
	8	989	843	719	623								
	12	934	893	748	687								
	Overall	953^a	846^b	714^c	631^d	21.1	749^b	794^a	816^a	15.1	< 0.0001	< 0.01	0.31
Total MUFA		987^a	877^b	743^c	657^d	22.3	778^b	823^a	847^a	15.8	< 0.0001	< 0.01	0.28

[1]Data are presented as least square means (LSM) ± standard error (SE) (n = 7 hens per treatment).
[2]Myristic (14:0), palmitic (16:0), stearic (18:0), palmitoleic (16:1), oleic (18:1) acid.
[a–d]Different superscripts between treatments (effect of diet) or periods (effect of week), within a row, are significantly different at $P < 0.05$.
[A,B] Different superscripts within a treatment(diet), within a column, for each parameter are significant different ($P < 0.05$), comparison of diet by week interaction.

Table 5.4: Total saturated (SFA) and monounsaturated (MUFA) fatty acids composition in egg yolk (mg/yolk) obtained from hens consuming diets containing increasing levels of hempseed oil (HO)[1]

Fatty acids[2]	Week (Wk)	Effect of diet				Effect of week				P - value		
		Control	4.5% HO	9.0%HO	SE	Wk 4	Wk 8	Wk 12	SE	Diet	Wk	Diet x Wk
14:0	4	8.98^A	7.02	8.15^B								
	8	8.34^{AB}	7.62	8.40^{AB}								
	12	7.96^B	7.93	8.90^A								
	Overall	8.43^a	7.52^b	8.48^a	0.21	8.05	8.12	8.26	0.17	< 0.01	0.61	< 0.01
16:0	4	750	642	649								
	8	789	710	724								
	12	753	737	747								
	Overall	764^a	697^b	707^{ab}	16.7	681^b	741^a	746^a	13.1	< 0.05	0.0001	0.098
18:0	4	281	284^B	311^B								
	8	292	319^A	347^A								
	12	274	321^A	349^A								
	Overall	282^b	308^b	335^a	7.63	292^b	319^a	315^a	5.64	< 0.001	0.0001	< 0.05
Total SFA		1055	1012	1051	21.2	980^b	1068^a	1068^a	17.4	0.31	0.0001	0.066
16:1	4	34.6	30.9	33.6								
	8	33.9	32.0	35.0								
	12	32.4	33.4	37.3								
	Overall	33.6	32.1	35.3	1.76	33.0	33.6	34.3	1.17	0.45	0.42	0.19
18:1	4	933	782	667								
	8	985	845	719								
	12	929	868	727								
	Overall	949^a	832^b	704^c	19.1	794^b	850^a	842^a	15.0	< 0.0001	< 0.01	0.25
Total MUFA		983^a	864^b	740^c	20.2	827^b	883^a	876^{ab}	15.8	< 0.0001	< 0.01	0.24

[1]Data are presented as least square means (LSM) ± standard error (SE) (n = 7 hens per treatment)
[2]Myristic (14:0), palmitic (16:0), stearic (18:0), palmitoleic (16:1), oleic (18:1) acid.
[a–c]Different superscripts between treatments (effect of diet) or periods (effect of week), within a row, are significantly different at $P < 0.05$.
[A,B] Different superscripts within a treatment (diet), within a column, for each parameter, are significant different ($P < 0.05$), comparison of diet by week interaction.

significantly ($P < 0.0001$) greater in eggs obtained from hens consuming higher levels of HS (30%) and HO (9.0%) compared to those fed the control diet or lower levels of the hempseed products. However, overall, the inclusion of either HS (Table 5.3) or HO (Table 5.4) in the laying hen diets did not affect the levels of total SFA in the egg yolk. The contents of total MUFA (palmitoleic and oleic acids) in the egg yolk significantly ($P < 0.0001$) decreased with increasing inclusions of both HS (Table 5.3) and HO (Table 5.4) in the laying hen diets, particularly due to a significant reduction ($P < 0.0001$) in the levels of oleic acid for both treatment modalities. However, a highly significant ($P < 0.0001$) period effect observed in the levels of oleic acid in the egg yolk did not result in a significant diet $\times$ week interaction within each treatment in either the HS or the HO treatment settings (Tables 5.3 and 5.4).

The inclusion of either HS (Table 5.5) or HO (Table 5.6) in the laying hen diets significantly influenced the total PUFA levels in the egg yolk, except for LA composition that was maintained at a similar level to the control. Although the levels of GLA in the total lipids of egg yolk were significantly ($P < 0.0001$) increased at higher levels of HS inclusions (at 20 and 30%) in the diets compared to the control or at lower levels of the HS (10%; Table 5.5), both levels of the HO (4.5 and 9.0%) were effective in increasing the levels of this fatty acid in the egg yolks compared to the control (Table 5.6). Arachidonic acid levels were significantly decreased in the total lipid of egg yolk for both the HS ($P < 0.0001$) and the HO ($P < 0.001$) treatment groups compared to the control. In general, there was no significant treatment effect on the total n-6 fatty acids level in the total lipid of egg yolk in both treatment modalities (data not presented).

A significant ($P < 0.0001$) increase in the n - 3 fatty acid contents of the eggs (ALA,

Table 5.5: Total polyunsaturated fatty acids (PUFA) composition in total lipid of egg yolk (mg/yolk) obtained from hens consuming diets containing increasing levels of hempseed (HS)[1]

Fatty acids[2]	Week (Wk)	Control	10% HS	20% HS	30% HS	SE	Wk 4	Wk 8	Wk 12	SE	Diet	Wk	Diet x Wk
			Effect of diet					Effect of week				P - value	
LA(18:2n-6)	4	823	810^B	818	810^B								
	8	923	915^A	882	905^{AB}								
	12	835	970^A	944	999^A								
	Overall	860	898	881	905	23.1	815^b	906^a	937^a	16.2	0.59	< 0.0001	< 0.05
GLA(18:3n-6)	4	5.49	6.08	7.87	9.37								
	8	6.44	6.99	8.49	10.1								
	12	5.91	7.45	8.97	10.9								
	Overall	5.94^c	6.84^c	8.44^b	10.1^a	0.35	7.20^b	8.01^a	8.30^a	0.21	< 0.0001	< 0.0001	0.26
ARA(20:4n-6)	4	68.6	59.3	55.8	52.6^B								
	8	72.8	63.4	56.7	55.8^{AB}								
	12	68.6	66.9	59.1	63.4^A								
	Overall	70.3^a	63.2^{bc}	57.3^{cd}	56.8^d	1.61	59.1^b	62.2^a	64.5^a	1.11	< 0.0001	< 0.001	< 0.05
ALA(18:3n-3)	4	13.4	53.2	99^B	135^B								
	8	13.7	59.2	110^{AB}	145^B								
	12	12.6	65.6	114^A	176^A								
	Overall	13.2^d	59.3^c	108^b	152^a	3.56	75.2^c	81.8^b	92.1^a	2.17	< 0.0001	< 0.0001	< 0.0001
EPA(20:5n-3)	4	0.00	1.01	1.80	2.57								
	8	0.00	1.07	1.84	2.57								
	12	0.00	1.12	1.87	2.66								
	Overall	0.00^d	1.07^c	1.84^b	2.60^a	0.060	1.34	1.37	1.41	0.038	< 0.0001	0.26	0.96
DPA(22:5n-3)	4	1.74	3.57	4.02	5.23								
	8	1.74	3.65	4.29	5.56								
	12	1.58	4.19	4.39	4.98								
	Overall	1.69^c	3.64^b	4.23^b	5.26^a	0.25	3.64	3.81	3.78	0.14	< 0.0001	0.33	0.066
DHA(22:6n-3)	4	17.0	41.1	39.6	39.8								
	8	16.7	41.1	41.2	42.8								
	12	14.1	42.9	42.8	41.7								
	Overall	16.2^b	41.0^a	41.3^a	41.0^a	1.57	33.8	35.5	35.4	0.93	< 0.0001	0.087	0.086

[1]Data are presented as least square means (LSM) ± standard error (SE) (n = 7 hens per treatment).
[2]Linoleic acid (LA), gamma-linolenic acid (GLA), arachidonic acid (ARA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA).
[a-d]Different superscripts between treatments (effect of diet) or periods (effect of week), within a row, are significantly different at $P < 0.05$.
[A-B] Different superscripts within a treatment (diet), within a column, for each parameter, are significant different ($P < 0.05$), comparison of diet by week interaction.

Table 5.6: Total polyunsaturated fatty acids (PUFA) composition in total lipid of egg yolk (mg/yolk) obtained from hens consuming diets containing increasing levels of hempseed oil (HO)[1]

Fatty acids[2]	Week (Wk)	Effect of diet				Effect of week				P - value		
		Control	4.5% HO	9.0% HO	SE	Wk 4	Wk 8	Wk 12	SE	Diet	Wk	Diet x Wk
LA(18:2n-6)	4	818	763^B	816^B								
	8	912	873^A	964^A								
	12	827	914^A	970^A								
	Overall	852	850	917	22.1	799^b	916^a	903^a	16.9	0.070	< 0.0001	< 0.05
GLA(18:3n-6)	4	5.48	6.27	8.61								
	8	6.43	7.53	10.4								
	12	5.90	7.59	10.3								
	Overall	5.93^c	7.13^b	9.78^a	0.28	6.78^b	8.12^a	7.94^a	0.19	< 0.0001	< 0.0001	0.069
ARA(20:4n-6)	4	68.4	57.4	55.9								
	8	72.6	61.7	62.7								
	12	68.3	63.2	62.4								
	Overall	69.8^a	60.8^b	60.3^b	1.42	60.6^b	65.7^a	64.7^a	1.14	< 0.001	< 0.01	0.24
ALA(18:3n-3)	4	13.3	65.7^B	135^B								
	8	13.5	78.0^A	165^A								
	12	12.4	83.5^A	167^A								
	Overall	13.1^c	75.7^b	156^a	2.42	71.4^b	85.5^a	87.6^a	1.87	< 0.0001	< 0.0001	< 0.0001
EPA(20:5n-3)	4	0.00	1.26	2.25								
	8	0.00	1.41	2.40								
	12	0.00	1.38	2.43								
	Overall	0.00^c	1.35^b	2.36^a	0.046	1.17^b	1.27^a	1.27^a	0.034	< 0.0001	< 0.05	0.24
DPA(22:5n-3)	4	1.68	3.45	5.07								
	8	1.67	3.89	5.07								
	12	1.52	3.77	5.35								
	Overall	1.62^c	3.70^b	5.17^a	0.25	3.40	3.54	3.55	0.16	< 0.0001	0.41	0.24
DHA(22:6n-3)	4	16.9	37.1	40.6^B								
	8	16.6	40.3	45.1^A								
	12	14.0	40.5	45.1^A								
	Overall	15.8^b	39.3^a	43.6^a	1.61	31.5^b	34.0^a	33.2^a	1.01	< 0.0001	< 0.01	< 0.001

[1]Data are presented as least square means (LSM) ± standard error (SE) (n = 7 hens per treatment).
[2]Linoleic acid (LA), gamma-linolenic acid (GLA), arachidonic acid (ARA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA).
[a-c]Different superscripts between treatments (effect of diet) or periods (effect of week), within a row, are significantly different at $P < 0.05$.
[A,B]Different superscripts within a treatment (diet), within a column, for each parameter, are significant different ($P < 0.05$), comparison of diet by week interaction.

EPA, DPA and DHA) in both the HS (Table 5.5) and HO (Table 5.6) treatment groups were measured. A major increase (by 13 fold) was observed in the contents of ALA achieving 152 ± 3.56 mg/yolk for HS and 156 ± 2.42 mg/yolk for HO treatments at the highest level of inclusion compared to control treatment. Compared to the control group, hens consuming hempseed products continued to deposit higher levels of ALA in the egg yolk, with the greatest levels observed after 12 and 8 weeks of feeding HS ($P < 0.0001$, Table 5.5) and HO ($P < 0.0001$, Table 5.6 and Figure 5.1), respectively. Among the long chain PUFA (LCPUFA), although EPA was not preferentially deposited in the egg yolk, upon feeding HS products to the laying hens, the levels of this fatty acid in the total lipid of egg yolk, showed a significant ($P < 0.001$) linear association (regression) to the levels of ALA in the total lipid of egg yolk for HS ($EPA_{yolk \ (mg/yolk)}$ $= 0.0159 \ ALA_{yolk \ (mg/yolk)} - 0.0575$; $R^2 = 0.95$, $P < 0.001$) and HO treatments ($EPA_{yolk \ (mg/yolk)} =$ $0.0159 \ ALA_{yolk \ (mg/yolk)} - 0.115$; $R^2 = 0.97$, $P < 0.001$), based on week 12 data.

The longer chain metabolites of ALA were significantly ($P < 0.0001$) increased in both the HS (Table 5.5) and HO (Table 5.6) treatment groups although proportional increases, particularly in the amount of DPA and DHA did not reflect the corresponding increase in the levels of dietary ALA (inclusion levels of HS or HO). The level of DHA increased from 16.2 to 41.3 ± 1.57 mg/yolk and from 15.8 to 43.6 ± 1.61 mg/yolk for HS and HO treatment groups, respectively at the highest level of inclusion. There was no significant period effect or an interaction with treatment in the accumulation levels of the n-3 LCPUFA of yolks derived from hens consuming either HS (Table 5.5) or HO (Table 5.6) products. The one exception was DHA in the HO treatment group, where there was a significant period ($P < 0.01$) and week $\times$ treatment interaction ($P < 0.001$, Table 5.6) in weeks 8 and 12 compared to that in week 4. The latter
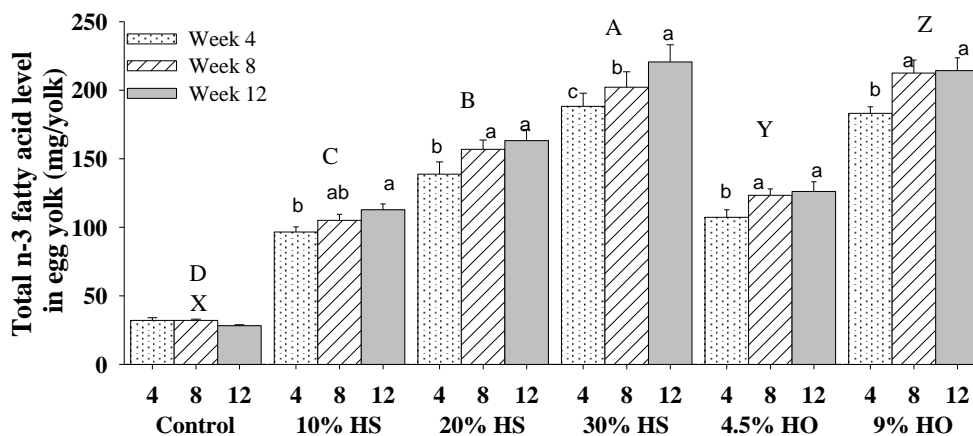
Figure 5.1. Total n-3 fatty acid, sum of ALA, EPA, DPA and DHA (mg/yolk) in egg yolk as a function of period (week 4, 8 and 12) of hens fed diets containing varying levels of hempseed (HS: 10, 20 and 30% of diet) or hempseed oil (HO: 4.5 and 9.0% of diet).

Data point in each period is a group mean ± SE (n = 7 hens per treatment). Letters denote significant differences ($P < 0.05$) between periods within a treatment and between diets within a treatment group, for either HS or HO vs. control.

observation may relate to the fact that the accumulation of DHA in the egg yolk attains a maximal level.

Over the 12 week feeding period, the total n-3 PUFA composition of egg yolk lipids reflected that of the laying hen diets (primarily due to that of ALA content) in both the HS (Table 5.5) and the HO (Table 5.6) treatments (Figure 5.1). Overall, the accumulation of total n-3 PUFA into the yolk significantly ($P < 0.0001$) increased as a function of increasing inclusion of the hempseed products in the laying hen diets. A period effect on the amounts of total n-3 PUFA in the egg yolks indicated a significant ($P < 0.001$, Figure 5.1) gradual increase for the HS containing diet (at 30% level), with the highest levels observed at week 12 compared to that in week 4. For HO groups, a week $\times$ treatment interaction was observed for both levels of HO (4.5 and 9.0%), as total n-3 PUFA were maintained significantly ($P < 0.05$) greater at week 8 compared to week 4 (Figure 5.1). As a result of the enhanced amount of n-3 PUFA concurrent with decreased ARA levels (Tables 5.5 and 5.6), the n-6 to n-3 ratio in the egg yolk obtained from HS and HO receiving hens, gradually decreased ($P < 0.0001$), with the lowest values observed at 30% HS and 9.0% HO (Figure 5.2). Although the n-6 to n-3 ratio in the egg yolk, as a function of hempseed product inclusion in the laying hen diets, was not influenced by period or the interaction with treatment effects, the control diet-fed hens produced eggs that showed a significant ($P < 0.0001$) period $\times$ treatment interaction in the n-6 to n-3 ratio in the egg yolk, - indicating an increase in the ratio as time of feeding increased.

5.4.3   *Fatty acid composition in the TAG and total PL lipid classes*

The levels of fatty acid composition in both the TAG and the total PL fractions of the yolk are presented for both the HS and HO treatment groups (Tables 5.7 and 5.8, respectively). Regardless of the percentage of inclusion of the hempseed products in the hen diets, the
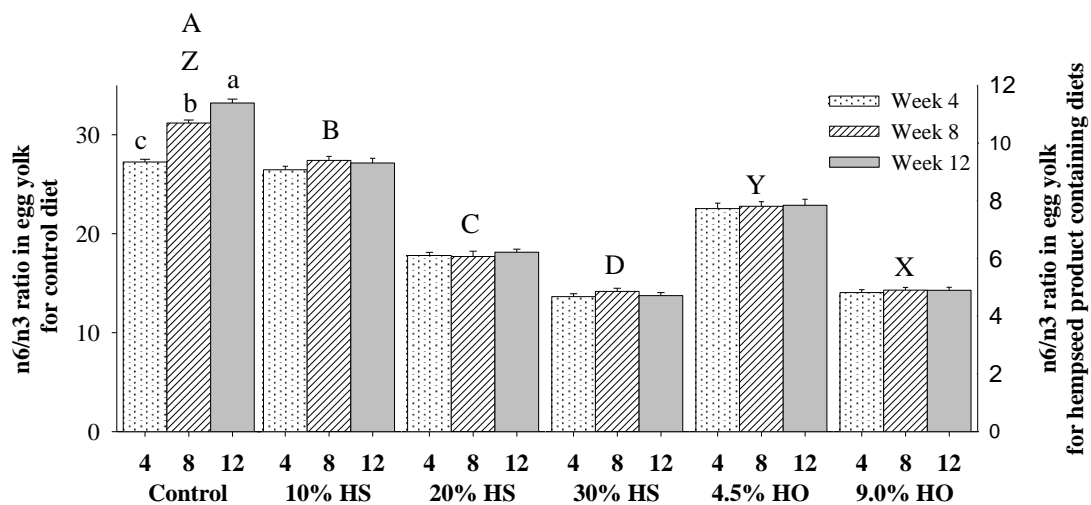
Figure 5.2. n6/n3 PUFA ratio in egg yolk as a function of period (week 4, 8 and 12) of hens fed diets containing varying levels of hempseed (HS: 10, 20 and 30% of diet) or hempseed oil (HO: 4.5 and 9.0% of diet).

Data point in each period is a group mean ± SE (n = 7 hens per treatment). Letters denote significant differences ($P < 0.05$) between periods within a treatment and between diets within a treatment group, for either HS or HO vs. control.

Table 5.7: Egg yolk triacylglycerol (TAG) fatty acid profiles (% of total fatty acid, by wt) after feeding hens with diets containing hempseed-product[1]

| Fatty acids | Hempseed (HS) | | | | | | Hempseed oil (HO) | | | | |
(%, by wt)	Control	10% HS	20% HS	30% HS	SE	*P* - value	Control	4.5% HO	9.0 % HO	SE	*P* - value
Total SFA[2]	31.6	31.7	32.1	32.5	0.48	0.50	31.6	31.4	33.0	0.52	0.082
Total MUFA[3]	35.2^a	31.8^b	28.8^c	22.5^d	0.76	< 0.0001	35.2^a	31.5^b	25.9^c	0.57	< 0.0001
PUFA											
LA(18:2n-6)	29.2^c	30.7^{bc}	31.4^b	34.3^a	0.67	< 0.001	29.2	30.0	31.6	0.74	0.093
GLA(18:3n-6)	0.189^c	0.216^c	0.261^b	0.326^a	0.012	< 0.0001	0.189^c	0.213^b	0.289^a	0.007	< 0.0001
ARA(20:4n-6)	0.478	0.413	0.433	0.390	0.023	0.078	0.478^a	0.403^b	0.364^b	0.021	< 0.01
ALA(18:3n-3)	0.445^d	2.27^c	3.76^b	6.69^a	0.25	< 0.0001	0.445^c	2.97^b	5.69^a	0.18	< 0.0001
EPA(20:5n-3)	0.022^c	0.055^b	0.068^b	0.102^a	0.008	< 0.0001	0.022^c	0.053^b	0.094^a	0.006	< 0.0001
DPA(22:5n-3)	0.043^b	0.090^a	0.099^a	0.092^a	0.009	< 0.001	0.043^c	0.079^b	0.108^a	0.008	< 0.0001
DHA(22:6n-3)	0.197	0.322	0.387	0.309	0.045	0.052	0.197	0.385	0.367	0.060	0.087
Total n-6	29.9^c	31.3^{bc}	32.0^b	35.0^a	0.69	< 0.001	29.9	30.6	32.3	0.74	0.097
Total n-3	0.71^d	2.68^c	4.72^b	7.15^a	0.28	< 0.0001	0.71^c	3.48^b	6.26^a	0.181	< 0.0001
Ratio n-6/n-3	44.9^a	11.7^b	7.56^c	4.92^c	0.81	< 0.0001	44.9^a	8.84^b	5.19^c	1.01	< 0.0001

[1]Data represents least square means (LSM) ± standard error (SE), n=7 per treatment. Different superscripts within a treatment group within a row are significantly different at $P < 0.05$.
[2]SFA: myristic, (14:0), palmitic (16:0) and stearic (18:0).
[3]MUFA: palmitoleic (16:1) and oleic (18:1).
[4]Total n-6: linoleic acid (LA), gamma-linolenic acid (GLA) and arachidonic acid (ARA).
[5]Total n-3: alpha-linolenic acid (ALA), EPA, docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA).

Table 5.8: Egg yolk total phospholipid (PL) fatty acid profiles (% of total fatty acid, by wt) after feeding hens with diets containing hempseed-product[1]

Fatty acids	Hempseed (HS)						Hempseed oil (HO)				
(%, by wt)	Control	10% HS	20% HS	30% HS	SE	*P* - value	Control	4.5% HO	9.0 % HO	SE	*P* - value
Total SFA[2]	48.0	48.2	48.0	48.2	0.38	0.98	48.0	47.3	48.2	0.35	0.27
Total MUFA[3]	18.9^a	18.2^a	18.4^a	17.2^b	0.29	< 0.01	18.9^a	18.9^a	17.6^b	0.32	< 0.05
PUFA											
LA(18:2n-6)	21.7	20.8	20.8	20.9	0.26	0.062	21.7^a	20.6^a	19.4^b	0.38	< 0.01
GLA(18:3n-6)	0.153^d	0.199^c	0.234^b	0.349^a	0.013	< 0.0001	0.153^c	0.216^b	0.325^a	0.012	< 0.0001
ARA(20:4n-6)	6.41	5.79	5.77	5.86	0.19	0.0788	6.41^a	6.03^b	5.49^b	0.16	< 0.01
ALA(18:3n-3)	0.139^d	0.339^c	0.494^b	0.723^a	0.009	< 0.0001	0.139^c	0.391^b	0.618^a	0.015	< 0.0001
EPA(20:5n-3)	0.000^d	0.039^c	0.060^b	0.088^a	0.003	< 0.0001	0.000^c	0.055^b	0.083^a	0.003	< 0.0001
DPA(22:5n-3)	0.126^c	0.268^{ab}	0.248^b	0.318^a	0.023	< 0.001	0.126^c	0.230^b	0.338^a	0.012	< 0.0001
DHA(22:6n-3)	1.46^b	3.82^a	4.04^a	3.85^a	0.18	< 0.0001	1.46^b	3.88^a	4.02^a	0.150	< 0.0001
Total n-6[4]	28.3^a	26.8^b	26.8^b	27.1^b	0.33	< 0.05	28.3^a	26.9^b	25.2^c	0.41	< 0.001
Total n-3[5]	1.73^b	4.47^a	4.84^a	4.98^a	0.21	< 0.0001	1.73^c	4.56^b	5.06^a	0.155	< 0.0001
Ratio n-6/n-3	16.7^a	6.02^b	5.54^b	5.52^b	0.41	< 0.0001	16.7^a	5.95^b	5.01^b	0.41	< 0.0001

[1]Data represents least square means (LSM) ± standard error (SE), n=7 per treatment. Different superscripts within a treatment group within a row are significantly different at *P* < 0.05.
[2]SFA: myristic, (14:0), palmitic (16:0) and stearic (18:0).
[3]MUFA: palmitoleic (16:1) and oleic (18:1).
[4]Total n-6: linoleic acid (LA), gamma-linolenic acid (GLA) and arachidonic acid (ARA).
[5]Total n-3: alpha-linolenic acid (ALA), EPA, docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA).

concentrations of SFA in yolk lipids (the TAG or the total PL) were not affected by treatment.

As the level of HS or HO inclusion in the diet increased, the concentrations of MUFA

significantly decreased in both the TAG ($P < 0.0001$) and the total PL ($P < 0.01$) lipid fractions

compared to the control diet fed group. However, in the latter fraction, the decrease was only

noted at higher levels of HS (30%) or HO (9.0%) inclusion. The level of LA significantly ($P <$

0.001) increased in the TAG fraction of the egg yolk for the HS treatment groups, but did not

change across the HO treatment groups. The reverse was true for LA levels in the total PL

fraction of the egg yolk (Tables 5.7 and 5.8). Gamma-linolenic acid increased significantly ($P <$

0.0001) with each additional increment of either HO or HS in both lipid fractions. While the

ARA levels in the egg yolk were not affected by the levels of HS in the diets of the hens for both

the TAG and the total PL fractions, decreased levels ($P < 0.01$) of the fatty acid were noted with

the HO treatments in both lipid fractions.

As a percentage of total fatty acids, the levels of ALA in the egg yolk represented a

greater ($P < 0.0001$) fraction of the TAG than the total PL in both the HS and HO treatment

group (Tables 5.7 and 5.8). Whereas the incorporation of DPA and DHA was observed mainly

in total PL fractions of the egg yolks from both the HS and HO treated hens, EPA was highly (P

< 0.0001) contained in the TAG fractions (data not presented). Although the DHA levels in the

TAG fraction of egg yolk were not affected by the levels of inclusion of the hempseed products

in the hen diets, this fatty acid reached a plateau in the total PLs of the egg yolk for both the HS

and HO groups.

The total n-6 PUFA level increased significantly ($P < 0.001$) with increasing levels of HS

in the egg yolk TAG fraction. A similar but non-significant pattern of increase was observed for

total n-6 PUFA level in the TAG of the egg yolk obtained upon HO feeding (Table 5.7). The

total n-6 PUFA levels in the total PL of egg yolk decreased significantly with increasing levels of either HS ($P < 0.05$) or HO ($P < 0.001$) in the total PL fraction (Table 5.8). Total n-3 PUFA increased significantly ($P < 0.0001$) due to the inclusion of hempseed products in both the TAG (Table 5.7) and total PL (Table 5.8) fractions of the egg in comparison to those obtained from the control fed hens. Furthermore, the inclusion of hempseed products (HS or HO) in the diets significantly ($P < 0.0001$) decreased the n-6 to n-3 PUFA ratio in both the TAG (Table 5.7) and total PL (Table 5.8) fractions of the egg in comparison to those obtained from the control fed hens. However, in the latter lipid fraction, no differences in the ratio were observed between the hempseed-containing diets. The highest reduction in the ratio, compared to the control, was achieved in TAG rather than the total PL fraction.

5.5 DISCUSSION

The present study was conducted to examine the effects of dose and duration of feeding hempseed products on the fatty acid composition of egg yolk lipid and major fractions. In general, increasing levels of hemp products in diets for the laying hen effectively increased the n-3 PUFA profile of the total lipid, TAG and total PL fractions of yolks. As has been well documented, the inclusion of plant-based sources of n-3 PUFA in laying hen diets leads to increases in the n-3 LCPUFA content of egg yolk total lipids (Caston and Leeson, 1990; Baucells et al., 2000; Souza et al., 2008). As ALA is the primary plant n-3 fatty acid, it stands to reason that this fatty acid should also reflect the major form of n-3 PUFA in the egg yolk. This has been previously observed for total egg yolk lipids for hens consuming hempseed products (Silversides and Lefrançois, 2005; Gakhar et al., 2012). Flaxseed oil contains about 53% ALA (Scheideler and Froning, 1996). On average, hens fed flaxseed up to 15% (Scheideler and Froning, 1996),

(Lewis et al., 2000) or up to 20% (Ferrier et al., 1995) in the diet produced eggs containing about 322 and 90 mg/egg of total n-3 and DHA, respectively (Aymond and Van Elswyk, 1995; Scheideler and Froning, 1996). On the other hand, hempseed oil and hempseed containing 18% and 7% ALA, respectively (Parker et al., 2003), incorporated approximately 160 to 250 mg/egg total n-3 and 41 to 50 mg/egg DHA, at 20 to 30% HS and 9.0 to 12% HO inclusions in the current and previous studies (Gakhar et al., 2012). This efficiency is related to the fact that DHA reaches a plateau, so further increases in ALA intake does not translate into subsequent proportional increase in the level of DHA. Although a comparative assessment of the economics between the two sources may also be useful, hempseed products have yet to be approved for utilization in poultry and other livestock diets.

In contrast, limited information exists as to the nature of the distribution of the various n-3 PUFA between the lipid classes in egg yolk as a function of dietary supply (Jiang et al., 1991). For total, as well as TAG and PL fractions, yolk ALA increased significantly in response to increasing dietary ALA from hemp products. The TAG was more responsive, increasing approximately 13- to 15-fold, over the control, at the highest level of HS or HO inclusion ($6.69 \pm 0.25\%$ and $5.69 \pm 0.18\%$ total fatty acids, respectively), consistent with the observed increases in total ALA content of egg yolk. As TAG represents approximately 65% of the total lipid in egg yolk (Anton and Gandemer, 1997), the close agreement in enrichment patterns is not unexpected. The ALA in the PL fraction, on the other hand, while enriched, was increased by only 4-fold over the control. This was in accordance with the findings by Jiang et al. (1991) were moderate increases in the levels of ALA were reflected in the major PL sub-classes but dominantly contained in the TAG fraction. Consistent with our findings at the highest levels of HS or HO inclusion, the latter authors observed similar amounts of ALA and total n-3 PUFA in the egg

yolk TAG fractions (6.9 and 7.1% total fatty acids, respectively). While not reported, the total n-3 fatty acid content of PL (PC and PE) fractions, particularly in the PC, were closely related when hens consumed hempseed products, PC: 4.08 ± 0.14 and PE: 10.8 ± 0.44 % of total fatty acids (unpublished data) to that of flaxseed PC: 6.1 and PE: 16.8 % of total fatty acids (Jiang et al., 1991).

While ALA enrichment of the yolk lipid fractions demonstrated dose-dependent increases, differential responses were observed for the n-3 LCPUFA in the 12-week feeding regime. The levels of EPA and DPA, both precursors for DHA synthesis, in general, increased in response to graded ALA intakes from hemp products, particularly in the PL fraction of egg yolk. The presence of EPA, DPA and DHA in yolk lipids reflects endogenous synthesis from ALA and deposition in the yolk, since the diets were essentially devoid of these fatty acids. Docosahexaenoic acid is derived from ALA through the sequential actions of desaturase and elongase enzymes, as well as β-oxidation with both EPA and DPA serving as intermediates (Sprecher, 2000). The observed pattern of responses in DHA accumulation provides insight into n-3 fatty acid metabolism within the hen. As previously demonstrated in total yolk lipids (Gakhar et al., 2012; Goldberg et al., 2012), increasing ALA intake by hens in response to dietary hemp inclusion results in increased DHA levels, but the response is saturable. The current data extends these results by providing evidence that both the TAG and PL fractions respond in a similar fashion. Further mechanistic studies are required to elucidate the biological rationale for this plateau. Previous research examining changes in n-3 LCPUFA in total egg yolk lipids, in response to graded levels of microencapsulated fish oil in a 21-day feeding trial, demonstrated significant capacity for the deposition of preformed DHA into total yolk lipids, with no evidence of a plateau being reached (Lawlor et al., 2010). Furthermore, even though

EPA intake exceeded DHA intake by a factor of 1.5 in the latter study, egg yolk deposition of EPA (total lipids) was approximately 25% of that observed for DHA, suggesting alternative hepatic handling of these preformed fatty acids. Such measures may include the possibility that EPA is metabolized in the liver to such a great degree that most of it gets converted to DHA and subsequently incorporated into yolk-destined VLDL.

Consumer demand for n-3 enriched eggs has driven interest in the examination of alternative dietary sources of n-3 PUFA for laying hens. As hemp oil, either as a component of hempseed or as the extracted oil, contains approximately 19% of its fatty acid profile as ALA, it can potentially serve as a source of dietary ALA for laying hens. The total n-3 PUFA in the egg yolk increased by about 7-fold when using 30% HS (209 ± 4.74 mg/yolk) and 9.0% HO (207 ± 3.88 mg/yolk) compared to the level observed when hens were fed the control diet (30.7 ± 4.74 mg/yolk) (Figure 5.1). Highest enrichment levels of total n-3 PUFA (primarily due to the changes of ALA content) in the eggs was achieved at week 8 for the HO (4.5 and 9.0% levels) treatment groups and at week 12 for HS (at 30% inclusion) although no statistical difference in enrichment beyond week 8 was observed at lower levels of HS inclusions. These results are in accordance with previous research findings (Carvalho et al., 2011) indicating maximum accumulation levels of n-3 PUFA in egg yolk after 8 weeks of experimental feeding; although, stability in egg yolk n-3 PUFA deposition may follow 4 week of feeding (Hargis et al., 1991).

With respect to the total n-6 PUFA, hempseed being relatively rich source of GLA, the egg content of this fatty acid in both the total lipids and the major lipid classes closely resembled the changes in the laying hen diets. Overall, for the n-6 PUFA in the total lipid of egg yolk, greater accumulations were primarily associated with prolonged feeding of the diets, as evident by significant diet by time interactions. Although dietary LA levels were similar between

treatments, LA accumulation was observed to increase in the TAG and decreased in the PL fraction, but did not reflect in similar changes with the levels in ARA, accumulating more in the PL fraction than the TAG of egg yolk. Given the absence of ARA in the laying hen diets, egg yolk ARA reflects endogenous synthesis in the liver and deposition to eggs. The levels of ARA in the various egg yolk lipid fractions decreased with increasing hemp product inclusion, indicating the two desaturase enzymes ($\Delta6$ and $\Delta5$) and one elongase (elongase 5) (Tu et al., 2010) also favor n-3 fatty acid metabolism as found in other animal model (Yeh et al., 1998).

On the other hand, while the PUFA profile of egg yolk was greatly influenced by the levels of the primary fatty acid in the hens' diets (i.e. increasing HS or the HO levels of inclusion in the diets reflecting corresponding increases in the amounts of dietary ALA), the total SFA in the egg yolk was not influenced by either the HS or the HO levels of inclusion. These results are in agreement with other studies utilizing flaxseed that provided evidence that the overall SFA levels in the egg yolk are not majorly influenced by dietary manipulations (Caston and Leeson, 1990; Scheideler and Froning, 1996). However, a significant impact on SFA content of egg yolk in other lipid feeding trials, has also been reported (Souza et al., 2008; Hayat et al., 2009). Although a previous study (Yalcyn et al., 2007) in which fish oil and flaxseed inclusion in hen diets showed greater reductions in the amount of palmitic acid (predominant SFA) during prolonged feeding, in the current study, no diet $\times$ week interaction was noted in this fatty acid in the total egg yolk lipid across the dietary treatments. Consistent with the total egg yolk lipid, the overall levels of total SFA in the both the TAG and PL lipid classes were not influenced by the dietary treatments.

The levels of MUFA in both the total lipid and the TAG fractions of egg yolk decreased with increasing levels of inclusion of either hemp product, by reflecting the fatty acids of the

diets. However, this was not the case for the PL fraction, where reductions were observed, relative to controls, only for the highest levels of HS and HO inclusion. In general, changes in MUFA occurred primarily via changes in oleic acid, the predominant MUFA in both the diets and the eggs in this study. These data may support previous findings that provided evidence of a depressive effect of PUFA-rich diets on the desaturation of palmitic (18:0) to oleic (18:1) acid by Δ9-desaturase (Garg et al., 1988). However, given the reduction in the oleic acid levels in the diet with increasing hemp product inclusion, the results may reflect a simple response to dose. In addition, based on diet compositions (Table 5.1), with the inclusion of HS and HO, the levels of other dietary ingredients changed; hence, changes in egg fatty acid contents could be due to changes in some of the other ingredients. Regardless, the PL fraction appears resistant to the manipulation of its MUFA content (Jiang et al., 1991) indicating the differential incorporation of the fatty acid between PL and TAG. Overall, palmitic acid, oleic acid, and LA represent the major fatty acids in yolk total lipid, TAG and PL fractions.

In summary, hempseed products compared to the control improved the nutritional value of the egg, resulting in decreased n-6 to n-3 PUFA ratio, increased total n-3 PUFA and selectively incorporated higher levels particularly of DHA into the PL fraction. Although, the utilization of hempseed products in laying hen diets, overall, results in maximum total n-3 PUFA enrichment achievable by week 8; egg enrichment with n-3 PUFAs is mainly due to the dosage of dietary inclusion rather than time effect (duration of feeding).

CHAPTER 6 MANUSCRIPT III

Increasing levels of dietary hempseed products leads to differential responses in the fatty acid

profiles of egg yolk, liver and plasma of laying hens

6.1 ABSTRACT

The limited efficiency with which dietary alpha-linolenic acid (ALA) is converted by hens into docosahexaenoic acid (DHA) for egg deposition is not clear. In this study, dietary ALA levels were increased, via hempseed (HS) and hempseed oil (HO) inclusion in hen diets, with the goal of assessing effects on fatty acid profiles of total and lipid classes in yolk, liver and plasma. Forty-eight hens were individually caged and fed 1 of 6 diets containing either HS:10, 20 or 30, HO:4.5 or 9.0 (%, diet) or a control (containing corn-oil), providing a range (0.1 to 1.28%, diet) of ALA. Fatty acid methyl esters of total and lipid classes, including phosphatidyl choline (PtdCho) and ethanolamine (PtdEtn) in yolk, plasma and liver were determined. Levels of n-3 Fatty acids in both total and lipid classes increased in all tissues. ALA and eicosapentaenoic acid (EPA) increased linearly, while docosapentaenoic acid and DHA increased quadratically. Fatty acid profiles of yolk closely reflected levels in both plasma and liver. While ALA was highly concentrated in the triacylglycerol, it was low but equally distributed between PtdCho and PtdEtn in all tissues; however, the net accumulation was lower ($P<0.0001$) in liver compared to yolk and plasma. Levels of EPA and ALA in yolk-PtdEtn were linearly ($P<0.0001$; $R^2 = 0.93$) associated, and reflected those in liver-PtdEtn ($P<0.0001$; $R^2 = 0.90$). In the liver, a strong inverse correlation ($P<0.0001$; r = -0.94) between PL-DHA and ALA/EPA ratio in PtdEtn supports theories of low substrate (ALA) availability, possibly limiting the conversion of ALA into DHA for egg enrichment.

Key words: egg yolk, liver, plasma, dietary ALA, fatty acids, lipid classes

6.2 INTRODUCTION

The omega-3 polyunsaturated fatty acid (PUFA), alpha-linolenic acid (ALA, 18:3n-3) has an established Adequate Intake (AI) value for adult humans of 1.1 and 1.6 g/d for females and males, respectively (Institute of Medicine, 2005). Despite calls for the establishment of specific requirement values for the n-3 long chain PUFA (LCPUFA), particularly eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) (Flock et al., 2013), no specific AI values have been established via the Dietary Reference Intake (DRI) process. Instead, the DRI process concludes that, on the basis of population intake patterns, EPA and DHA can replace up to 10% of the requirement for ALA. No specific mention is made on docosapentaenoic (DPA, 22:5n-3), perhaps due to the numerous health benefits attributed to EPA and DHA (Yashodhara et al., 2009; Fraeye et al., 2012).

The identification or development of alternative dietary sources of the omega-3 LCPUFA beyond traditional sources, such as fatty fish, has gained substantial attention over the past two decades. Because of their wide acceptability in the human diet, chicken eggs can represent a significant source of the omega-3 LCPUFA. Since laying hens have the capacity to endogenously convert dietary ALA into the omega -3 LCPUFA and deposit these into yolk lipids (González-Esquerra and Leeson, 2001), numerous dietary manipulation has been attempted to increase n-3 LCPUFA using either ALA from plant seed and oils (Cherian and Sim, 1991; Scheideler and Froning, 1996; Lewis et al., 2000), or preformed n-3 LCPUFA from either fish/marine oils (Hargis et al., 1991; Herber and Van Elswyk, 1996) or microalgal sources (Harel et al., 2002; Lawlor et al., 2010; Lemahieu et al., 2013). Previous research has documented the effect of increasing the levels of hemp (*Cannabis sativa* L.) products, including hemp seed and hemp oil, on egg yolk lipid profiles, as a function of the duration of feeding to laying hens

(Gakhar et al., 2012). Hemp seed oil contains 19% of ALA in the total fatty acid (Callaway, 2004), and its consumption either as the extracted oil or via milled whole hemp seed, leads to linear increases in egg yolk ALA content in both the triacylglycerol (TAG) and total phospholipids (PL) (Neijat et al., 2016a). However, the enrichment of DHA from dietary hemp was saturable in the egg yolk lipid, as has been observed previously (Gakhar et al., 2012; Goldberg et al., 2012). It is unknown how this differential deposition of omega -3 ALA and DHA takes place. Since liver is a major synthesis site of ALA to DHA, following hepatic, plasma and egg yolk lipid pools may answer some aspects of the question. In addition, the accumulation of n-3 LCPUFA in tissues depends on substrate availabilities for biosynthesis, and the competition among substrates for the enzymes involved in desaturation and elongation (Sprecher, 2000; Tu et al., 2010).

Hence, it was hypothesized in this study that the amount and distribution of fatty acids in different lipid classes would provide a basis for the regulation of LCPUFA biosynthesis. Therefore, the objectives of the current study included the detailed assessment of the omega -3 PUFA profiles of egg yolk, plasma and liver from hens consuming graded levels of ALA from hemp products. These results aim to provide a more comprehensive view of the factors regulating n-3 LCPUFA deposition into egg yolk, a potential source of dietary DHA for humans.

6.3 MATERIALS AND METHODS

6.3.1 *Animals and diets*

All animal management procedures used in this study (the hens used and the diets fed are the same as described by Neijat et al., 2014) were in accordance with recommendations established by the Canadian Council on Animal Care (1993) reviewed and approved by the

University of Manitoba's Animal Care Protocol Management and Review Committee. Forty

eight laying hens (Lohmann LSL-Classic), 19 weeks of age, were individually housed in cages

and maintained in an environmentally controlled room. The experimental diets (wheat-barley-

soybean basal ingredients) were formulated to be isocaloric, isonitrogenous and isolipidic

(2800kcal AMEn/kg diet; 17% CP and 11% crude fat) and fed to eight hens each for 12 weeks.

The diets included a control diet containing no hempseed product (corn-oil supplemented, 9.86%

of diet), three diets providing 10, 20, or 30% of diet hempseed (HS; containing $32.8 \pm 1.05\%$

lipid) and two diets with 4.5 or 9.0% of diet hempseed oil (HO) as a lipid source, resulting in

diets containing ALA levels of 0.01, 0.50, 0.66, 0.90, 1.22 and 1.28% of diet (by wt; Table 6.1,

reproduced from Neijat et al., 2014). Although hempseed also contains some stearidonic acid

(18:4n-3; Petrović et al., 2015), the content of this fatty acid in the hempseed products used in

this study was not determined, as lipid standards for its identification were not included in the

analysis. The detailed nutrient compositions of the diets and hempseed products sourced from

Hemp Oil Canada (St. Agathe, MB) used in this study, have been previously described by Neijat

et al. (2014) and Gakhar et al. (2012), respectively.

Diet (duplicates per treatment) and tissue (egg yolk, liver and plasma, n=8) samples were

obtained after feeding 12 weeks of the diets. Eggs were collected during the last 3 days of the

experimental period, and 1 egg per hen (n=8) per treatment was subjected to breaking, careful

yolk separation from the white using an egg separator, and the resultant yolks individually

weighed and stored in plastic bags at $-20^{\circ}C$ until analyzed. Blood samples were collected from

the wing vein in heparinized syringes (4 mL vol), using 25G x 5/8 (0.5mm x 16mm) needles,

transferred to lithium heparinized vacutainers, centrifuged (2800 RCF, 20 min, $4^{\circ}C$) and the

Table 6.1: Description, composition and fatty acid profile of experimental laying hen diets containing increasing levels of alpha-linolenic acid (ALA) from hempseed products[1,2]

Source of ALA	Control	10%HS	4.5%HO	20%HS	30%HS	9.0%HO
ALA content (% of diet)	0.10	0.50	0.66	0.90	1.22	1.28
Diet composition (%)						
Wheat	35.0	33.4	35.0	31.9	30.3	35.0
Barley	15.0	15.0	15.0	15.0	15.0	15.0
Soybean meal	21.5	16.9	21.5	12.2	7.59	21.5
Hemp seed	0.00	10.0	0.00	20.0	30.0	0.00
Hemp oil	0.00	0.00	4.50	0.00	0.00	9.00
Corn oil	9.68	6.48	5.18	3.28	0.07	0.68
Vitamin-mineral premix	2.50	2.50	2.50	2.50	2.50	2.50
Limestone	13.9	13.5	13.9	13.0	12.4	13.9
Dicalcium phosphate	1.88	1.60	1.88	1.56	1.52	1.88
Sodium chloride	0.34	0.35	0.34	0.35	0.35	0.34
DL-Methionine	0.116	0.095	0.116	0.073	0.051	0.116
L-Lysine-HCl	0.083	0.128	0.083	0.173	0.218	0.083
L-Threonine	0.005	0.000	0.005	0.000	0.000	0.005
Fatty acid profile[3] (mg/g of diet)						
Total SFA	9.74	9.09	8.42	7.88	6.46	7.29
Total MUFA	17.8	14.4	12.7	10.5	6.09	7.70
18:2n-6 (linoleic, LA)	38.1	39.2	39.0	38.6	36.3	40.9
18:3n-6 (gamma-linolenic, GLA)	0.01	0.92	1.30	1.83	2.57	2.70
18:3n-3 (alpha-linolenic, ALA)	0.96	5.00	6.64	8.95	12.2	12.8
Total PUFA	39.1	45.1	46.9	49.4	51.0	56.5

[1]Diets contain increasing levels of ALA (% of diet) supplied by hemp-derived products (hempseed, HS or hempseed oil, HO).
[2]Calculated nutrient contents in each diet included: 2800 AMEn (Kcal/kg), 17 CP (%) and 11 crude fat (%). Vitamin-mineral premix composition and analyzed nutrient values were as previously described by Neijat et al. (2014).
[3]Saturated (SFA = myristic (14:0), palmitic (16:0), stearic (18:0)); monounsaturated (MUFA = palmitoleic (16:1), oleic (18:1)) and polyunsaturated fatty acids (PUFA = LA, GLA, ALA).

plasma was separated and stored at -20°C until analyzed. Birds were then euthanized by cervical

dislocation and liver samples were quickly removed, rinsed in cold saline and frozen in liquid

nitrogen for storage at -80°C until analyzed.

6.3.2 *Lipid extraction of samples*

Approximately 1 g of ground feed, 3 g of yolk, 100 µL of plasma and 0.5 g of liver tissue

were used for the extraction of total lipids using chloroform-methanol (2:1, by vol) according to

Folch et al. (1957). The extracted total lipids were dried under nitrogen and weighed. The feed

and egg yolk extracts were first dissolved in hexane to a volume of 25mL to obtain aliquots of a

known volume (containing 40 - 50 mg lipid). The dried lipid extracts for all samples were

dissolved in 1 mL toluene and esterified by heating in the presence of methanol containing 2%

(by vol) sulphuric acid (Burdge et al., 2000). The resulting fatty acid methyl esters (FAME)

were extracted into iso-octane for determining fatty acid profile using gas chromatography (GC).

Separation of the major lipid classes (TAG, PL and cholesteryl ester (CE)) and further

fractionation of the total PL into phosphatidylcholine (PtdCho) and phosphatidylethanolamine

(PtdEtn) of egg yolk, plasma and liver were conducted by thin layer chromatography (TLC) on

silica gel (Analtech, Inc, Newark, DE, USA) G and H plates, respectively, according to the

method described by (Suh et al., 1994) with slight modification. A solvent system of petroleum

ether/diethyl ether/acetic acid (80:20:1, by vol) was used for TAG, CE and total PL while

chloroform/methanol/2-propanol/0.25% KCl/triethylamine (41.5:12:34:8:24.5, by vol) was used

for PtdEtn and PtdCho. For both procedures, approximately 2.4 mg of lipid extract was applied

on the TLC plates. Lipid classes were visualized with 0.1% (wt/vol) 2′, 7′-dichlorofluorescein in

methanol under UV light. Prior to methylation, the neutral lipids (TAG and CE fractions) were

saponified according to the method previously described (Hartman, 1973). All lipid fractions

were then methylated using 14 % boron trifluoride in methanol, and fatty acids quantified by GC. Although total lipid fatty acid analysis was based on a sample size of n=8 for each tissue type, the sample size TLC analysis was $4 \leq n \leq 7$; the latter depended on the amounts of tissue samples. For both total and lipid classes, C17:1 (Nu-Chek Prep Inc., Elysian, MN) was used as the external standard.

6.3.3 Fatty acid analysis

Fatty acid methyl esters of total lipids and lipid classes derived from yolk, liver and plasma were analyzed using a Varian 450 GC with flame ionization detector (FID) and equipped with a DB225MS column (30 m × 0.25 mm diameter and 0.25 μm film thickness, Agilent Technologies Canada Inc., Mississauga, Ontario). The GC program settings used for the analysis were as described previously (Neijat et al., 2016a). Each fatty acid was identified by comparing its retention time to authentic standard samples of known composition (Lipid standards, Nu-Chek Prep, Inc., Elysian, MN, USA). The peak area for an individual fatty acid was expressed as a percentage of the total peak areas for all identified fatty acids (g fatty acid/100g total fatty acid), and then converted to mol% using the molecular weight of the FAME.

6.3.4 Statistical analysis

The fatty acid compositions of total and each lipid class from each tissue were analyzed applying a statistical model using diet type (dietary ALA level or intake of hempseed products) as fixed effects using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC) accounting for the random effect of hens. Least squares means (LSM), adjusted using Tukey's significant difference test, were compared for significant difference ($P < 0.05$). Normality of the data distribution was assessed using the Shapiro-Wilk test and data points with studentized residuals

below or above ±3.0 were considered outliers and excluded from the analysis. Using the same statistical package, Proc Reg and Proc Corr procedures were used to evaluate the significance of the linear and quadratic terms; and the Pearson Correlation Coefficients (r), respectively.

6.4 RESULTS

6.4.1   Experimental diets

The fatty acid compositions of the diets are presented in Table 6.1. The linoleic acid (LA, 18:2n-6) and ALA in the HS and HO products in the laying hen diets were 17.0 and 5.2% for HS; and 54.2 and 18.9% for HO, respectively, as indicated previously (Neijat et al., 2014). The higher the inclusion of hempseed products in the diets of the laying hens, the greater the ALA content supplied; however, the LA levels in all diets were similar within the range of 3.87 ± 0.15%, leading to a corresponding reductions in the LA to ALA ratio in the diets. As the inclusion of hemp product increased, the ALA levels in the diets increased in the order 0.10, 0.50, 0.66, 0.90, 1.22 and 1.28 % of diet (by wt); correspondingly, the hens consumed increasing ALA levels of 85.6 ± 1.74, 449 ± 10.3, 585 ± 10.2, 795 ± 10.1, 1109 ± 28.5 and 1132 ± 25.8 mg/hen/day, respectively. Feed intake by the laying hens was reported in our previous work and was found to be similar between treatments (Neijat et al., 2014). Although the total saturated fatty acids (SFA) in the diets were in very close range, monounsaturated fatty acids (MUFA) decreased, mainly due to a reduction in oleic acid content. The levels of gamma-linolenic acid (GLA, 18:3n-6) in the diets also increased (from 0.001 to 0.27 % of diet) in a similar trend to that of the ALA. No LCPUFA (arachidonic acid (ARA, 20:4n-6), EPA, DPA and DHA) were detected in the diets.

6.4.2 *Plasma, egg yolk and liver lipids*

6.4.2.1 *Saturated (SFA) and monounsaturated fatty acids (MUFA)*

The total SFA and MUFA profiles of total lipids, TAG and total PL in the egg yolk, liver and plasma are shown in Tables 6.2, 6.3 and 6.4, respectively. While significant effects of diet on SFA in total lipids (fluctuations) were observed in the liver ($P < 0.01$) and plasma ($P < 0.05$), mainly due to the palmitic (16:0) and stearic (18:0) acids (Table 6.2), there was no effect on egg yolk lipid. For individual lipid classes, the overall levels of SFA in the TAG or the total PL of the egg yolk, liver and plasma were not affected by the diets' lipid composition (Tables 6.3 and 6.4, respectively).

Increasing levels of ALA in the diets of the laying hens, particularly at higher levels of inclusion (1.22 and 1.28% of diet), significantly lowered the levels of total MUFA in the total lipids ($P < 0.0001$), TAG ($P < 0.0001$) and the total PL ($P < 0.01$) in the egg yolk, liver and plasma (a tendency ($P < 0.09$) in the latter lipid fraction for plasma; Tables 6.2, 6.3 and 6.4, respectively), due primarily to changes in the level of oleic acid (18:1). However, these changes, may relate to the fact that the MUFA content of the diets decreased as the level of HS or HO increased. In addition, decreases of MUFA in PL, although significant, were not very large, and in the liver differences were only observed between negative control and the diets containing HS or HO. Furthermore, the trends in accumulation of MUFA in the egg yolk, liver and plasma were observed to be in the opposite direction to that of the total PUFA in the total lipids and TAG but not in the PL fraction (Table 6.2, 6.3 and 6.4, respectively). However, overall average

Table 6.2: Total saturated (SFA), total monounsaturated (MUFA) and total polyunsaturated (PUFA) fatty acids profile (mol%) of total lipids in egg yolk, liver and plasma after feeding hens diets with varying levels of alpha-linolenic acid (ALA, % of diet) derived from hempseed products[1]

Source of ALA	Control	10%HS	4.5%HO	20%HS	30%HS	9.0%HO					
ALA content (% of diet)	0.10	0.50	0.66	0.90	1.22	1.28	SE	P - value	Linear	Quadratic	Adj R^2
Egg yolk											
14:0 (myristic)	0.33	0.31	0.31	0.32	0.30	0.34	0.01	0.09			
16:0 (palmitic)	27.1^a	25.9^{ab}	25.6^{ab}	25.5^b	24.8^b	25.6^{ab}	0.34	< 0.01	< 0.01	0.07	0.28
18:0 (stearic)	8.91^d	9.59^{cd}	10.2^{bc}	10.2^{bc}	11.3^a	10.8^{ab}	0.22	< 0.0001	< 0.05	0.50	0.56
Total SFA	36.3	35.8	36.1	36.0	36.4	36.8	0.30	0.27			
16:1 (palmitoleic)	1.29^a	1.18^{ab}	1.16^{ab}	1.11^{ab}	1.00^b	1.29^a	0.07	< 0.05	0.09	0.17	
18:1 (oleic)	30.5^a	27.3^b	27.7^b	24.6^c	21.0^d	22.8^{cd}	0.57	< 0.0001	< 0.05	0.50	0.77
Total MUFA	32.0^a	28.4^b	28.9^b	25.7^c	22.0^d	24.0^{cd}	0.60	< 0.0001	< 0.05	0.58	0.76
Total PUFA^2	32.0^e	35.7^{cd}	35.1^{de}	38.4^{bc}	41.6^a	39.2^{ab}	0.73	< 0.0001	< 0.05	0.88	0.65
Liver											
14:0 (myristic)	0.24	0.22	0.22	0.22	0.21	0.23	0.01	0.71			
16:0 (palmitic)	24.7^a	23.2^b	23.1^b	22.8^b	22.1^b	22.2^b	0.27	< 0.0001	< 0.001	0.07	0.55
18:0 (stearic)	14.9^b	15.1^{ab}	14.9^b	15.8^{ab}	16.7^a	15.3^{ab}	0.39	< 0.05	0.94	0.56	
Total SFA	39.9^a	38.4^{ab}	38.2^{ab}	38.8^{ab}	39.1^{ab}	37.7^b	0.38	< 0.01	< 0.05	0.08	0.12
16:1 (palmitoleic)	0.64	0.60	0.63	0.60	0.58	0.65	0.05	0.91			
18:1 (oleic)	24.2^a	21.5^b	21.4^b	19.9^{bc}	17.8^{cd}	17.1^d	0.59	< 0.0001	< 0.05	0.79	0.67
Total MUFA	24.8^a	22.1^b	22.1^b	20.5^{bc}	18.4^{cd}	17.7^d	0.61	< 0.0001	< 0.05	0.80	0.66
Total PUFA^2	35.3^d	39.5^c	39.7^c	40.8^{bc}	42.5^{ab}	44.6^a	0.58	< 0.0001	< 0.001	0.36	0.71
Plasma											
14:0 (myristic)	0.32	0.29	0.33	0.29	0.30	0.31	0.01	0.40			
16:0 (palmitic)	25.8^a	24.1^b	24.7^{ab}	23.8^b	23.6^b	23.8^b	0.32	< 0.001	< 0.01	0.06	0.36
18:0 (stearic)	8.46^d	9.25^{cd}	9.98^{abc}	9.67^{bc}	10.8^a	10.4^{ab}	0.23	< 0.0001	< 0.05	0.37	0.50
Total SFA	34.6^{ab}	33.6^c	35.0^a	33.8^{bc}	34.8^a	34.4^{abc}	0.33	< 0.05	0.30	0.25	
16:1 (palmitoleic)	1.07^{ab}	0.87^c	1.07^{ab}	0.96^{abc}	0.91^{b}c	1.10^a	0.06	< 0.05	0.19	0.20	
18:1 (oleic)	30.6^a	28.0^b	28.6^b	25.1^c	21.3^d	23.1^d	0.46	< 0.0001	0.07	0.05	0.82
Total MUFA	31.7^a	28.9^b	29.8^{ab}	26.0^c	22.2^d	24.2^{cd}	0.47	< 0.0001	0.07	0.08	0.81
Total PUFA^2	33.7^d	37.5^c	35.2^{cd}	40.2^b	43.0^a	41.3^{ab}	0.61	< 0.0001	0.06	0.43	

[1]Data are presented as least square means (LSM) ± SE. [a-e]Different superscripts between treatments within a row for each tissue type (yolk, n =7; liver, n = 5; plasma, n = 4 per treatment), are significantly different at $P < 0.05$. Coefficient of determination (R^2) indicated when linear or quadratic effects are significant ($P < 0.05$).

[2]Total PUFA = LA, GLA, ARA, ALA, EPA, DPA and DHA.

Table 6.3: Total saturated (SFA), total monounsaturated (MUFA) and total polyunsaturated (PUFA) fatty acids profile (mol%) of triacylglycerol (TAG) in egg yolk, liver and plasma after feeding hens diets with varying levels of alpha-linolenic acid (ALA, % of diet) derived from hempseed products[1]

Source of ALA ALA content (% of diet)	Control 0.10	10%HS 0.50	4.5%HO 0.66	20%HS 0.90	30%HS 1.22	9.0%HO 1.28	SE	P - value	Linear	Quadratic	Adj R²
Egg yolk											
14:0 (myristic)	0.37	0.37	0.35	0.38	0.36	0.41	0.02	0.18			
16:0 (palmitic)	26.3	25.8	24.7	25.4	24.7	25.5	0.56	0.29			
18:0 (stearic)	6.44^c	6.96^{bc}	7.74^{ab}	7.70^{abc}	8.82^a	8.49^a	0.30	< 0.0001	0.08	0.73	
Total SFA	33.1	33.1	32.8	33.6	33.8	34.3	0.53	0.30			
16:1 (palmitoleic)	1.45	1.56	1.45	1.49	1.24	1.65	0.13	0.35			
18:1 (oleic)	32.9^a	29.6^b	29.4^b	26.8^b	20.8^b	23.7^c	0.61	< 0.0001	0.11	0.18	
Total MUFA	34.4^a	31.1^b	30.8^b	28.2^b	22.1^d	25.3^c	0.65	< 0.0001	0.07	0.25	
Total PUFA[2]	29.9^d	33.4^{cd}	33.4^{cd}	35.7^{bc}	41.4^a	37.7^b	0.88	< 0.0001	0.11	0.56	
Liver											
14:0 (myristic)	0.50	0.45	0.42	0.46	0.59	0.47	0.06	0.45			
16:0 (palmitic)	26.3	23.9	23.8	23.8	21.5	22.7	1.09	0.09			
18:0 (stearic)	8.80	8.14	9.62	10.3	9.81	10.5	0.79	0.29			
Total SFA	35.6	32.5	33.9	34.6	31.9	33.7	1.63	0.63			
16:1 (palmitoleic)	1.26	0.99	1.09	1.03	1.20	1.18	0.15	0.77			
18:1 (oleic)	35.7^a	29.8^{bc}	30.3^b	27.6^{bc}	25.4^{cd}	22.9^d	1.10	< 0.0001	< 0.05	0.57	0.69
Total MUFA	36.9^a	30.8^b	31.4^b	28.7^{bc}	26.6^{bc}	24.1^c	1.19	< 0.0001	< 0.05	0.54	0.65
Total PUFA[2]	26.4^d	33.1^{bc}	31.6^{cd}	33.3^{bc}	37.8^{ab}	39.1^a	1.22	< 0.0001	0.05	0.81	
Plasma											
14:0 (myristic)	0.33	0.29	0.30	0.31	0.30	0.34	0.02	0.29			
16:0 (palmitic)	23.6	22.1	22.4	22.8	22.2	22.3	0.74	0.78			
18:0 (stearic)	6.84	9.56	8.39	7.87	8.28	8.69	0.91	0.59			
Total SFA	30.8	31.9	31.0	31.0	30.8	31.3	1.28	0.99			
16:1 (palmitoleic)	0.99	0.78	1.06	1.25	1.10	1.11	0.15	0.45			
18:1 (oleic)	33.1^a	29.2^{ab}	32.3^a	25.4^{bc}	23.0^c	23.9^{bc}	1.19	< 0.0001	0.52	0.37	
Total MUFA	34.0^a	30.0^{ab}	33.4^a	26.6^{bc}	24.1^c	25.0^{bc}	1.21	< 0.0001	0.54	0.38	
Total PUFA[2]	33.2^c	36.3^{bc}	33.4^c	40.1^{ab}	42.8^a	41.7^{ab}	1.24	< 0.0001	0.51	0.46	

[1]Data are presented as least square means (LSM) ± SE. [a-d]Different superscripts between treatments within a row for each tissue type (yolk, n =7; liver, n = 5; plasma, n = 4 per treatment), are significantly different at $P < 0.05$. Coefficient of determination (R^2) indicated when linear or quadratic effects are significant ($P < 0.05$).

[2]Total PUFA = LA, GLA, ARA, ALA, EPA, DPA and DHA.

Table 6.4: Total saturated (SFA), total monounsaturated (MUFA) and total polyunsaturated (PUFA) fatty acids profile (mol%) of total phospholipid (PL) in egg yolk, liver and plasma after feeding hens diets with varying levels of alpha-linolenic acid (ALA, % of diet) derived from hempseed products[1]

Source of ALA	Control	10%HS	4.5%HO	20%HS	30%HS	9.0%HO	SE	*P* - value	Linear	Quadratic	Adj R²
ALA content (% of diet)	0.10	0.50	0.66	0.90	1.22	1.28					
Egg yolk											
14:0 (myristic)	0.13	0.14	0.12	0.15	0.14	0.15	0.01	0.38			
16:0 (palmitic)	31.4ᵃ	31.0ᵃ	30.2ᵃᵇ	30.9ᵃᵇ	28.8ᵇ	29.6ᵃᵇ	0.52	< 0.01	0.79	0.50	
18:0 (stearic)	18.1ᶜ	18.8ᵇᶜ	18.7ᵇᶜ	18.7ᵇᶜ	20.8ᵃ	20.1ᵃᵇ	0.36	< 0.0001	0.86	0.12	
Total SFA	49.7	49.9	49.1	49.8	49.7	49.9	0.38	0.62			
16:1 (palmitoleic)	0.37	0.37	0.40	0.41	0.34	0.42	0.04	0.55			
18:1 (oleic)	18.2ᵃ	17.5ᵃᵇ	18.2ᵃ	17.7ᵃᵇ	16.6ᵇ	17.0ᵇ	0.26	< 0.001	0.74	0.13	
Total MUFA	18.6ᵃᵇ	17.9ᵃᵇᶜ	18.6ᵃ	18.1ᵃᵇ	17.0ᶜ	17.4ᵇᶜ	0.27	< 0.001	0.69	0.12	
Total PUFA²	29.4ᵃᵇ	29.9ᵃᵇ	30.1ᵃᵇ	29.8ᵃᵇ	30.8ᵃ	29.0ᵇ	0.39	< 0.05	0.52	0.66	
Liver											
14:0 (myristic)	0.25	0.21	0.22	0.21	0.18	0.21	0.03	0.68			
16:0 (palmitic)	28.5ᵃ	24.1ᵇ	24.8ᵇ	24.2ᵇ	22.7ᵇ	23.9ᵇ	0.63	< 0.0001	< 0.001	< 0.01	0.56
18:0 (stearic)	21.4ᵃᵇ	21.3ᵃᵇ	20.7ᵇ	21.5ᵃᵇ	22.7ᵃ	22.0ᵃᵇ	0.43	< 0.05	0.31	0.10	
Total SFA	46.2	45.6	45.7	45.8	45.6	46.1	0.27	0.52			
16:1 (palmitoleic)	0.50	0.38	0.40	0.39	0.35	0.37	0.05	0.40			
18:1 (oleic)	19.4ᵃ	15.9ᵇ	16.4ᵃᵇ	16.1ᵇ	15.8ᵇ	15.3ᵇ	0.72	< 0.01	< 0.01	0.05	0.37
Total MUFA	19.9ᵃ	16.3ᵇ	16.8ᵃᵇ	16.5ᵇ	16.2ᵇ	15.6ᵇ	0.74	< 0.01	< 0.01	0.05	0.38
Total PUFA²	36.4	34.2	33.8	33.7	34.2	34.6	0.85	0.34			
Plasma											
14:0 (myristic)	0.16	0.17	0.22	0.16	0.17	0.16	0.02	0.24			
16:0 (palmitic)	31.9ᵃ	28.1ᵇ	29.1ᵃᵇ	29.7ᵃᵇ	28.7ᵇ	29.5ᵃᵇ	0.55	< 0.01	< 0.01	< 0.01	0.38
18:0 (stearic)	19.8	18.9	20.1	19.1	18.6	18.9	0.37	0.06			
Total SFA	49.3	46.7	49.0	48.6	48.2	47.8	1.04	0.55			
16:1 (palmitoleic)	0.34ᵃᵇ	0.29ᵇ	0.48ᵃ	0.39ᵃᵇ	0.38ᵃᵇ	0.46ᵃ	0.03	< 0.01	0.52	0.82	
18:1 (oleic)	19.8	18.9	20.1	19.1	18.6	18.9	0.37	0.06			
Total MUFA	20.1	19.2	20.5	19.5	19.0	19.4	0.38	0.09			
Total PUFA²	28.2	31.6	27.9	29.3	30.2	30.2	0.95	0.13			

[1]Data are presented as least square means (LSM) ± SE. . ᵃ⁻ᶜDifferent superscripts between treatments within a row for each tissue type (yolk, n =7; liver, n = 5; plasma, n = 4 per treatment), are significantly different at *P* < 0.05. Coefficient of determination (R²) indicated when linear or quadratic effects are significant (*P* < 0.05).
[2]Total PUFA = LA, GLA, ARA, ALA, EPA, DPA and DHA.

of total MUFA were significantly ($P < 0.0001$) higher in the TAG compared to that in the PL for egg yolk (28.7 vs. 17.9 ± 0.20), liver (29.8 vs. 16.9 ± 0.40) and plasma (29.4 vs. 19.6 ± 0.27), respectively (data not shown).

6.4.2.2 Polyunsaturated fatty acids (PUFA)

The fatty acid profiles of the total lipids (Table 6.5) and the individual classes (Tables 6.6–6.11) of lipids in the egg yolk, liver and plasma followed similar patterns.

Total lipids: Linoleic acid was the major PUFA found in the total lipid of egg yolk, liver and plasma (Table 6.5). Although in the egg yolk, the increase in the levels of GLA in the total lipids was only evident at higher levels of hempseed product inclusions, 30% HS and 9.0% HO in the diets (containing highest levels of GLA as well as ALA), the level of GLA in the liver and plasma was more closely related to its composition in the diets (Table 6.1). The second abundant PUFA, ARA, significantly decreased in a linear fashion in the egg yolk ($P < 0.0001$; $R^2 = 0.48$), liver ($P < 0.05$; $R^2 = 0.20$) and plasma ($P < 0.05$; $R^2 = 0.28$, Table 6.5). A quadratic effect ($P < 0.001$) was also noted for egg yolk, highest at lowest level of ALA intake (negative control). The total n-6 PUFA levels in total lipids in the egg yolk were reflective of dietary LA levels.

For the n-3 PUFA (ALA, EPA, DPA and DHA), increasing intakes of ALA by the laying hens effectively ($P < 0.0001$) increased their concentrations in the total lipids of the egg yolk, liver and plasma (Table 6.5). Although the total lipid content of ALA in all the tissues increased significantly ($P < 0.0001$), it increased at a lower rate (lower coefficient gradient) in the liver compared to that in the egg yolk or plasma (Figure 6.1a). The accumulation responses of ALA (mol%) in the three tissues were represented by the following regression equations: $ALA_{yolk} = 0.0049x - 0.0598$; $R^2 = 0.97$, $P < 0.001$; $ALA_{liver} = 0.0037x - 0.1408$; $R^2 = 0.95$, $P < 0.001$; $ALA_{plasma} = 0.0051x - 0.1093$; $R^2 = 0.97$, $P < 0.001$; where x is the inclusion or intake level of

Table 6.5: Polyunsaturated fatty acids profile (PUFA, mol%) of total lipids in egg yolk, liver and plasma after feeding hens diets with varying levels of alpha-linolenic acid (ALA) derived from hempseed products [1]

Source of ALA ALA content (% of diet)	Control 0.10	10%HS 0.50	4.5%HO 0.66	20%HS 0.90	30%HS 1.22	9.0%HO 1.28	SE	P - value	Linear	Quadratic	Adj R^2
Egg yolk											
18:2n-6 (LA)	28.8^b	30.3^{ab}	29.1^b	31.1^{ab}	32.4^a	30.5^{ab}	0.56	< 0.001	0.53	0.75	
18:3n-6 (GLA)	0.22^c	0.24^{bc}	0.24^c	0.30^{ab}	0.34^a	0.33^a	0.01	< 0.0001	0.41	0.15	
20:4n-6 (ARA)	2.09^a	1.93^b	1.87^b	1.80^b	1.87^b	1.81^b	0.03	< 0.0001	< 0.0001	< 0.0001	0.48
18:3n-3 (ALA)	0.46^e	2.16^d	2.59^d	3.78^c	5.87^a	5.01^b	0.14	< 0.0001	< 0.0001	0.44	0.94
20:5n-3 (EPA)	0.00^e	0.03^d	0.04^d	0.06^c	0.08^a	0.07^b	0.002	< 0.0001	< 0.0001	< 0.01	0.94
22:5n-3 (DPA)	0.05^c	0.12^{ab}	0.10^b	0.12^{ab}	0.14^{ab}	0.14^a	0.01	< 0.0001	< 0.0001	< 0.01	0.64
22:6n-3 (DHA)	0.44^b	1.13^a	1.11^a	1.22^a	1.16^a	1.22^a	0.05	< 0.0001	< 0.0001	< 0.0001	0.82
Total n-6	31.1^b	32.4^{ab}	31.2^b	33.2^{ab}	34.7^a	32.6^{ab}	0.57	0.001	0.70	0.60	
Total n-3	0.95^d	3.51^c	3.84^c	5.18^b	7.28^a	6.53^a	0.18	< 0.0001	< 0.0001	0.12	0.94
n6:n3	36.1^a	9.80^b	8.18^c	6.43^d	4.81^e	5.02^e	0.20	< 0.0001	< 0.0001	< 0.0001	0.95
Liver											
18:2n-6 (LA)	26.6^b	28.3^b	27.9^b	27.8^b	28.6^{ab}	30.4^a	0.47	0.0001	0.48	0.68	
18:3n-6 (GLA)	0.22^e	0.37^d	0.48^c	0.55^c	0.70^b	0.82^a	0.03	< 0.0001	0.001	0.68	0.83
20:4n-6 (ARA)	7.03^a	5.94^{ab}	5.95^{ab}	6.01^{ab}	6.06^{ab}	5.36^b	0.27	< 0.01	< 0.05	0.14	0.20
18:3n-3 (ALA)	0.26^e	1.55^d	2.02^{cd}	2.56^c	3.62^b	4.75^a	0.15	< 0.0001	< 0.001	0.57	0.86
20:5n-3 (EPA)	0.05^d	0.06^d	0.09^{cd}	0.11^{bc}	0.15^a	0.14^{ab}	0.01	< 0.0001	0.10	0.65	
22:5n-3 (DPA)	0.08^b	0.17^a	0.15^{ab}	0.19^a	0.23^a	0.23^a	0.02	< 0.0001	< 0.01	0.12	0.43
22:6n-3 (DHA)	1.25^b	3.10^a	3.16^a	3.50^a	3.20^a	3.07^a	0.21	< 0.0001	< 0.0001	< 0.0001	0.59
Total n-6	33.9^b	34.6^{ab}	34.3^b	34.4^b	35.3^{ab}	36.5^a	0.48	< 0.01	0.71	0.19	
Total n-3	1.45^e	4.88^d	5.42^d	6.35^c	7.19^b	8.02^a	0.19	< 0.0001	< 0.0001	< 0.0001	0.93
n6:n3	21.8^a	7.12^b	6.36^c	5.45^d	4.94^{de}	4.57^e	0.16	< 0.0001	< 0.0001	< 0.0001	0.92
Plasma											
18:2n-6 (LA)	30.4^{bc}	31.6^{ab}	29.0^c	32.2^{ab}	32.9^a	31.8^{ab}	0.49	< 0.0001	0.61	0.17	
18:3n-6 (GLA)	0.20^d	0.40^c	0.41^c	0.63^b	0.78^a	0.79^a	0.02	< 0.0001	< 0.0001	0.31	0.91
20:4n-6 (ARA)	2.17^a	1.97^{ab}	1.97^{ab}	1.87^b	1.86^b	1.86^b	0.06	< 0.01	< 0.05	0.14	0.28
18:3n-3 (ALA)	0.50^e	2.16^d	2.45^d	3.97^c	6.11^a	5.36^b	0.13	< 0.0001	< 0.0001	0.15	0.94
20:5n-3 (EPA)	0.00^d	0.03^c	0.04^{bc}	0.05^b	0.06^a	0.06^a	0.003	< 0.0001	< 0.0001	<.0001	0.91
22:5n-3 (DPA)	0.04^b	0.10^a	0.10^a	0.12^a	0.13^a	0.13^a	0.01	< 0.0001	< 0.0001	< 0.01	0.65
22:6n-3 (DHA)	0.36^b	1.29^a	1.29^a	1.38^a	1.29^a	1.35^a	0.07	< 0.0001	< 0.0001	< 0.0001	0.74
Total n-6	32.8^{bc}	33.9^{ab}	31.4^c	34.7^{ab}	35.6^a	34.4^{ab}	0.50	< 0.0001	0.57	0.12	
Total n-3	0.90^d	3.57^c	3.89^c	5.52^b	7.39^a	6.91^a	0.16	< 0.0001	< 0.0001	< 0.05	0.96
n6:n3	32.8^a	9.52^b	8.14^c	6.32^d	4.83^e	4.99^e	0.18	< 0.0001	< 0.0001	< 0.0001	0.95

[1]Data are presented as least square means (LSM) ± SE (n=8 per treatment). [a-e]Different superscripts between treatments within a row of each tissue type are significantly different at $P < 0.05$. Coefficient of determination (R^2) indicated when linear or quadratic effects are significant ($P < 0.05$).
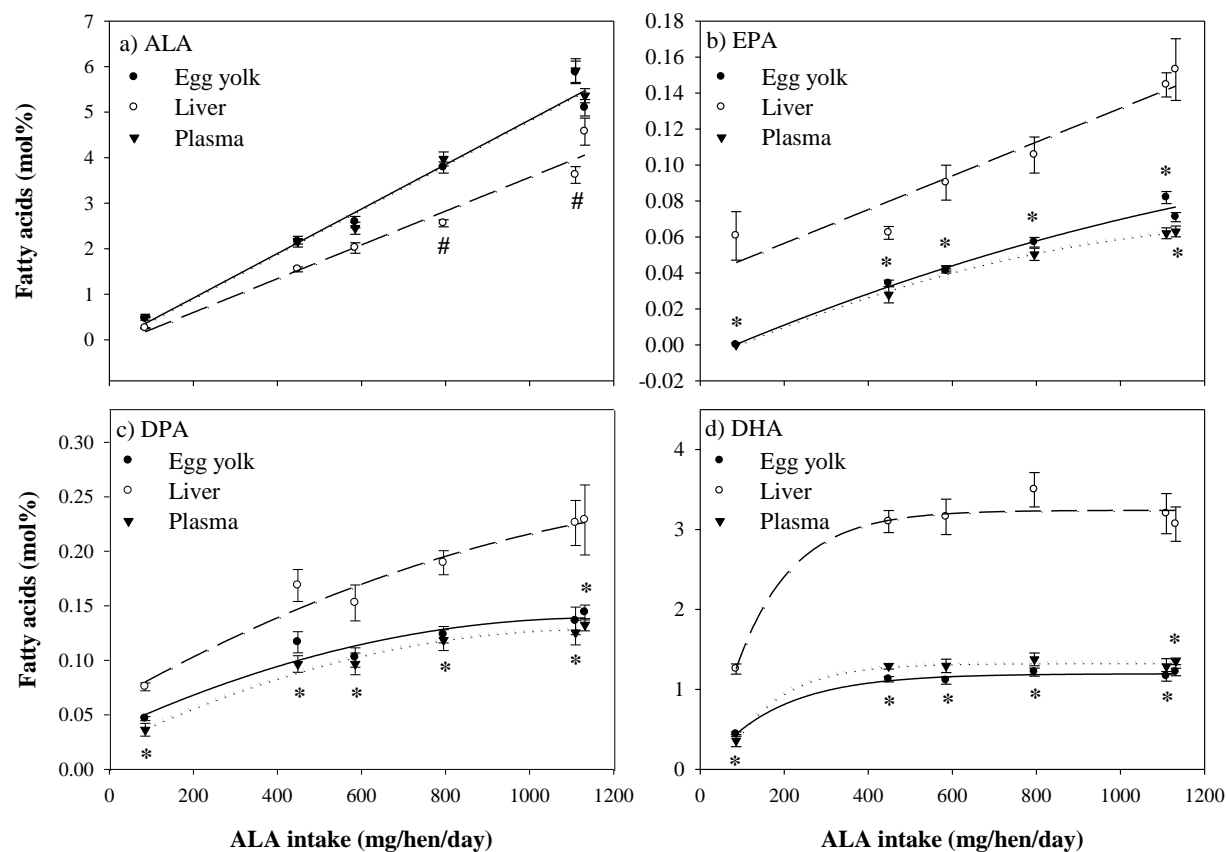
Figure 6.1. Trend analysis of fatty acid levels (mol%) of total lipid in egg yolk, liver and plasma of laying hens as a function of alpha-linolenic acid intake (ALA, mg/hen/day) derived from hempseed products. Displayed are: a) alpha-linolenic acid, ALA; b) eicosapentaenoic acid, EPA; c) docosapentaenoic acid, DPA; and d) docosahexaenoic acid, DHA. Each data point are group means ± SE (n = 8 per treatment). Symbols denote: #, fatty acid in the liver less than that in the egg yolk and plasma;*, fatty acid in the liver greater than in the egg yolk and plasma.

hempseed products in diets of hens. Among the n-3 LCPUFA, levels of EPA were lowest in the total lipids of egg yolk, liver and plasma compared to the levels of DPA and DHA. Except for a gradual increase in the levels of EPA in the total lipids of the liver, significant linear ($P < 0.001$; $R^2 = 0.43$ to 0.94) and quadratic (highest for DHA, $P < 0.0001$; $R^2 = 0.59$ to 0.82) responses were observed for the n-3 LCPUFA in the three tissues as a function of ALA intakes by the laying hens fed hempseed products (Table 6.5). Overall, the n-3 LCPUFA (EPA, DPA and DHA), were maintained at significantly ($P < 0.0001$) greater proportions in the total lipids in the liver compared to that in the egg yolk or the plasma at all levels of ALA intakes, with no significant differences between the latter two tissues (Figure 6.1b to 6.1d). Consequently, changes in the total n-3 PUFA and the n-6 to n-3 ratio in the egg yolk, liver and plasma showed linear ($P < 0.0001$) and quadratic ($P < 0.05$) effects ($R^2 > 0.90$).

Neutral (CE and TAG) and polar (total PL, PtdCho and PtdEtn) lipids: Although the combination of SFA and MUFA constituted the majority of the CE proportion in the egg yolk, liver and plasma, among the PUFA, the CE in all the tissues was mainly characterized by a high content of LA; this fatty acid, did not change with treatment (Table 6.6). Gamma-linolenic acid, ARA, ALA and DHA were detected in both the liver and plasma CE but not in the egg yolk CE. As a function of ALA intakes by the laying hens, a significant increase in the levels of ALA in the liver and plasma CE ($P < 0.01$ and $P < 0.0001$, respectively) were noted. In addition, a significant ($P < 0.001$) linear and quadratic changes where observed in the levels of DHA in the plasma CE (Table 6.6).

The PUFA profiles of the TAG and total PL of the egg yolk, liver and plasma, are illustrated in Tables 6.7 and 6.8, respectively. Overall, the levels LA were significantly ($P < 0.0001$) higher in the TAG than in the total PL for egg yolk, liver and plasma (Tables 6.7 and

Table 6.6: Fatty acid (mol%) profiles of cholesteryl ester (CE) in egg yolk, liver and plasma after feeding hens diets with varying levels of alpha-linolenic acid (ALA) derived from hempseed products[1]

Source of ALA	Control	10%HS	4.5%HO	20%HS	30%HS	9.0%HO					
ALA content (% of diet)	0.10	0.50	0.66	0.90	1.22	1.28	SE	P - value	Linear	Quadratic	Adj R^2
Egg Yolk											
Total SFA[2]	34.6	38.3	22.0	37.8	40.4	32.7	5.36	0.17			
Total MUFA[3]	15.8	17.2	13.1	16.0	14.9	12.0	2.53	0.67			
Total PUFA[4]	17.7	15.2	19.7	19.1	14.3	12.6	2.68	0.35			
18:2n-6 (LA)	15.5	14.3	13.2	16.8	17.1	10.6	3.21	0.73			
Liver											
Total SFA[2]	24.0	22.0	23.0	21.2	20.2	23.8	1.21	0.24			
Total MUFA[3]	42.1	39.3	35.6	39.7	39.9	35.0	4.43	0.85			
Total PUFA[4]	16.8	19.8	21.5	16.8	21.4	22.4	2.79	0.58			
18:2n-6 (LA)	14.9	16.4	17.2	12.2	16.5	16.9	2.28	0.63			
18:3n-6 (GLA)	0.08	0.54	0.71	0.97	0.80	0.97	0.20	0.08			
20:4n-6 (ARA)	0.69	0.71	0.78	0.86	0.69	0.61	0.18	0.94			
18:3n-3 (ALA)	0.80[b]	1.93[ab]	2.45[ab]	2.27[ab]	3.32[a]	3.74[a]	0.45	< 0.01	0.13	0.78	
22:6n-3 (DHA)	0.37	0.15	0.42	0.28	0.15	0.27	0.08	0.18			
Plasma											
Total SFA[2]	21.0	21.8	24.5	22.2	19.0	22.9	2.55	0.62			
Total MUFA[3]	32.2	32.3	30.1	29.9	31.5	29.9	1.51	0.74			
Total PUFA[4]	39.3	39.7	34.2	41.7	40.2	39.2	2.19	0.16			
18:2n-6 (LA)	36.7	36.0	31.0	36.3	34.6	34.3	2.12	0.33			
18:3n-6 (GLA)	0.85[b]	1.00[ab]	0.98[b]	1.36[ab]	1.55[a]	1.44[ab]	0.14	< 0.01	0.34	0.97	
20:4n-6 (ARA)	0.90	0.87	0.87	0.83	0.87	0.74	0.11	0.94			
18:3n-3 (ALA)	0.60[c]	1.16[bc]	1.29[b]	1.97[a]	2.48[a]	2.15[a]	0.13	< 0.0001	< 0.01	0.54	0.80
22:6n-3 (DHA)	0.24[b]	0.67[a]	0.70[a]	0.74[a]	0.66[a]	0.58[ab]	0.08	< 0.01	< 0.0001	< 0.001	0.59

[1]Data represents least square means (LSM) ± SE, n=7 (egg yolk), n=5 (liver), n=4 (plasma). [a-c]Different superscripts between treatments within a row of each tissue type are significantly different at $P < 0.05$. Coefficient of determination (R^2) indicated when linear or quadratic effects are significant ($P < 0.05$). [2]Total saturated fatty acid (SFA): for egg yolk, palmitic (16:0) and stearic (18:0); for liver and plasma, myristic, (14:0), palmitic (16:0) and stearic (18:0). [3]Total monounsaturated fatty acid (MUFA): for egg yolk, oleic (18:1); for liver and plasma, palmitoleic (16:1) and oleic (18:1). [4]Total polyunsaturated fatty acid (PUFA): for egg yolk, LA; for liver and plasma, LA, GLA, ARA, ALA and DHA.

Table 6.7: Polyunsaturated fatty acids profile (PUFA, mol%) of triacylglycerol (TAG) in egg yolk, liver and plasma after feeding hens diets with varying levels of alpha-linolenic acid (ALA) derived from hempseed products [1]

Source of ALA	Control	10%HS	4.5%HO	20%HS	30%HS	9.0%HO	SE	P - value	Linear	Quadratic	Adj R^2
ALA content (% of diet)	0.10	0.50	0.66	0.90	1.22	1.28					
Egg yolk											
18:2n-6 (LA)	28.6^b	30.1^b	29.4^b	30.8^{ab}	33.6^a	31.0^{ab}	0.70	< 0.001	0.63	0.52	
18:3n-6 (GLA)	0.19^d	0.21^{cd}	0.21^d	0.26^{bc}	0.32^a	0.29^{ab}	0.01	< 0.0001	0.35	0.16	
20:4n-6 (ARA)	0.43^a	0.37^{ab}	0.37^{ab}	0.39^{ab}	0.35^{ab}	0.33^b	0.02	< 0.05	0.11	0.42	
18:3n-3 (ALA)	0.44^d	2.24^c	2.93^c	4.01^b	6.61^a	5.61^a	0.25	< 0.0001	< 0.001	0.66	0.89
20:5n-3 (EPA)	0.02^d	0.05^c	0.05^c	0.06^{bc}	0.09^a	0.09^{ab}	0.02	< 0.0001	< 0.05	0.50	0.60
22:5n-3 (DPA)	0.04^b	0.08^a	0.07^a	0.09^a	0.07^a	0.09^a	0.01	< 0.0001	< 0.001	< 0.05	0.42
22:6n-3 (DHA)	0.22	0.27	0.28	0.33	0.26	0.33	0.04	0.43			
Total n-6	29.3^b	30.7^b	30.0^b	31.4^{ab}	34.3^a	31.6^{ab}	0.71	< 0.001	0.66	0.50	
Total n-3	0.66^d	2.72^c	3.37^c	4.57^b	7.11^a	6.10^a	0.26	< 0.0001	< 0.0001	0.92	0.89
Liver											
18:2n-6 (LA)	25.5^c	30.4^{ab}	28.5^{bc}	29.5^{abc}	31.5^{ab}	32.6^a	0.93	< 0.001	0.07	0.47	
18:3n-6 (GLA)	0.15^c	0.32^{bc}	0.38^b	0.44^b	0.75^a	0.73^a	0.05	< 0.0001	0.15	0.30	
20:4n-6 (ARA)	0.34	0.32	0.35	0.34	0.37	0.31	0.04	0.93			
18:3n-3 (ALA)	0.39^c	1.96^b	2.24^b	2.82^b	4.96^a	5.30^a	0.27	< 0.0001	< 0.05	0.21	0.87
20:5n-3 (EPA)	ND	ND	0.02(±0.001)	ND	0.03(±0.008)	0.03(±0.008)	-	-			
22:5n-3 (DPA)	0.03	0.04	0.03	0.03	0.04	0.04	0.01	0.93			
22:6n-3 (DHA)	0.04^b	0.11^a	0.12^a	0.13^a	0.15^a	0.13^a	0.01	< 0.01	< 0.001	< 0.01	0.55
Total n-6	26.0^c	31.0^{ab}	29.2^{bc}	30.3^{abc}	32.6^{ab}	33.6^a	0.98	< 0.001	0.08	0.53	
Total n-3	0.43^c	2.08^b	2.40^b	2.98^b	5.17^a	5.50^a	0.27	< 0.0001	< 0.05	0.28	0.87
Plasma											
18:2n-6 (LA)	31.9^{ab}	33.0^{ab}	29.7^b	34.2^a	34.5^a	34.0^a	0.93	< 0.01	0.72	0.32	
18:3n-6 (GLA)	0.18^c	0.35^c	0.35^c	0.64^b	0.87^a	0.76^{ab}	0.05	< 0.0001	0.08	0.65	
20:4n-6 (ARA)	0.49^a	0.48^{ab}	0.43^{abc}	0.33^{bc}	0.33^c	0.40^{abc}	0.08	< 0.01	0.14	0.48	
18:3n-3 (ALA)	0.49^d	2.25^c	2.66^c	4.71^b	6.81^a	6.21^a	0.32	< 0.0001	< 0.01	1.00	0.87
20:5n-3 (EPA)	0.03^c	0.04^{bc}	0.04^{bc}	0.05^{abc}	0.06^{ab}	0.07^a	0.005	< 0.001	0.17	0.89	
22:5n-3 (DPA)	0.02^b	0.06^{ab}	0.05^{ab}	0.05^{ab}	0.06^a	0.07^a	0.01	< 0.05	0.10	0.48	
22:6n-3 (DHA)	0.06^b	0.17^a	0.15^a	0.16^a	0.15^a	0.17^a	0.01	< 0.001	< 0.01	< 0.01	0.51
Total n-6	32.6^{ab}	33.8^{ab}	30.5^b	35.2^a	35.7^a	35.1^a	0.96	< 0.01	0.77	0.32	
Total n-3	0.58^d	2.51^c	2.90^c	4.95^b	7.07^a	6.51^a	0.33	< 0.0001	< 0.01	0.91	0.87

[1]Data are presented as least square means (LSM) ± SE, n=7 (egg yolk), n=5 (liver), n=4 (plasma). [a-d]Different superscripts between treatments within a row of each tissue type are significantly different at P < 0.05. Coefficient of determination (R^2) indicated when linear or quadratic effects are significant (P < 0.05). ND = not detectable.

Table 6.8: Polyunsaturated fatty acids profile (PUFA, mol%) of total phospholipids (PL) in egg yolk, liver and plasma after feeding hens diets with varying levels of alpha-linolenic acid (ALA) derived from hempseed products[1]

Source of ALA	Control	10%HS	4.5%HO	20%HS	30%HS	9.0%HO					
ALA content (% of diet)	0.10	0.50	0.66	0.90	1.22	1.28	SE	*P* - value	Linear	Quadratic	Adj R^2
Egg yolk											
18:2n-6 (LA)	21.4^a	20.6^a	20.4^{ab}	20.6^a	20.7^a	19.2^b	0.31	< 0.001	0.13	0.41	
18:3n-6 (GLA)	0.17^c	0.20^{bc}	0.21^{bc}	0.23^b	0.35^a	0.32^a	0.01	< 0.0001	0.80	< 0.05	0.76
20:4n-6 (ARA)	5.85^a	5.29^{ab}	5.51^{ab}	5.27^{ab}	5.37^{ab}	5.03^b	0.15	< 0.05	0.05	0.20	0.20
18:3n-3 (ALA)	0.14^f	0.34^e	0.39^d	0.49^c	0.72^a	0.62^b	0.01	< 0.0001	< 0.0001	0.51	0.93
20:5n-3 (EPA)	0.00^d	0.04^c	0.05^b	0.06^b	0.08^a	0.08^a	0.003	< 0.0001	< 0.0001	< 0.001	0.92
22:5n-3 (DPA)	0.12^c	0.23^{ab}	0.20^b	0.21^b	0.27^{ab}	0.29^a	0.02	< 0.0001	< 0.05	0.39	0.53
22:6n-3 (DHA)	1.24^b	3.25^a	3.30^a	3.44^a	3.28^a	3.42^a	0.15	< 0.0001	< 0.0001	< 0.0001	0.74
Total n-6	27.5^a	26.1^{ab}	26.1^a	26.1^{ab}	26.4^a	24.6^b	0.36	< 0.001	0.05	0.21	0.20
Total n-3	1.48^b	3.85^a	3.93^a	4.19^a	4.35^a	4.40^a	0.16	< 0.0001	< 0.0001	< 0.0001	0.82
Liver											
18:2n-6 (LA)	22.6^a	20.0^{ab}	19.1^b	19.2^b	19.3^b	19.7^{ab}	0.67	< 0.05	< 0.001	< 0.01	0.40
18:3n-6 (GLA)	0.18b	0.19b	0.24ab	0.22ab	0.26ab	0.33a	0.02	< 0.01	0.68	0.58	
20:4n-6 (ARA)	10.7^a	8.88^b	8.83^b	8.37^b	8.89^b	8.48^b	0.35	< 0.01	< 0.001	< 0.01	0.47
18:3n-3 (ALA)	0.14^d	0.30^c	0.31^{bc}	0.39^b	0.53^a	0.52^a	0.02	< 0.0001	< 0.001	0.93	0.88
20:5n-3 (EPA)	0.02^d	0.06^c	0.08^c	0.11^b	0.15^a	0.18^a	0.005	< 0.0001	< 0.001	0.99	0.89
22:5n-3 (DPA)	0.16^b	0.28^a	0.27^a	0.31^a	0.33^a	0.33^a	0.02	< 0.0001	< 0.001	< 0.05	0.62
22:6n-3 (DHA)	1.91^b	4.47^a	4.97^a	5.17^a	4.73^a	5.01^a	0.19	< 0.0001	< 0.0001	< 0.0001	0.90
Total n-6	34.1^a	29.1^b	28.2^b	27.7^b	28.5^b	28.5^b	0.86	< 0.001	< 0.0001	< 0.001	0.55
Total n-3	2.23^c	5.10^b	5.62^{ab}	5.99^a	5.74^{ab}	6.03^a	0.19	< 0.0001	< 0.0001	< 0.0001	0.93
Plasma											
18:2n-6 (LA)	21.4^a	20.4^{ab}	18.3^b	19.9^{ab}	20.5^{ab}	20.2^{ab}	0.54	< 0.05	< 0.05	< 0.05	0.13
18:3n-6 (GLA)	0.13^c	0.19^{bc}	0.15^c	0.25^{ab}	0.31^a	0.30^a	0.02	< 0.0001	0.29	0.47	
20:4n-6 (ARA)	5.53	6.18	3.71	5.00	5.11	5.16	0.62	0.16			
18:3n-3 (ALA)	0.14^d	0.35^c	0.29^{cd}	0.50^b	0.71^a	0.65^{ab}	0.04	< 0.0001	< 0.05	0.51	0.85
20:5n-3 (EPA)	0.01^b	0.05^a	0.05^a	0.06^a	0.07^a	0.07^a	0.005	< 0.0001	< 0.0001	< 0.001	0.80
22:5n-3 (DPA)	0.25	0.25	0.22	0.33	0.31	0.30	0.04	0.45			
22:6n-3 (DHA)	1.20^c	4.13^a	2.55^{bc}	3.23^{ab}	3.24^{ab}	3.60^{ab}	0.34	< 0.001	< 0.01	< 0.05	0.30
Total n-6	27.0	26.8	22.9	25.2	25.9	25.6	0.96	0.09			
Total n-3	1.60^c	4.77^a	2.89^{bc}	4.12^{ab}	4.33^{ab}	4.62^a	0.38	< 0.001	< 0.05	0.07	0.38

[1]Data are presented as least square means (LSM) ± SE, n=7 (egg yolk), n=5 (liver), n=4 (plasma). [a-f]Different superscripts between treatments within a row of each tissue type are significantly different at *P* < 0.05. Coefficient of determination (R^2) indicated when linear or quadratic effects are significant (*P* < 0.05).

6.8, respectively). Significant increases in the levels of GLA were observed in the TAG (Table 6.7) and total PL (Table 6.8) of egg yolk ($P < 0.0001$), liver ($P < 0.0001$ for TAG; $P < 0.01$ for total PL) and plasma ($P < 0.0001$) as a function of hempseed products inclusions in the laying hen diets. On average, the level of egg yolk-GLA in the TAG versus that in the total PL was not significantly different (TAG: 0.246 vs. PL: 0.248 ± 0.005 mol%); conversely, in the liver and plasma, the GLA levels (overall average) were greater ($P < 0.0001$) in the TAG compared to that in the total PL (liver: 0.457 vs. 0.256 ± 0.012 mol%; and plasma: 0.514 vs. 0.221 ± 0.014 mol%, respectively; data not shown). Although the levels of ARA in the TAG of the liver were not influenced by treatment, levels fluctuated in the TAG of the egg yolk and plasma. In the total PL of the liver, there was a linear ($P < 0.001$) and quadratic ($P < 0.01$) decrease in the level of ARA as a function of ALA intakes by the hens.

The levels of ALA, the primary precursor for the n-3 LCPUFA, were significantly ($P < 0.0001$) increased in both the TAG (Table 6.7) and total PL (Table 6.8), with levels being more enriched in the former than the latter lipid class of the egg yolk, liver and plasma (Table 6.5). The pattern of ALA enrichment in the lipid classes followed a similar trend as that observed in the total lipids (Figure 6.1). In the egg yolk, the ALA content contained in the TAG contributed to more than two-thirds the amount in the total lipid portion (Figure 6.2a). Generally, higher levels of inclusion of hempseed products in the diets of the laying hens significantly increased the accumulation of n-3 LCPUFA. In the case of EPA, yolk-EPA levels significantly ($P < 0.0001$) increased linearly in both the TAG ($P < 0.05$, $R^2 = 0.60$; Table 6.7) and total PL ($P < 0.0001$, $R^2 = 0.92$; Table 6.8). A quadratic effect was also observed for the PL. Overall averages (data extracted from Tables 6.7 and 6.8) indicated that, the proportion of EPA in the egg yolk was significantly ($P < 0.01$) higher in the TAG than in the total PL class as a mol% 0.060 vs.
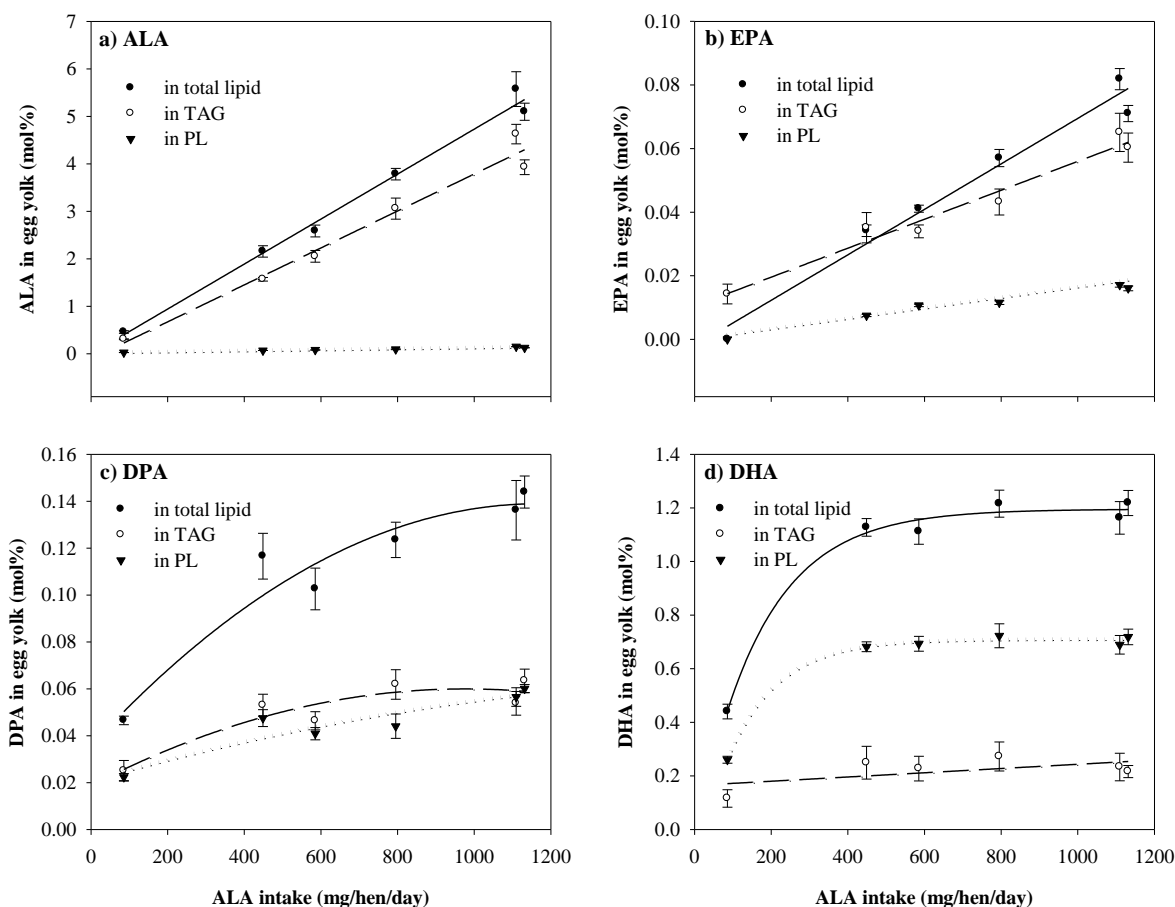
Figure 6.2. Trends in the accumulations of a) alpha-linolenic acid, ALA; b) eicosapentaenoic acid, EPA; c) docosapentaenoic acid, DPA; and d) docosahexaenoic acid, DHA levels in total lipids, TAG and total PL fractions of egg yolk as a function of alpha-linolenic acid intake (ALA, mg/hen/day) derived from hempseed products.

Each data point are group means ± SE (n = 8 hens/ treatment). Values at each data points were adjustments based on 70% and 21% contributions by the fatty acids in the TAG and PL fractions, respectively of the total lipid portion in the egg yolk.

0.049 ± 0.002, respectively and as a percentage contribution of total yolk lipid (Figure 6.2b). In the liver, the TAG-EPA levels were not detectable, particularly at lower levels of dietary ALA intakes (Table 6.7); this fatty acid was mainly contained in the total PL and significantly ($P < 0.0001$) increased linearly in relation to increasing dietary ALA intakes ($P < 0.001$, $R^2 = 0.89$). In the plasma, there was a significant gradual increase ($P < 0.001$, Table 6.7) in the levels of EPA in the TAG; and showed a linear and quadratic effects ($P < 0.0001$ and $P < 0.001$; $R^2 = 0.80$, respectively, Table 6.8) in the total PL.

The DPA levels in the egg yolk were significantly ($P < 0.0001$) increased in both the TAG (linear, $P < 0.001$ and quadratic effects, $P < 0.05$; $R^2 = 0.42$, Table 6.7) and in the total PL (linearly, $P < 0.05$; $R^2 = 0.53$, Table 6.8). In the egg yolk, the levels of DPA in relation to increases in dietary ALA intakes were uniformly distributed between the TAG and total PL contributing equally to the total yolk lipid (Figure 6.2c); although in relative values (mol%), DPA is highly incorporated in the total PL than that in the TAG. In the liver, the levels of accumulation of DPA in the TAG of the lipids were not different between treatments; however, linear and quadratic responses ($P < 0.001$ and $P < 0.05$, respectively, $R^2 = 0.62$) were observed in the total PL in response to changes in the dietary ALA intakes by the hens. Although the levels of DPA were significantly ($P < 0.05$) increased in the TAG of the plasma at higher levels of ALA intakes compared to the control, the levels of this fatty acid in the total PL pool in the plasma were not significantly different between treatments although the absolute levels were much greater than in the TAG fractions. Docosahexaenoic acid was the dominant n-3 LCPUFA of the egg yolk, liver and plasma as observed in all lipid classes (TAG, Table 6.7; total PL, Table 6.8). No significant increase in DHA in TAG in egg yolk, in contrast to the increases ($P < 0.001$) found in liver ($R^2 = 0.53$) and plasma ($R^2 = 0.51$) as the level of ALA in the diet increased

(Table 6.7). Linear and quadratic effects were also noted in the PL-DHA levels in the egg yolk

(Table 6.8). Unlike EPA and DPA, the PL-DHA contributes to more than 50% of the DHA in

the total lipids of the egg yolk (Figure 6.2d).

The trends in the levels of n-3 PUFA in PtdCho and PtdEtn were consistent in the egg

yolk, liver and plasma (Tables 6.9, 6.10 and 6.11; and Appendix I). The LCPUFAs incorporated

more into PtdEtn than the PtdCho sub-class in the egg yolk, liver and plasma, DHA exhibiting a

plateau in all tissues. In the PtdEtn fraction of the liver, a highly significant ($R^2 = 0.87$, $P <$

0.0001; Figure 6.3a) linear association was noted between the levels of ALA and EPA and a non-

proportional response between ALA and DHA ($R^2 = 0.71$, $P < 0.0001$; Figure 6.3b). Our results

also showed a significant inverse linear and quadratic ($R^2 = 0.92$; $P < 0.0001$) effects of

increasing dietary ALA levels on the ratio of ALA to EPA (Figure 6.3c), unlike DHA (Figure

6.3d), in the PtdEtn fraction of the liver lipid which was in correlation (r = - 0.94; $P < 0.0001$)

with the amount of DHA in the total PL fraction of the liver.

Table 6.9: Polyunsaturated fatty acid profile in the lipid fraction of egg yolk (mol%) a) phosphatidylcholine (PtdCho) and b) phosphatidylethanolamine (PtdEtn.) after feeding hens diets with varying levels of alpha-linolenic acid (ALA) derived from hempseed products [1]

Source of ALA	Control	10%HS	4.5%HO	20%HS	30%HS	9.0%HO					
ALA content (% of diet)	0.10	0.50	0.66	0.90	1.22	1.28	SE	P - value	Linear	Quadratic	Adj R^2
a) Egg yolk - PtdCho											
18:2n-6 (LA)	23.5[a]	23.1[ab]	21.7[bc]	23.0[ab]	22.8[ab]	21.6[c]	0.30	< 0.001	0.13	0.34	
18:3n-6 (GLA)	0.17[d]	0.22[c]	0.22[c]	0.28[b]	0.38[a]	0.35[a]	0.01	< 0.0001	0.14	0.34	
20:4n-6 (ARA)	4.24[a]	3.81[ab]	3.66[b]	3.68[ab]	3.91[ab]	3.71[ab]	0.13	< 0.05	< 0.01	< 0.01	0.24
18:3n-3 (ALA)	0.13	0.36	0.38	0.54	0.75	0.67	0.01	< 0.0001	< 0.0001	0.38	0.94
20:5n-3 (EPA)	0.00[c]	0.04[b]	0.04[b]	0.04[b]	0.06[a]	0.05[a]	0.002	< 0.0001	< 0.0001	< 0.001	0.93
22:5n-3 (DPA)	0.06[d]	0.12[bc]	0.11[c]	0.13[abc]	0.15[ab]	0.17[a]	0.01	< 0.0001	< 0.01	0.17	0.65
22:6n-3 (DHA)	0.87[b]	2.44[a]	2.27[a]	2.68[a]	2.49[a]	2.66[a]	0.12	< 0.0001	< 0.0001	< 0.0001	0.74
Total n-6	28.0[a]	27.1[ab]	25.8[bc]	27.0[ab]	27.1[a]	25.7[c]	0.30	< 0.0001	< 0.05	0.07	0.19
Total n-3	1.06[d]	2.92[bc]	2.78[c]	3.35[abc]	3.45[ab]	3.55[a]	0.13	< 0.0001	< 0.0001	< 0.0001	0.84
b) Egg yolk - PtdEtn											
18:2n-6 (LA)	18.3[a]	16.1[b]	16.2[b]	16.1[b]	16.7[b]	15.5[b]	0.34	< 0.0001	< 0.001	< 0.001	0.35
18:3n-6 (GLA)	0.15[d]	0.18[cd]	0.18[cd]	0.22[bc]	0.27[a]	0.25[ab]	0.01	< 0.0001	0.21	0.41	
20:4n-6 (ARA)	14.1[a]	12.5[b]	12.6[b]	11.4[b]	12.1[b]	12.0[b]	0.30	< 0.0001	< 0.0001	< 0.001	0.49
18:3n-3 (ALA)	0.18[f]	0.38[e]	0.45[d]	0.51[c]	0.73[a]	0.65[b]	0.01	< 0.0001	< 0.0001	0.11	0.93
20:5n-3 (EPA)	0.00[d]	0.08[c]	0.14[b]	0.16[b]	0.24[a]	0.23[a]	0.01	< 0.0001	< 0.0001	< 0.05	0.88
22:5n-3 (DPA)	0.25[c]	0.50[ab]	0.46[b]	0.52[ab]	0.59[ab]	0.66[a]	0.04	< 0.0001	< 0.01	0.14	0.53
22:6n-3 (DHA)	3.16[b]	8.24[a]	7.99[a]	8.03[a]	7.76[a]	8.02[a]	0.37	< 0.0001	< 0.0001	< 0.0001	0.69
Total n-6	32.6[a]	28.8[bc]	29.0[bc]	28.6[bc]	29.1[b]	27.8[c]	0.30	< 0.0001	< 0.0001	< 0.0001	0.70
Total n-3	3.61[b]	9.42[a]	9.05[a]	9.25[a]	9.31[a]	9.58[a]	0.42	< 0.0001	< 0.0001	< 0.0001	0.72

[1]Data represents least square means (LSM) ± standard errors (SE), n=7 per treatment. [a-f]Different superscripts between treatments within a row for each lipid class are significantly different at $P < 0.05$. Coefficient of determination (R^2) indicated when linear or quadratic effects are significant ($P < 0.05$).

Table 6.10: Polyunsaturated fatty acid profile in the lipid fraction of liver (mol%) a) phosphatidylcholine (PtdCho) and b) phosphatidylethanolamine (PtdEtn.) after feeding hens diets with varying levels of alpha-linolenic acid (ALA) derived from hempseed products[1]

Source of ALA	Control	10%HS	4.5%HO	20%HS	30%HS	9.0%HO					
ALA content (% of diet)	0.10	0.50	0.66	0.90	1.22	1.28	SE	P - value	Linear	Quadratic	Adj R^2
a) Liver - PtdCho											
18:2n-6 (LA)	19.8	20.5	19.6	19.6	19.4	19.3	0.52	0.69			
18:3n-6 (GLA)	0.21^b	0.24^b	0.30^{ab}	0.28^{ab}	0.32^{ab}	0.39^a	0.03	< 0.01	0.33	0.99	
20:4n-6 (ARA)	4.42^a	4.02^{ab}	3.91^{ab}	3.78^b	4.17^{ab}	3.79^b	0.13	< 0.05	< 0.01	< 0.05	0.24
18:3n-3 (ALA)	0.10^c	0.28^b	0.28^b	0.35^b	0.46^a	0.48^a	0.02	< 0.0001	< 0.001	0.30	0.84
20:5n-3 (EPA)	0.00^d	0.02^c	0.02^{bc}	0.03^{bc}	0.04^{ab}	0.05^a	0.003	< 0.0001	< 0.01	0.28	0.79
22:5n-3 (DPA)	0.05^b	0.12^a	0.12^a	0.15^a	0.16^a	0.17^a	0.01	< 0.0001	< 0.001	< 0.05	0.70
22:6n-3 (DHA)	1.13^b	3.14^a	3.30^a	3.58^a	3.36^a	3.33^a	0.16	< 0.0001	< 0.0001	< 0.0001	0.88
Total n-6	24.4	24.7	23.8	23.6	23.9	23.6	0.51	0.58			
Total n-3	1.29^b	3.56^a	3.72^a	4.11^a	4.01^a	4.10^a	0.15	< 0.0001	< 0.0001	< 0.0001	0.92
b) Liver - PtdEtn											
18:2n-6 (LA)	14.3	15.4	14.5	13.2	12.6	15.3	1.19	0.51			
18:3n-6 (GLA)	0.13^b	0.17^{ab}	0.20^{ab}	0.17^{ab}	0.18^{ab}	0.27^a	0.03	< 0.05	0.73	0.74	
20:4n-6 (ARA)	19.4^a	16.4^b	16.2^b	16.1^b	16.7^b	15.3^b	0.51	< 0.001	< 0.001	< 0.01	0.54
18:3n-3 (ALA)	0.21^b	0.28^{ab}	0.27^b	0.31^{ab}	0.45^a	0.45^a	0.04	< 0.001	0.78	0.22	
20:5n-3 (EPA)	0.02^d	0.15^c	0.20^c	0.29^b	0.40^a	0.41^a	0.01	< 0.0001	< 0.0001	0.62	0.95
22:5n-3 (DPA)	0.19^b	0.37^a	0.40^a	0.47^a	0.46^a	0.48^a	0.04	< 0.0001	< 0.0001	< 0.01	0.65
22:6n-3 (DHA)	3.35^b	7.81^a	8.91^a	9.02^a	7.56^a	8.65^a	0.41	< 0.0001	< 0.0001	$<.0001$	0.84
Total n-6	33.8	32.0	30.9	30.8	29.4	30.8	0.94	0.06			
Total n-3	3.72^b	8.61^a	9.78^a	10.1^a	8.91^a	10.0^a	0.42	< 0.0001	< 0.0001	< 0.0001	0.87

[1]Data represents least square means (LSM) ± standard errors (SE), n=5 per treatment. [a-d]Different superscripts between treatments within a row for each lipid class are significantly different at $P < 0.05$. Coefficient of determination (R^2) indicated when linear or quadratic effects are significant ($P < 0.05$).

Table 6.11: Polyunsaturated fatty acid profile in the lipid fraction of plasma (mol%) a) phosphatidylcholine (PtdCho) and b) phosphatidylethanolamine (PtdEtn.) after feeding hens diets with varying levels of alpha-linolenic acid (ALA) derived from hempseed products[1]

Source of ALA	Control	10%HS	4.5%HO	20%HS	30%HS	9.0%HO					
ALA content (% of diet)	0.10	0.50	0.66	0.90	1.22	1.28	SE	P - value	Linear	Quadratic	Adj R^2
a) Plasma - PtdCho											
18:2n-6 (LA)	24.2	22.4	22.2	23.1	23.1	22.8	0.44	0.07			
18:3n-6 (GLA)	0.16^b	0.20^b	0.22^b	0.31^a	0.37^a	0.35^a	0.02	< 0.0001	0.06	0.45	0.83
20:4n-6 (ARA)	4.33	4.49	3.67	4.04	4.02	3.81	0.21	0.11			
18:3n-3 (ALA)	0.14^d	0.33^{cd}	0.34^c	0.56^b	0.77^a	0.69^{ab}	0.04	< 0.0001	< 0.05	0.27	0.89
20:5n-3 (EPA)	0.00^c	0.04^b	0.03^b	0.04^{ab}	0.05^a	0.05^a	0.003	< 0.0001	< 0.0001	< 0.01	0.83
22:5n-3 (DPA)	0.06^c	0.14^{ab}	0.11^{bc}	0.14^{ab}	0.16^a	0.16^{ab}	0.01	< 0.001	< 0.05	0.24	0.53
22:6n-3 (DHA)	0.92^b	2.96^a	2.37^a	2.94^a	2.72^a	2.81^a	0.20	< 0.0001	< 0.0001	< 0.0001	0.61
Total n-6	28.7^a	27.1^{abc}	26.1^c	27.4^{abc}	27.5^{ab}	26.9^{bc}	0.32	< 0.001	< 0.01	< 0.01	0.33
Total n-3	1.12^c	3.47^{ab}	2.87^b	3.68^a	3.70^a	3.71^a	0.20	< 0.0001	< 0.0001	< 0.0001	0.73
b) Plasma - PtdEtn											
18:2n-6 (LA)	18.6^a	16.0^b	15.4^b	16.4^b	16.8^b	15.9^b	0.37	< 0.0001	< 0.0001	< 0.01	0.41
18:3n-6 (GLA)	0.15^c	0.16^{bc}	0.18^{bc}	0.23^{ab}	0.24^a	0.25^a	0.02	< 0.001	0.35	0.67	
20:4n-6 (ARA)	13.7	12.3	12.5	12.2	11.4	11.6	0.55	0.14			
18:3n-3 (ALA)	0.23^d	0.42^c	0.42^c	0.61^b	0.81^a	0.71^{ab}	0.03	< 0.0001	< 0.01	0.79	0.81
20:5n-3 (EPA)	0.02^d	0.10^c	0.12^{bc}	0.15^b	0.19^a	0.20^a	0.01	< 0.0001	< 0.0001	< 0.05	0.93
22:5n-3 (DPA)	0.23^b	0.40^{ab}	0.40^{ab}	0.42^{ab}	0.50^a	0.51^a	0.05	< 0.05	0.10	0.57	
22:6n-3 (DHA)	3.48^b	8.74^a	8.55^a	8.19^a	7.70^a	8.68^a	0.60	0.0001	0.0001	< 0.001	0.54
Total n-6	32.4^a	28.5^b	28.1^b	28.8^b	28.4^b	27.7^b	0.50	0.0001	< 0.0001	< 0.001	0.60
Total n-3	3.95^b	9.66^a	9.48^a	9.37^a	9.17^a	10.1^a	0.62	< 0.0001	< 0.0001	< 0.001	0.61

[1]Data represents least square means (LSM) ± standard errors (SE), n=4 per treatment. [a-d]Different superscripts between treatments within a row for each lipid class are significantly different at $P < 0.05$. Coefficient of determination (R^2) indicated when linear or quadratic effects are significant ($P < 0.05$).
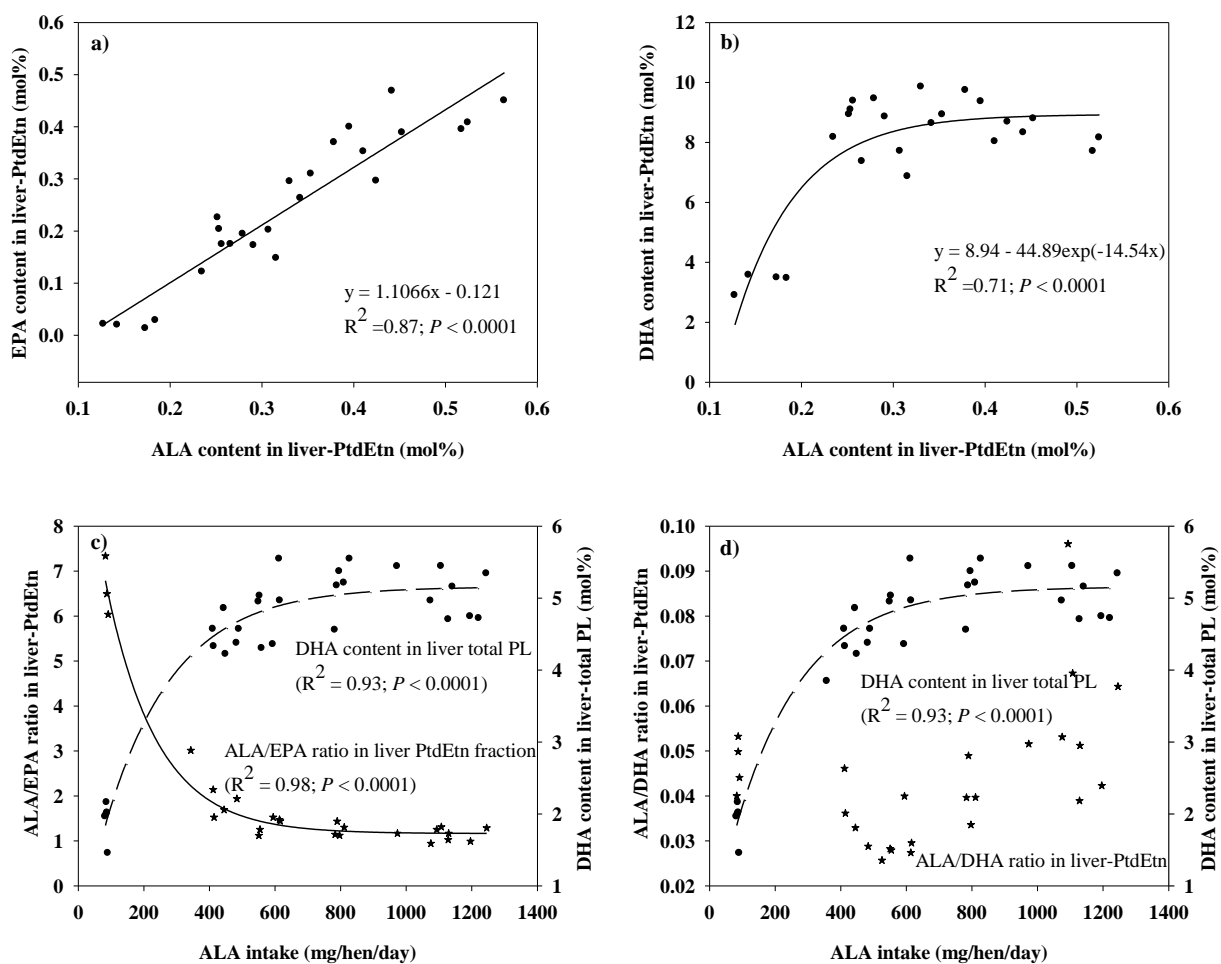
Figure 6.3. Relationship between accumulation of alpha-linolenic acid (ALA) versus a) eicosapentaenoic acid (EPA) and b) docosahexaenoic acid (DHA) in liver phosphatidylethanolamine (PtdEtn). Relationship between the ratio of either c) ALA to EPA or d) ALA to DHA (closed stars, solid line) in the liver- PtdEtn and accumulation of docosahexaenoic acid (DHA) (closed circles, long-dash line) in liver total phospholipid (PL) fraction.

Individual data points (n = 5 per treatment) are presented and regression line/curves have been fitted to the data.

6.5 DISCUSSION

This study was designed to understand the mechanism(s) regulating n-3 LCPUFA deposition in egg yolk by analyzing comprehensive fatty acid profiles in total and major lipid classes by following the hen's liver-circulating plasma-egg yolks. This was achieved by providing hens with increasing ALA levels in the diets, via inclusion of HS or HO. Overall, as a function of ALA intakes, the inclusion of hempseed products in diets of laying hens greatly increased the levels of the n-3 PUFA in the egg yolk, liver and plasma. More specifically this study found that ALA increased linearly as function of ALA in all lipid classes in all biological samples tested but DHA increased in a non-proportional manner.

The influence of dietary ALA was most evident with respect to the n-3 PUFA metabolites. In this study, as the dietary ALA intakes increased from 85.6 ± 1.74 to 1132 ± 25.8 mg/hen/day, the levels of the n-3 PUFA (ALA, EPA, DPA and DHA) increased in the total lipids of the egg yolk, liver and plasma. This observation supports the well-established phenomenon that laying hens have the ability to convert dietary ALA to its long chain metabolites (González-Esquerra and Leeson, 2001). The liver, sensitive to dietary n-3 PUFA, regulates the synthesis of LCPUFA metabolites from its primary precursor ALA through the process of elongation and desaturation (Garg et al., 1988; Portolesi et al., 2007). In the present study, except for ALA, the levels of the n-3 PUFA (mol%) in total lipids in the liver were higher than that in the egg yolk and the plasma. The lipid contents (wet weight), in the liver and egg yolk are approximately 3% to 5% (Pita et al., 2011) and 33% (of total yolk weight) (Leskanich and Noble, 1997), respectively. Based on these lipid proportions, the total PL and TAG contents in the liver are ~35% and 64%, respectively; however, the proportions depend on the liver fat content, being ~7% and 92%, respectively with fatty liver hemorrhage condition (~80% DM fat in liver) (Ivy

and Nesheim, 1973). In the egg yolk, the total PL and TAG contents are ~21% and 70%, respectively (Leskanich and Noble, 1997). On the other hand, the lipoproteins (VLDL fraction, main component of yolk lipid) of the hen plasma contains 87% lipids, containing approximately 66 - 68% TAG and 23 - 26% total PL (McIndoe, 1971; Evans et al., 1977). Hence, the higher levels (mol%, Figure 1) of the LCPUFA in the liver compared to that in the egg yolk (or the plasma) of the hens, may be attributable to the differences in the proportions of their lipid fractions, particularly that of the total PL fraction. In addition, Elkin et al. (2015, 2016) made similar observations in laying hens and broilers, respectively, and attributed this to the liver's ability to elongate and desaturate ALA and the preference for n-3 LCPUFA in hepatic PL membranes.

Although the majority of the ALA in the liver was incorporated into the TAG fraction, the study also showed that dietary ALA gets converted to the n-3 LCPUFA and these can be enriched in the hepatic PL. However, of the three metabolites, EPA is least accumulated in all tissues. Different authors have reported that the conversion of ALA into EPA is limited in both animal and humans (Scheideler and Froning, 1996; Portolesi et al., 2007; Palmquist, 2009; Fraeye et al., 2012) or EPA could have been converted to eicosanoids for other functions *in vivo*. In poultry, a weak relationship between dietary ALA content and tissue EPA content has been suggested to reflect limitations in the accumulation of EPA in edible tissues (Rymer and Givens, 2005). In a human study, using uniformly labeled [^{13}C] ALA, limited incorporation of dietary ALA into the hepatic PL pool was thought to contribute to the low net hepatic conversion of ALA into EPA (Goyens et al., 2005). Examining the levels of EPA in the current study, the synthesis and accumulation of EPA in relation to increasing ALA intakes by the laying hens was limited in the TAG fraction in the liver. A linear increase of the fatty acid in the PL fraction was

reflected in a similar linear increase in the total lipids in the liver. However, the very high proportion of EPA in the TAG compared to total PL of the egg yolk observed in the current study, was unexpected because it had been widely reported previously that EPA, as for DPA and DHA are mainly deposited in the total PL fraction of the egg yolk (Jiang et al., 1991; Leskanich and Noble, 1997; Surai et al., 1999). Others (Fredriksson et al., 2006) using rapeseed or corn oil in control diet in a study involving marine microalgae, reported similar levels of EPA in both the TAG and total PL of the yolk lipid derived from the hens fed these diets. Comparing the lipid fractions of chicken edible products, in skeletal muscles, depending on the dietary EPA content, EPA is accumulated to a greater extent in the PL fraction (Rymer and Givens, 2005). Because the lipid component in the egg yolk (33% of the total weight of egg yolk) is TAG-rich (70%) (Leskanich and Noble, 1997), there is a substantial pool available for EPA deposition in egg yolk TAG.

The liver plays a key role in lipid metabolism in poultry (Leveille et al., 1975) similar to humans (Patel et al., 1975). Moreover, in the formation of complex lipids, primarily involving glycerol-3-phosphate and activated fatty acids (Dircks and Sul, 1999), EPA (and its CoA derivative, EPA-CoA) is a poor substrate for diacylglycerol acyltransferase, an enzyme involved in the esterification of 1, 2-diacylglycerol. Therefore, a decrease in hepatic TAG synthesis (Berge et al., 1999) may provide a possible explanation for the undetectable levels of EPA in the TAG fraction of the liver, especially at lower levels of ALA intakes in the current study. Alternatively, it could be possible that EPA (at lower dietary ALA intake levels) is also readily converted to DHA; hence, translating into lower detection levels of EPA at lower inclusion levels of ALA in the laying hen diets.

In the current study, DPA levels were intermediate between those for EPA and DHA. Furthermore, DPA accumulation was limited (observed to plateau) in the PL of the liver. This limitation in DPA accumulation likely translated to a similar trend for DHA, given the fact that DPA occupies an intermediate position in the interconversion of EPA to DHA (Sprecher, 2000), and a temporary storage form for the latter fatty acid (Tapiero et al., 2002). As a final end-point product in the metabolism of dietary ALA, DHA was shown to be highly enriched in the total lipids of the egg yolk, liver and plasma. Docosahexaenoic acid (together with ARA) is a prominent PUFA in cellular PL (Salem et al., 2001). However, unlike the case for EPA, DHA (and its CoA derivative) does not inhibit diacylglycerol acyltransferase (Madsen et al., 1999), providing a potential explanation for the accumulation of this fatty acid in the neutral lipids (TAG and CE) of all tissues, except the CE of egg yolk (because the latter contained only traces of CE). Despite this ability to accumulate in the TAG of all tissues, there is minimal DHA in the TAG fraction of egg yolk, possibly because it is preferentially oxidized for metabolic processes. Except in the TAG fraction of the egg yolk, DHA was shown to attain saturation levels in the polar lipids as well as the TAG fraction of all tissues. Hence, these results may also provide support for the contention that the accumulation of endogenously synthesized DHA was not necessarily limited by the size of the PL pool in the liver (Portolesi et al., 2007), because the accumulation of DHA also reached a plateau in the PL fraction of plasma as well as the egg yolk. However, as DHA is mainly confined to the total PL of the egg yolk rather than the TAG, its accumulation may be restricted by the limited PL pool size in the egg yolk lipid. Although the accumulation of DHA in egg yolk may be explained on the basis of the efficiency of biosynthesis (involving the conversion of dietary ALA) and transfer, preferential accumulation sites/pools may determine it metabolic fate.

A significant finding in the current study was the strong inverse correlation between the ratio of ALA to EPA in the hepatic PtdEtn lipid and the level of DHA in the total PL (Figure 6.3). The first phase of the graph may indicate increased hepatic conversion of ALA to EPA as dietary ALA increased, which subsequently would lower the ratio of liver ALA/EPA; thereafter, the plateau of the ratio may indicate the impact of prevailing levels/concentration of EPA (and possibly not DHA) in regulating LCPUFA biosynthesis, limiting the conversion of either ALA and/or EPA to DHA when derived via endogenous synthesis from plant-based ALA. As a function of ALA intakes, in part, these factors may include the regulation of long chain acyl-CoA use for β-oxidation (Coleman and Lee, 2004), indicating the need to control hepatic metabolism of LCPUFA and maintain lipid homeostasis (Jump, 2008). Although other dietary derived PUFA are also β-oxidized to similar percentages as ALA (Barceló-Coblijn and Murphy, 2009), β-oxidation in the mitochondria is known to be stimulated more in the presence of EPA in the PL (Liu et al., 2013). In studies utilizing products with preformed LCPUFAs, such as fish oils containing a ratio of EPA to DHA of 1.13 (Millet et al., 2006) and 1.50 (Lawlor et al., 2010) in diets of laying hens, linear increases were observed in the enrichment of eggs with both fatty acids when graded levels of these products were used, possibly because fish oils contains only low (~1-1.5 %) levels of ALA. In the current study, the ratio as an indicator of hepatic conversion of either ALA to EPA or ALA to DHA in the liver PtdEtn fraction, resulting in a strong association in the former relationship, may imply that factor(s) regulating LCPUFA biosynthesis as a function of ALA intake may be closer to EPA than DHA.

For the n-6 PUFA, the increase in the levels of LA observed in the total lipids of the egg yolk, liver and plasma in this study was in accordance with reports by Caston and Leeson (1990) who noted increases in the n-3 fatty acids as well as the n-6 fatty acids in egg yolk in a feeding

trial using flaxseed, and more so at higher levels of inclusion. Similarly, the increase in the levels of ALA in diets of the laying hens, due to the inclusion of the hempseed products, was accompanied by an overall decrease in ARA concentration in the total, the TAG and the polar lipids in all tissues. Previous studies (Van Elswyk et al., 1985; Cherian and Sim, 1991) have shown similar responses in total lipids in egg yolk. The changes in the levels of accumulation of GLA in the egg yolk, liver and plasma were indicative of the levels of this fatty acid in the diets. Higher levels of GLA observed in the TAG than in the total PL of both the liver and plasma, may imply a preferential acylation of GLA into the neutral lipids (TAG and CE), unlike its subsequent metabolite ARA, dominant in the PL fraction (Salem et al., 2001; Gładkowski et al., 2011). However, the egg yolk contained equal amounts of GLA in both the TAG and total PL, possibly due to a limited CE fraction in the egg yolk (Vieira et al., 1995).

The SFA content of the hen's egg is fairly resistant to alteration by dietary means (Cherian and Sim, 1991; Baucells et al., 2000). The latter findings also extended to the total SFA levels in the TAG and total PL fractions of the egg yolk. As a function of increased ALA intakes by the laying hens, *de novo* synthesis of fatty acids (primarily, palmitate) in the liver is inhibited (Naber and Squires, 1993; Ayerza et al., 2002), not only influencing the formation of the SFA (Sims and Qi, 1995) but also the MUFA (Brenner, 1989). Furthermore, decreased hepatic fatty acid synthesis is also known to lower the incorporation of cholesterol into yolk precursors (vitellogenin and lipoprotein components), which may also have a similar effect on its esters, the CE fraction (Sinanoglou et al., 2011). In part, this may explain the observed limited distribution of fatty acids into the CE fraction of the egg yolk unlike that in the liver or the plasma, most likely due to the fact that most (>90%) of the cholesterol in egg yolk exists in free

form (Kuksis, 1992). The CE fraction in the egg yolk represents 1.3% of the yolk mass (Vieira et al., 1995), and is therefore not a major contributor of the fatty acid pool.

In summary, increasing dietary ALA levels in laying hen diets significantly increased the levels of n-3 PUFA in the total and the major lipid classes of the egg yolk, liver and plasma; however, these increases were differentially expressed. Firstly, although the levels of the primary precursor, ALA, increased in both the TAG and the PL fractions in all tissues, higher levels accumulated in the former than the latter fraction. Secondly, the LCPUFA, particularly DHA, accumulated to a high degree in the PL, mainly in the PtdEtn lipids in all tissues. The levels and distribution of DPA in the total lipids as well as the lipid classes of the tissues were intermediate between those of EPA and DHA; hence, exhibiting a central role, such as being a temporary storage form of the long chain metabolites. Docosahexaenoic acid exhibited a plateau in both total and individual lipid classes with a strong inverse correlation between PL-DHA and the ratio of ALA to EPA in the liver PtdEtn fraction. The latter association and the non-proportional response between ALA and DHA liver PtdEtn fraction supports the link between low substrate (ALA) availability in the liver and the limitation in converting ALA into DHA. In addition, when ALA serves as the primary dietary n-3 source for hens, the n-3 LCPUFA, DHA is deposited primarily into the PL fraction, a limited pool size in egg lipids when compared to TAG. Future work comparing endogenously-derived versus exogenously-supplied DHA in laying hen diets, coupled with molecular analysis, will elucidate substrate differences as to the potential for the enrichment of DHA in eggs.

CHAPTER 7 MANUSCRIPT IV

Effect of flaxseed oil and microalgae DHA on the production performance, fatty acids and

total lipids of egg yolk and plasma in laying hens

7.1 ABSTRACT

The incorporation of omega-3 polyunsaturated fatty acids (PUFA) in the egg is dependent on both the transfer efficiency of preformed dietary omega-3 PUFA to the eggs as well as endogenous PUFA metabolism and deposition. Employing an experimental design consisting of 70 Lohmann LSL-Classic hens in a 6-week feeding trial, we examined the impact of graded levels of either flaxseed oil (alpha-linolenic acid, ALA) or algal DHA (preformed docosahexaenoic acid, DHA), each supplying 0.20, 0.40 and 0.60% total omega-3 PUFA. The control diet was practically low in omega-3 fatty acids. Study parameters included monitoring the changes of fatty acid contents in yolk, measures of hen performance, eggshell quality, total lipids and fatty acid contents of plasma. Data were analyzed as a complete randomized design using Proc Mixed procedure of SAS. No significant differences were observed between treatments with respect to hen performance, eggshell quality and cholesterol content in plasma and yolk. Individual and total omega-3 PUFA in the yolk and plasma increased ($P < 0.0001$) linearly as a function of total omega-3 PUFA intake. At the highest inclusion levels, DHA-fed hens incorporated 3-fold more DHA in eggs compared with ALA-fed hens (179 ± 5.55 vs. 66.7 ± 2.25 mg/yolk, respectively). In both treatment groups, maximal enrichment of total n-3 PUFA was observed by week-2, declined by week-4 and leveled thereafter. In addition, accumulation of DHA in eggs showed linear ($P < 0.0001$) and quadratic ($P < 0.05$) effects for flaxseed oil (R^2 =0.89) and algal DHA (R^2 =0.95). The current data, based on defined level of total omega-3 PUFA in the background diet, provides evidence to suggest that exogenous as well as endogenous synthesis of DHA may be subject to a similar basis of regulation, and serve to highlight potential regulatory aspects explaining the limitations in the deposition of endogenously produced omega-3 LCPUFA.

Key words: egg yolk; fatty acid profile; flaxseed oil and algal DHA

7.2 INTRODUCTION

Egg enrichment with omega-3 polyunsaturated fatty acids (n-3 PUFA) is achieved by feeding hens diets rich in these fatty acids. As a result, the enriched eggs offer a potential way to increase the content of important PUFA, including those implicated in the protection against nutrition-related chronic diseases, in the human diet. The inclusion of omega-3 PUFA into yolk lipids can be achieved by feeding hens with plant-derived sources of alpha-linolenic acid (ALA) (Baucells et al., 2000; Gakhar et al., 2012; Goldberg et al., 2012; Neijat et al., 2016a). However, it is primarily the ALA and, to some extent, the docosahexaenoic acid (DHA) and more limited eicosapentaenoic acid (EPA) levels that are enriched (Cherian and Sim, 1991; Gakhar et al., 2012; Neijat et al., 2016a). As such, there exists inefficiency in the conversion of ALA to its long chain PUFA (LCPUFA) metabolites. Alternatively, preformed omega-3 LCPUFA sources such as fish oils and marine algae (rich in EPA and/or DHA) have been used to directly incorporate the long chain omega-3 PUFA into the egg yolk (Van Elswyk, 1997; Cachaldora et al., 2005; Lawlor et al., 2010). Both source and level of PUFA inclusion in laying hen diets greatly modify the level and type of PUFA retained in the eggs without affecting the total lipid content (Guenter et al., 1971; Hargis et al., 1991).

Although the hen has an inherent ability to synthesize omega-3 PUFA, differential deposition of various components of the lipids (such as fatty acids) in the egg as well as other tissues may be influenced by the difference in the rate of accretion among various omega-3 PUFA (Herber and Van Elswyk, 1996). The metabolism of omega-3 PUFA and its final deposition into the egg, in part, can be related to differences in diet and ingredient composition (González-Esquerra and Leeson, 2001), in particular, the total PUFA content of the basal diet (Gibson et al., 2013). Consequently, the latter may influence both the transfer efficiency of

preformed dietary omega-3 fatty acids to the eggs as well as endogenous PUFA metabolism and deposition.

The objective of the current study was to determine the impact of including graded levels of either flaxseed oil (provider of precursor ALA) or algal DHA (a source of preformed docosahexaenoic acid, DHA) on the enrichment of omega-3 fatty acids in egg yolk using a basal diet, low in total omega-3 fatty acid. Based on previous studies in our lab, utilizing hempseed products (Gakhar et al., 2012; Neijat et al., 2016a; b), 500 to 600 mg/hen/day was the most optimal range of intake of total omega-3 PUFA, primarily ALA (indicating, optimal supplementation dose for hens consuming 100g feed per day) to reach the highest DHA enrichment level in eggs. Although, flaxseed/oil results in greater incorporation of DHA into egg yolk (up to 90 mg/yolk) (Scheideler and Froning, 1996; Baucells et al., 2000) compared to using hempseed products (~50 mg/yolk) (Gakhar et al., 2012; Neijat et al., 2016a), the efficiency of deposition of DHA with the former starts to decline within a similar range of intake levels of ALA as for hempseed ingredients (Cherian and Sim, 1991; Scheideler and Froning, 1996; Baucells et al., 2000). Therefore, for the current study, a daily intake of 600 mg/hen was used as a guide in considering the highest supplementation level to the diet of the laying hens. The study also aimed to determine the potential interaction between the length of period of feeding with the dietary level of omega-3 fatty acid and the resultant DHA levels in the eggs. In this comparative assessment, the impact of providing graded dietary levels of either the precursor ALA or preformed DHA on egg yolk and plasma levels of cholesterol as well as plasma triacylglycerol (TAG) level were also determined.

7.3 MATERIALS AND METHODS

7.3.1   Experimental birds and diets

A total of 70, 34-week-old, Lohmann LSL-Classic laying hens, were individually caged

with separate feeding troughs and drinkers. For the first week, the hens were fed a commercial

layer diet without supplemental omega-3 PUFA or the control diet followed by a 50:50,

commercial layer diet to experimental diet, in the second week before feeding 100% of

experimental diet. Following the adaptation period, the hens were assigned randomly 1 of 7

experimental diets (10 hens/treatment) containing graded levels of total omega-3 fatty acid (0.20,

0.40 and 0.60%, by wt of diet) derived from either flaxseed oil (provider of precursor ALA,

contained in 0.36, 0.77 and 1.19% by wt flaxseed oil) or algal DHA (a source of preformed

DHA, 1.00, 2.18 and 3.36% by wt algal DHA), on a cereal-based diet practically low in omega-3

fatty acids (included as a control, 0.03% total omega-3 fatty acid; contained in 1.35% by wt of

diet almond oil). All diets were formulated (based on analysed data) to be isolipidic, isocaloric

and isonitrogenous, containing 4% crude fat, 2800 kcal/kg energy and 16% crude protein,

respectively. The use of almond oil in laying hen diets is not a practical approach, however, the

current study was also linked to another part of the study designed to address, as a separate study

(Neijat et al., unpublished), potential control point(s) that may be involved in the regulation of

endogenous synthesis and enrichment of eggs with DHA the same substrates used in the current

study. Hence, in both parts of the study, almond oil (low in ALA) formed the basis of the

background diet, and was used in order to remove the confounding effect of ALA, particularly

on the preformed DHA containing diets. The ingredients and nutrient composition of the

experimental diets are shown in Table 7.1. The flaxseed oil was sourced from Dyets, Inc.

(Bethlehem, PA, USA) and the algal DHA (DHAgold[TM] S17-B, dried whole-cell algae product).

Table 7.1: Ingredients and nutrient composition of laying hen diets

	Control	Flaxseed oil			Algal DHA		
Total omega-3 fatty acid (% of diet)	0.03	0.20	0.40	0.60	0.20	0.40	0.60
Ingredient (%, by wt)							
Soybean meal	25.1	25.1	25.1	25.1	25.1	24.8	24.5
Wheat	2.10	2.10	2.10	2.11	0.00	0.00	0.00
Corn	56.9	56.9	56.9	56.9	58.4	58.3	58.3
Vitamin-mineral premix[1]	2.50	2.50	2.50	2.50	2.50	2.50	2.50
Sodium chloride	0.39	0.39	0.39	0.39	0.40	0.40	0.40
Limestone	9.70	9.70	9.70	9.70	9.59	9.14	8.70
Dicalcium phosphate	1.90	1.90	1.90	1.90	1.91	1.91	1.92
L-Lysine-HCl	0.038	0.038	0.038	0.038	0.041	0.049	0.057
DL-Methionine	0.092	0.092	0.092	0.092	0.093	0.095	0.097
Flaxseed oil	0.00	0.36	0.77	1.19	0.00	0.00	0.00
DHAgold[TM] S17-B	0.00	0.00	0.00	0.00	1.00	2.18	3.36
Almond oil	1.35	0.99	0.58	0.16	0.97	0.55	0.14
Calculated Nutrients							
AMEn (Poultry; Kcal/kg)	2850	2850	2850	2850	2850	2850	2850
Crude Protein (%)[2]	16.5	16.5	16.5	16.5	16.5	16.5	16.5
Total Fat (%)	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Analyzed Nutrients							
MEn (Poultry; Kcal/kg)	2821	2812	2808	2796	2813	2815	2840
Crude protein (%)[2]	15.3	15.5	15.7	16.1	15.4	16.1	15.7
Total fat (%)	4.04	4.18	3.93	3.74	3.68	3.59	3.75
Crude fiber (%)	2.46	2.46	2.81	3.45	2.77	2.71	2.67
Calcium (%)	4.39	4.22	4.39	4.27	4.30	4.11	3.82
Phosphorus (%)	0.70	0.72	0.69	0.70	0.71	0.70	0.74
Fatty Acids(%, by wt)[2]							
Calculated							
18:2n-6 (LA)	1.30	1.29	1.28	1.27	1.25	1.17	1.10
18:3n-3 (ALA)	0.03	0.20	0.40	0.60	0.03	0.03	0.03
22:6n-3 (DHA)	0.00	0.00	0.00	0.00	0.17	0.37	0.57
Total omega 3-fatty acids[3]	0.03	0.20	0.40	0.60	0.20	0.40	0.60
Analyzed							
Total SFA[1]	0.34	0.36	0.38	0.39	0.43	0.55	0.67
Total MUFA[2]	1.00	0.96	0.83	0.70	0.83	0.67	0.49
Ratio n-6/n-3	25.6	6.7	3.8	2.5	5.8	3.0	1.9
18:2n-6 (LA)	1.23	1.34	1.36	1.32	1.15	1.15	1.04
18:3n-3 (ALA)	0.05	0.20	0.36	0.53	0.05	0.05	0.05
20:5n-3 (EPA)	-	-	-	-	0.006	0.012	0.017
22:5n-3 (DPA)	-	-	-	-	0.002	0.004	0.006
22:6n-3 (DHA)	0.00	0.00	0.00	0.00	0.145	0.332	0.504
Total omega 3-fatty acids[3]	0.05	0.20	0.36	0.53	0.20	0.40	0.60

[1]Provided per kilogram of diet, vitamin-mineral premix contained: 11,000 IU of vitamin A; 3,000 IU of vitamin D_3, 150 IU of vitamin E, 3 mg of vitamin K_3 (as menadione), 0.02 mg of vitamin B_{12}, 0.2 mg of biotin, 6.5 mg of riboflavin, 4 mg of folic acid, 10 mg of calcium pantothenate, 39.9 mg of niacin, 2.2 mg of thiamine, 4.5 mg of pyridoxine, 1000 mg of choline chloride, 125mg antioxidant (ethoxyquin), 66 mg of manganese oxide, 70 mg of zinc oxide, 80 mg of ferrous sulfate, 10 mg of copper sulfate, 0.3 mg of sodium selenite, 0.4 mg of calcium iodate, 0.67 mg of sodium chloride (salt).
[2]Nutrients expressed as a percentage by wt of diet.
[3]Total omega-3 fatty acids = sum of ALA, EPA DPA and DHA.

was obtained (a generous gift) from DSM Nutritional Products (North America, Parsippany, NJ, USA); the almond oil was obtained from a local commercial source. The composition of these ingredients is shown in Table 7.2. All lipid ingredients prior to mixing and mixed diets were stored at -20°C. However, for immediate use, feed was stored under cool, dry conditions, for a maximum of 3 weeks. All diets were formulated to meet minimum nutrient specifications according to published NRC recommendations (NRC, 1994) and as indicated in management guide for Lohmann hens within Phase 1 of the production cycle (egg lay to 40 weeks of age) consuming ~105 g/hen/day feed, (Lohmann LSL-Lite, North American Edition). Vitamin E, in addition to synthetic antioxidants (total of 150 IU/kg diet) was included in the diet. Feed and water were supplied to allow *ad libitum* consumption during the experimental period. A lighting program of 16 h of light and 8 h of dark was used for the entire experiment. The protocol and use of the animals during this study were in accordance with recommendations established by the Canadian Council on Animal Care (1993) which were reviewed and approved by the University of Manitoba's Animal Care Protocol Management and Review Committee.

7.3.2   *Sampling and measurements*

Feed intake, egg production and egg weight were recorded continuously throughout the experiment, and weekly averages were analysed. For feed intake, a 7-day feeding record was considered by supplying 400 g of diets to each hen at intervals of 2 days to maintain freshness of the diets. At the end of each week, unconsumed feed from each hen (n = 10 per treatment) was weighed (weigh-back) to determine the daily rate of feed intake of each hen and finally expressed as averages by dividing by the number of hens per treatment. This was done for each period. Hen body weight was recorded at the beginning of the experiment (36 weeks of age) and thereafter weekly to the end of the feeding trial (42 weeks of age). On the last 3 consecutive

Table 7.2: Composition of flaxseed oil, DHAgold^{TM} S17-B (algal DHA) and almond oil used in the formulation of the diets of laying hens

Composition	Flaxseed oil[1]	DHAgold^{TM} S17-B[2]	Almond oil[3]
Analysed (%)[4]			
Crude protein	-	11.3 ± 0.06	-
Total fat	100	39.9 ± 4.14	100
Calcium	-	0.05 ± 0.001	-
Total phosphorus	-	1.04 ± 0.01	-
Fatty acids			
14:0	0.051	3.39 ± 0.052	0.056
14:1		0.032 ± 0.025	
15:0	0.030	0.165 ± 0.003	0.010
16:0	5.56	8.65 ± 0.024	5.30
16:1	0.067	0.096 ± 0.004	0.118
17:0	0.064	0.071 ± 0.009	0.047
18:0	3.34	0.235 ± 0.002	2.78
18:1	19.2	0.565 ± 0.022	65.6
18:2n-6 (LA)	17.1	0.461 ± 0.022	24.3
18:3n-6 (GLA)		0.097 ± 0.004	
18:3n-3 (ALA)	53.2	0.038 ± 0.004	0.196
20:0	0.160	0.052 ± 0.004	0.272
20:1	0.257		0.267
20:2	0.053		
20:3n-6		0.632 ± 0.005	
20:3n-3	0.103	0.013 ± 0.031	
20:4		0.170 ± 0.002	
20:5n-3 (EPA)		0.403 ± 0.005	
22:0	0.136	0.041 ± 0.003	0.733
22:1	0.195		
22:4		0.029 ± 0.010	
22:5n-6		6.45 ± 0.063	
22:5n-3 (DPA)		0.123 ± 0.007	
22:6n-3 (DHA)		17.96 ± 0.054	
24:0		0.122 ± 0.018	0.269

[1]Flaxseed oil obtained from Dyets, Inc. (Bethlehem, PA, USA). Specification provided (%): Fat, 16:0, 18:0, 18:1, LA and ALA are 100, 5.0, 4.0, 18.0, 16.0 and 57.0, respectively.
[2]DHAgold^{TM} S17-B dried whole-cell algae product supplied by DSM Nutritional Products (North America, Parsippany, NJ, USA). Specification provided (%): Fat and DHA are 40 and 17, respectively.
[3]Almond oil obtained from commercial source.
[4]Analyzed as per materials and methods section, values (means ± SD, of duplicate analysis per sample) and expressed as % by weight of product (derived from % total fatty acids of all fatty acids identified)

days of week 2, 4 and 6, one egg per hen per day was collected for lipid analysis and for measurements of yolk weight, shell weight/index and shell thickness. Within each week, only one egg per hen per treatment (n=10) was used for lipid analysis, however, for the last three parameters, daily individual analysis was conducted on each egg, then expressed as averages of the three days per treatment (n=10) for each experimental period. Other eggs were also collected for 3 consecutive days (day 2, 3 and 4 of the week) during week 3 and 6 for the determination of specific gravity and Haugh units. Eggs for lipid analysis (fatty acid and cholesterol) were first weighed and cracked, and yolk separated using an egg separator and yolk fresh weights were recorded and the yolks stored at -20^oC until analysed. Individual eggshells were washed with tap-water and allowed to air-dry for 2 days and shell dry weight was recorded. Shell thickness, index, and Haugh unit scores were determined by methods reported by Neijat et al. (2014).

Subsamples of dietary oils and algae biomass were analyzed for total fat, following method 920.39 (AOAC, 1990), and for nitrogen using a Leco analyzer (NS-2000, Corp., St. Joseph, MI). Calcium and phosphorus levels were determined by inductively coupled plasma mass spectrometry (Varian Inc., Palo Alto, CA) after samples were ashed for 12 h and digested according to AOAC (1990) procedures (method 990.08). Proximate analyses of mixed diets were conducted by the Central Testing Laboratory ltd. (Winnipeg, MB, Canada). Samples of blood (~4 mL vol) were obtained via the wing vein, using a heparinised syringe, with 25G x 5/8 (0.5mm x 16mm) needles. The blood was split into 2 parts while transferring each part into lithium heparinised vacutainer tubes. The first 2 mL blood sample was transferred on ice to the Manitoba Veterinary Services Laboratory (Winnipeg, MB, Canada) for the determination of plasma cholesterol and TAG levels analysed using an automated analyzer (Cell-Dyn 3500 System, Abbott Laboratories, Abbott Park, IL). The rest of the blood sample was

centrifuged (2800 RCF, 20 min, 4°C) and the plasma was separated and stored at $-20°C$ pending

fatty acid profile analyses.

7.3.3   *Extraction of fatty acids and analysis*

Diet (1 g, finely ground), egg yolk (1 g) and plasma (200 µL) samples (egg yolk and

blood samples n = 9 per treatment) from each treatment were used for the extraction of total

lipids using chloroform/methanol (2:1, by vol) containing 0.01% butylated hydroxytoluene

(antioxidant) according to Folch et al. (1957). The extracted total lipids were weighed and

reconstituted in hexane to a volume of 8 mL. From each extract, aliquots of a known volume (to

contain 40 to 50 mg lipid) were dried under nitrogen, and methylated using 3mL, 3N methanolic

HCl including C17:1 (Nu-Chek Prep Inc., Elysian, MN) as an external standard (used for

quantification of fatty acids), by heating for 2 h at 80°C. The resulting fatty acid methyl esters

were extracted into iso-octane and analyzed using a Varian 450 GC with flame ionization

detector (FID) and equipped with a DB225MS column (30 m $\times$ 0.25 mm diameter and 0.25 µm

film thickness, Agilent Technologies Canada Inc., Mississauga, Ontario). The GC program

settings used for the analysis were as described previously (Neijat et al., 2016a). Each fatty acid

was identified by comparing its retention time to authentic standard samples of known

composition (Lipid standards, Nu-Chek Prep, Inc., Elysian, MN, USA).

7.3.4   *Extraction and analysis of yolk cholesterol*

Egg cholesterol was determined by a saponification method after lipid extraction

according to Van Elswyk et al. (1991), with slight modifications. Briefly, egg total lipid was

first extracted (~ 600 mg) using the Folch et al. (1957) method. The total lipid was mixed with

50 mL methanolic KOH (2M) and 1 mL internal standard (used for quantification), dotriacontane

($C_{32}H_{66}$) dissolved in hexane:iso-propanol mixture (93:7 by vol, 7 mg/mL). The mixture was

refluxed on water bath at 100°C for 1 h. After cooling to room temperature, the refluxed

mixtures were transferred into a 500-mL separatory funnel and three sequential extractions each

time using 30 mL deionized water and 50 mL diethyl ether were conducted. The combined ether

extracts were filtered (Whatmann filter paper #4) over anhydrous sodium sulphate. The solvent

was evaporated to dryness using a rotary evaporator in water bath at 35°C. The sterol extract

was then dissolved in 2 mL hexane:iso-propanol mixture (93:7 by vol), of this, 1mL was

derivatized using 1 mL BSTFA (N,O-bis (trimethylsilyl) trifluoroacetamide; Sigma-Aldrich Co.,

St. Louis, MO, USA) by heating at 70°C for 30 min. The reaction mixture was directly injected

onto the capillary column of a gas chromatograph, Bruker 450GC FID, equipped with Agilent

DB-5ms column (30m $\times$ 0.25mm diameter $\times$ 0.25µm film thickness, Agilent Technologies

Canada Inc., Mississauga, Ontario) and cholesterol was identified against a standard, 3β-

Hydroxy-5-cholestene,5-Cholesten-3β-ol, cholesterol (Sigma-Aldrich Co., St Louis, MO, USA).

The program was run as follows: initial temperature was set at 200°C, held for 2 min, increased

at 30°C/min to 270°C held for 0 min. Then increased at the rate of 10°C/min to 320°C and held

for 10 min. Total run time was 19.33 min. Hydrogen was used as the carrier gas with a column

flow rate of 1.0 mL/min, using 100 split and 1µL of sample injection. The temperature of the

injector and detector were held at 270 and 325°C, respectively.

7.3.5   Statistical analysis

Data were analysed as a completely randomized design using the MIXED procedure of

SAS (SAS Institute Inc., Cary, NC) considering dietary treatment as the fixed effects and hen (an

experimental unit), with 10 hens/treatment, as the random effect. Weekly data (hen

performance, egg quality indices and fatty acid composition of egg yolk (expressed as mg/yolk))

were analysed as repeated measures. Least squares means (LSM), adjusted using Tukey's

significant difference test, were compared for significant difference ($P < 0.05$). Orthogonal

contrasts were used to compare treatment means between different dietary sources of total

omega-3 fatty acids. For egg yolk fatty acids, expressed as mg/g yolk (only week 6 data), the

corresponding values of feed intake were used as a covariate. Using the same statistical

program, the Proc Reg procedure was used to evaluate the significance of the linear and

quadratic terms considering each treatment group at 4 levels of total omega-3 PUFA (with a

separate control). Normality of the data distribution was assessed using the Shapiro-Wilk test.

7.4 RESULTS

7.4.1 *Hen performance and egg quality*

There were no significant effects of dietary source and amount of omega-3 on hen

performance (feed intake, body weight gain (BWG) and rate of egg production; Table 7.3).

Averaged, across diets, hens consumed 108 g/hen/day (~1.78 g feed/g egg mass), gained 6.18

g/week of body weight, had a hen-day egg lay rate of 98.4% and produced eggs weighing 60.6 g.

A significant ($P < 0.0001$) main effect of week on feed intake indicated a decline in intake by

week 2 and again in week 6 compared to the hen's initial intake in week 1. However, at most

times during the study, intake remained relatively constant within the range of 105 – 109

g/hen/day. Although there was no significant diet $\times$ week interaction for BWG, a significant (P

< 0.0001) loss in body weight by week 4 may have been a consequence of the significant drop in

feed intake in preceding weeks compared to that in week 1. Nonetheless, the hens gained weight

in weeks 5 and 6. A steady gradual increase ($P < 0.0001$) in egg weight was noted throughout

the study. Egg quality measurements, including egg yolk weights, Haugh unit scores and indices

of eggshell quality (specific gravity, shell weight or index and shell thickness) were not

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Table 7.3: Performance and egg quality of hens (from 36 to 42 weeks of age) consuming diets containing increasing levels of total omega-3 fatty acids derived from flaxseed oil or algal DHA[1]

Dietary total omega-3 (% of diet)	Feed intake (g/hen/day)	BWG (g/week)	Rate of lay (%)	Egg weight (g/egg)	Egg yolk (g/egg)	Shell index[2] (g/cm^2)	Shell thickness (microns)	Specific Gravity	Haugh Unit
Diet effect									
0.03 (Control diet)[3]	105	3.81	98.8	61.4	17.3	8.12	387	1.084	91.6
Flaxseed oil diets[4]									
0.20	111	8.28	98.0	60.6	16.6	8.09	387	1.084	89.3
0.40	108	4.00	99.3	60.3	16.8	8.13	389	1.085	90.7
0.60	111	4.53	98.1	61.4	16.6	8.32	397	1.086	90.3
Algal DHA diets[4]									
0.20	112	5.77	98.4	61.6	16.8	8.12	388	1.084	89.9
0.40	104	6.27	97.6	59.1	16.6	8.02	384	1.086	91.6
0.60	107	10.6	98.9	59.8	16.8	8.22	394	1.085	90.9
SE	2.08	2.26	0.76	0.89	0.26	0.12	4.78	0.001	1.20
Week effect									
wk 1	112^a	27.2^a	97.2	59.5^c	-	-	-	-	-
wk 2	107^{bc}	3.81^b	98.6	60.2^{bc}	16.4^b	8.19	397^a	-	-
wk 3	109^b	11.9^{ab}	98.8	60.8^{ab}	-	-	-	1.085	90.7
wk 4	109^b	-26.6^c	98.6	60.8^{ab}	16.7^b	8.15	390^b	-	-
wk 5	107^{bc}	16.0^{ab}	99.1	61.0^a	-	-	-	-	-
wk 6	105^c	4.73^b	98.4	61.4^a	17.2^a	8.09	381^c	1.085	90.5
SE	0.97	3.63	0.55	0.37	0.13	0.05	2.10	0.001	0.53
P-values									
Diet	0.06	0.29	0.73	0.38	0.49	0.65	0.53	0.51	0.82
Week	< 0.0001	< 0.0001	0.17	< 0.0001	< 0.0001	0.11	< 0.0001	0.44	0.77
Diet x week	0.98	0.26	0.89	0.75	0.56	0.42	0.58	0.34	0.82

[1]Data are presented as least square means (LSM) ± standard errors (SE), n =10 per treatment.
[a–c]Means with different superscripts within each parameter are significantly different at $P < 0.05$.
[2]Eggshell weight expressed per unit of surface area.
[3]Contained 1.35 g/100 g of diet almond oil.
[4]Levels correspond to 0.36, 0.77 and 1.19 g/100 g of diet flaxseed oil or 1.00, 2.18 and 3.36 g/100g of diet algal DHA, respectively.

influenced by the inclusion of either flaxseed oil or algal DHA to the diets of the laying hens. However, there was a significant ($P < 0.0001$) main effect of week on egg yolk weight and eggshell thickness; while egg yolk weight increased, shell thickness decreased during the study period (Table 7.3). The latter phenomenon of shell quality is well explained by Roland et al. (1975).

7.4.2 *Egg yolk and plasma fatty acid composition*

The total saturated fatty acids (SFA; myristic (14:0); palmitic (16:0) and stearic (18:0) acids) composition in the total lipid of egg yolk did not vary significantly as a function of increasing levels of either flaxseed oil (Table 7.4) or algal DHA (Table 7.5). Unlike the ALA (flaxseed oil)-fed hens, the feeding of preformed DHA to hens resulted in a significant ($P < 0.01$; Table 7.5) decrease, compared to the lower level or control, in the level of total monounsaturated fatty acid (MUFA; palmitoleic (16:1) and oleic (18:1) acids) in the eggs, at both 0.40 and 0.60% of dietary total omega-3 fatty acid level in the diet.

The PUFA (omeag-6 and omega-3 fatty acid series) levels in the total lipid of egg yolk, particularly the LCPUFA, showed a highly significant ($P < 0.0001$) treatment effects for both the flaxseed oil- and algal DHA-fed hens (Tables 7.4 and 7.5, respectively). The composition of the omega-3 PUFA in the egg was greatly ($P < 0.0001$) influenced by the source of omega-3 fatty acids in the diet, with flaxseed oil-fed hens accumulating an overall 10-fold ALA and 2.5-fold DHA over that of the control. The algal DHA treatment, which supplied predominantly DHA, increased the level of DHA in the egg by 7-fold over the control level and by 3-fold over that of highest flaxseed oil-fed hens (Tables 7.4 and 7.5).

In general, in the current study, a significant ($P < 0.0001$) period effect in the deposition of all fatty acids (except EPA in the ALA-fed groups) in the egg yolk in both treatment groups

Table 7.4: Fatty acid composition of egg yolk (mg/yolk) as a function of increasing levels of total omega-3 fatty acids using flaxseed oil in diets of hens fed from 36 to 42 weeks of age[1]

	Week (wk)	Diet effect Total omega-3 in diet (%) 0.03	0.20	0.40	0.60	SE	Week effect wk 2	wk 4	wk 6	SE	P-value diet	wk	diet x wk
TSFA[2]	2	1623	1544	1527	1616								
	4	1406	1381	1325	1332								
	6	1377	1377	1325	1359								
	Overall	1469	1434	1415	1436	49.0	1577^a	1378^b	1359^b	34.0	0.90	<0.0001	0.88
TMUFA[2]	2	2027	1953	1849	1900								
	4	1729	1767	1729	1519								
	6	1705	1703	1543	1631								
	Overall	1820	1807	1707	1683	62.3	1932^a	1686^b	1645^b	43.1	0.31	<0.0001	0.48
LA	2	564	555	544	584								
	4	494	491	508	473								
	6	483	493	477	497								
	Overall	514	513	510	518	19.2	562^a	492^b	488^b	13.1	0.99	<0.0001	0.75
ARA	2	88.6	74.4	62.9	65.1								
	4	81.4	65.3	57.9	49.7								
	6	79.5	68.4	56.2	51.4								
	Overall	83.2^a	69.4^b	59.0^c	55.4^c	1.81	72.8^a	63.6^b	63.8^b	1.28	<0.0001	<0.0001	0.29
ALA	2	12.7	37.2	71.8^A	114^A								
	4	10.4	33.3	61.5^B	88.7^C								
	6	10.3	33.7	58.2^B	97.2^B								
	Overall	11.2^d	34.7^c	63.8^b	99.9^a	2.33	58.9^a	48.5^b	49.9^b	1.58	<0.0001	<0.0001	< 0.05
EPA	2	0.28	0.89	2.56	3.90								
	4	0.19	0.85	2.12	3.67								
	6	0.24	0.97	2.23	3.40								
	Overall	0.24^d	0.91^c	2.30^b	3.66^a	0.11	1.91	1.71	1.71	0.093	<0.0001	0.21	0.57
DPA	2	2.29	4.78	6.49	8.05								
	4	1.96	3.83	5.40	6.36								
	6	2.16	3.75	5.63	6.40								
	Overall	2.14^d	4.12^c	5.84^b	6.94^a	0.24	5.40^a	4.39^b	4.48^b	0.17	<0.0001	<0.0001	0.26
DHA	2	29.6	54.0	68.0	74.7								
	4	25.9	47.5	62.8	64.2								
	6	24.7	49.4	59.9	66.9								
	Overall	26.7^c	50.3^b	63.6^a	68.6^a	1.61	56.6^a	50.1^b	50.2^b	1.22	<.0001	<.001	0.74
Total n-6	2	659	635	611	654								
	4	582	566	561	527								
	6	568	566	538	541								
	Overall	603	587	570	574	21.2	640^a	558^b	553^b	14.5	0.71	<.0001	0.80
Total n-3	2	44.2	96.5	148^A	214^A								
	4	38.3	85.4	130^B	167^B								
	6	37.6	88.2	121^B	174^B								
	Overall	40.1^d	90.0^c	133^b	185^a	3.90	126^a	105^b	105^b	2.57	<0.0001	<0.0001	<0.001
n-6/n-3 ratio	2	15.1	6.50	4.35	3.36								
	4	15.6	6.57	4.42	3.16								
	6	15.2	6.41	4.27	3.20								
	Overall	15.3^a	6.49^b	4.35^c	3.24^d	0.074	7.33	7.45	7.28	0.056	<0.0001	0.063	0.078

[1]Data are presented as least square means (LSM) ± standard error (SE), n = 9 per treatment.
[2]Total saturated fatty acids (TSFA): myristic (14:0), palmitic (16:0), stearic (18:0); total monounsaturated fatty acids (TMUFA): palmitoleic (16:1) and oleic (18:1); Total n-6 fatty acids = sum of LA, GLA and ARA; Total n-3 fatty acids = sum of ALA, EPA, DPA and DHA.
[a–d]Different superscripts between treatments (effect of diet) or periods (effect of week), within a row, are significantly different at $P < 0.05$.
[A–C]Different superscripts within a column for each parameter, are significantly different ($P < 0.05$), represents a diet by week interaction.

Table 7.5: Fatty acid composition of egg yolk (mg/yolk) as a function of increasing levels of total omega-3 fatty acids using algal DHA in diets of hens fed from 36 to 42 weeks of age[1]

	Week	Diet effect					Week effect				P-value		
		Total omega-3 in diet (%)											
	(wk)	0.03	0.20	0.40	0.60	SE	wk 2	wk 4	wk 6	SE	Diet	wk	Diet x wk
TSFA[2]	2	1623	1667	1607	1719								
	4	1406	1485	1368	1460								
	6	1377	1457	1362	1497								
	Overall	1469	1536	1446	1559	39.8	1654^a	1430^b	1423^b	29.0	0.16	<0.0001	0.99
TMUFA[2]	2	2027	1967	1753	1760								
	4	1729	1740	1525	1524								
	6	1705	1715	1484	1598								
	Overall	1820^a	1808^a	1587^b	1628^b	55.6	1877^a	1630^b	1626^b	37.7	<0.01	<0.0001	0.90
LA	2	563	563	548	514								
	4	494	493	458	446								
	6	482	480	449	436								
	Overall	514^a	512^a	485^{ab}	466^b	11.2	547^a	473^b	462^b	10.1	<0.05	<0.0001	1.00
ARA	2	88.6	70.4	56.9	51.1								
	4	81.4	60.8	44.9	41.8								
	6	79.5	58.6	46.3	42.0								
	Overall	83.2^a	63.3^b	49.3^c	44.9^c	1.52	66.7^a	57.2^b	56.6^b	1.09	<0.0001	<0.0001	0.89
ALA	2	12.7	14.7	16.7	18.4								
	4	10.4	12.8	14.0	15.8								
	6	10.3	12.8	14.0	15.3								
	Overall	11.2^d	13.5^c	14.9^b	16.5^a	0.41	15.6^a	13.3^b	13.1^b	0.38	<0.0001	<0.0001	1.00
EPA	2	0.30	2.75	4.95	7.94								
	4	0.17	2.16	3.99	6.63								
	6	0.23	2.30	4.21	6.91								
	Overall	0.23^d	2.40^c	4.38^b	7.16^a	0.21	3.98^a	3.24^b	3.42^b	0.13	<0.0001	<0.0001	0.21
DPA	2	2.29	2.74^A	3.63^A	6.21^A								
	4	1.96	2.19^B	3.05^B	4.87^B								
	6	2.15	2.38^{AB}	2.99^B	4.88^B								
	Overall	2.13^c	2.44^c	3.22^b	5.32^a	0.14	3.72^a	3.02^b	3.10^b	0.10	<0.0001	<0.0001	<.05
DHA	2	29.6	110^A	145^A	206^A								
	4	25.9	95.7^B	124^B	177^B								
	6	24.7	99.4^B	130^B	173^B								
	Overall	26.7^d	102^c	133^b	185^a	3.03	123^a	106^b	107^b	2.15	<0.0001	<0.0001	<.01
Total n-6	2	657	558	609	569								
	4	582	558	507	491								
	6	568	543	498	481								
	Overall	602^a	579^a	538^b	513^b	11.9	618^a	534^b	522^b	10.8	<0.0001	<0.0001	1.00
Total n-3	2	44.2	130^A	170^A	239^A								
	4	38.3	113^B	146^B	205^B								
	6	37.6	117^B	151^B	201^B								
	Overall	40.1^d	120^c	156^b	215^a	3.21	146^a	125^b	127^b	2.45	<0.0001	<0.0001	<0.05
n-6/n-3 ratio	2	15.2	4.94	3.55	2.38								
	4	15.5	4.97	3.48	2.48								
	6	15.1	4.64	3.28	2.35								
	Overall	15.3^a	4.85^b	3.44^c	2.40^d	0.07	6.52^a	6.61^a	6.33^b	0.06	<0.0001	<0.05	0.64

[1]Data are presented as least square means (LSM) ± standard error (SE), n = 9 per treatment.

[2]Total saturated fatty acids (TSFA): myristic (14:0), palmitic (16:0), stearic (18:0); total monounsaturated fatty acids (TMUFA): palmitoleic (16:1) and oleic (18:1); Total n-6 fatty acids = sum of LA, GLA and ARA; Total n-3 fatty acids = sum of ALA, EPA, DPA and DHA.

[a–d]Different superscripts between treatments (effect of diet) or periods (effect of week), within a row, are significantly different at $P < 0.05$.

[A,B]Means with different superscripts within a column for each parameter, are significantly different ($P < 0.05$), represents a diet × week interaction.

(Tables 7.4 and 7.5) was noted, being lower in weeks 4 and 6 compared with week 2. This translated in a significant ($P < 0.05$) diet $\times$ week interaction in the accumulation of ALA and total omega-3 PUFA (ALA; EPA; docosapentaenoic acid, DPA; and DHA) for flaxseed oil treatment group (Table 7.4); DPA, DHA and total omega-3 PUFA for the algal DHA treatment group (Table 7.5) were observed in the egg yolk (mg/yolk). The results indicated a maximal enrichment in egg yolk of these fatty acids being reached by week 2, a decline in week 4 and leveling thereafter (Tables 7.4 and 7.5). Although feed intake showed a highly significant ($P < 0.0001$) diet $\times$ week interaction, there was a tendency for a diet effect ($P = 0.06$) (Table 7.3). Hence, based on a possible confounding effect of feed intake, it was used as a covariate in the analysis of egg yolk fatty acid contents. In addition, due to a possible impact of the changes in the weight of egg yolk over a period of time ($P < 0.0001$, diet $\times$ week interaction), yolk weight was also used as a covariate in the analysis of egg yolk fatty acids (hence, expressed as mg/g yolk). However, despite these adjustments, similar patterns of interaction effects were observed in the accumulation of ALA, DHA and total omega-3 PUFA in the egg yolk (mg/g yolk, Figure 7.1). Furthermore, it was noted that for the ALA-fed hens stability in the accumulation of ALA (by week 4, $P < 0.05$; Table 7.4 and Figure 7.1a) was only evident at higher levels of flaxseed oil inclusion (providing 0.40 and 0.60% levels of total omega-3 fatty acids in the diet) compared to lower levels. On the other hand, the DHA-fed hens deposited highest levels of DHA in week 2 ($P < 0.01$; Figure 7.1b), with stabilization evident in week 4. The latter was observed at all levels of algal DHA inclusion in the laying hen diets. A similar trend of enrichment of total omega-3 PUFA in egg yolk was also observed for both treatment groups (Figure 7.1c). Eicosapentaenoic acid level in the egg yolk in both treatment groups (Tables 7.4 and 7.5) did not achieve a maximal enrichment level (lack of diet $\times$ week interaction).

a) ALA

b) DHA

c) Total omega-3 PUFA

Total omega-3 fatty acids (%, diet)
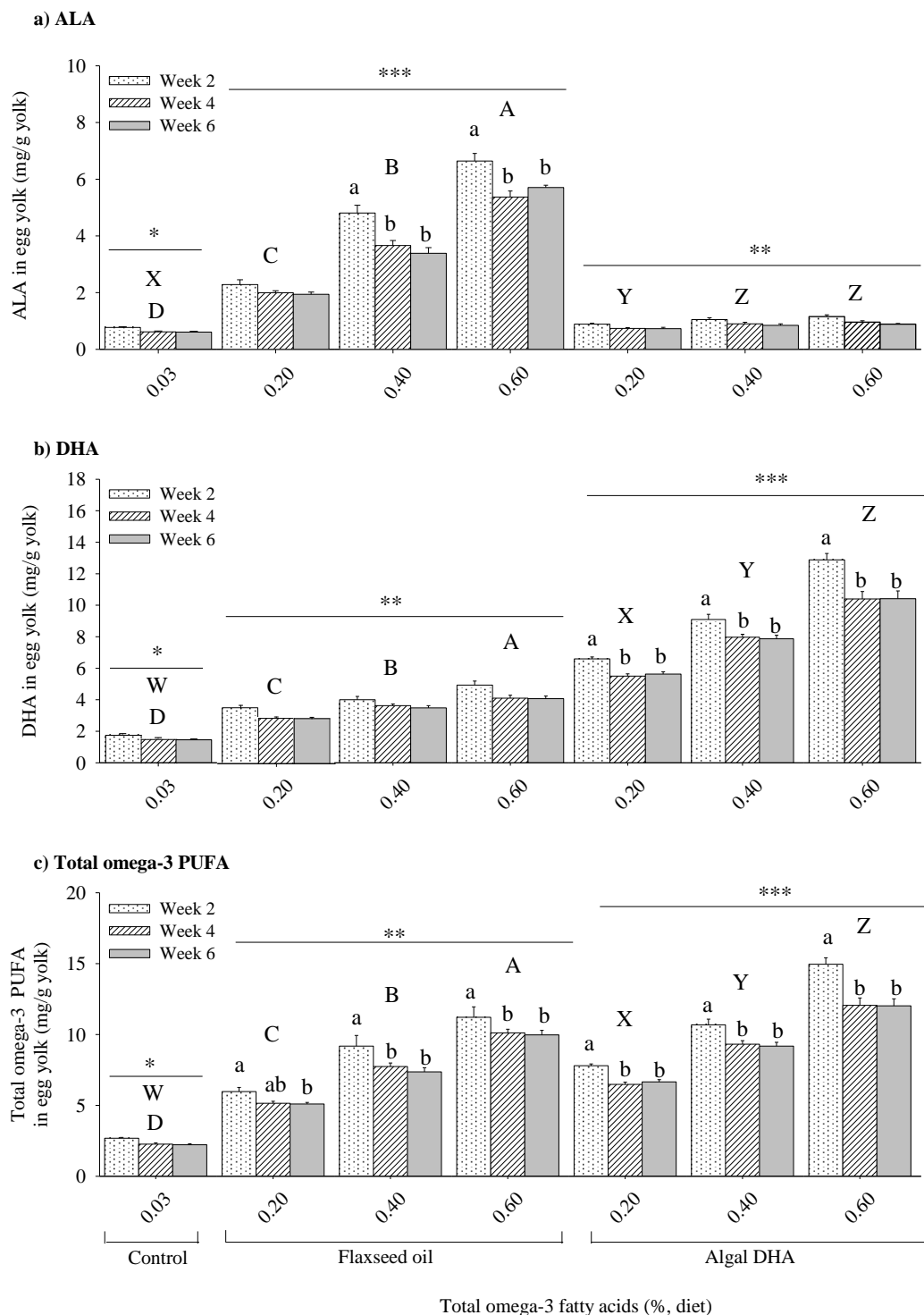
Figure 7.1. Levels of a) Alpha-linolenic acid (ALA), b) docosahexaenoic acid (DHA) and c) total n-3 fatty acid (sum of ALA, EPA, DPA and DHA) in egg yolk (mg/g yolk) as a function of period (week 2, 4 and 6) in hens (from 36 to 42 weeks of age) fed diets containing varying levels of total omega-3 fatty acids (0.03, 0.20, 0.40 and 0.60%, diet) derived from either flaxseed oil or algal DHA.

Data point in each period is a group mean ± SE (n = 9 per treatment). Letters denote significant differences ($P < 0.05$) between periods within a treatment (lower case) and between diets within a treatment group (upper case, considering separate control); symbols denote differences between treatment groups for either flaxseed oil or algal DHA vs. control.

The accumulation of synthesized DHA from ALA (flaxseed oil-fed hens) in the egg yolk did not reflect a diet × week interaction effect (Table 7.4 and Figure 7.1a). It was also noted that, although the ALA levels in the diets containing algal DHA were similar to that of the control (Table 7.1), the preformed DHA-fed hens, particularly at highest inclusion level of algal DHA, accumulated slightly more ALA in the eggs ($P < 0.0001$; Table 7.5 and Figure 7.1a) compared to the control, with the overall average increasing from 11.2 to 16.5 ± 0.41 mg/yolk, respectively. Although a significant week effect ($P < 0.0001$, Table 7.5) was evident, in a similar pattern as previously described, no interaction effect was noted. By contrast (utilizing week 6 data only), while a significant ($P < 0.01$, Table 7.6) amount of ALA accumulated in the egg yolk for DHA-fed hens compared to that of the control, plasma levels of ALA did not indicate such differences between the control- and the DHA-fed hens (Table 7.7). However, overall, the assessment of plasma fatty acid profile alongside that of the egg yolk for both the flaxseed oil and algal DHA treatment groups indicated similar trends of accumulation fatty acids, particularly for the omega-3 PUFA (Figure 7.2). The egg yolk and plasma levels of ALA for flaxseed-fed hens increased linearly (egg yolk: $P < 0.0001$, $R^2 = 0.96$, Table 6 and plasma: $P < 0.05$, $R^2 = 0.74$, Table 7.7; Figure 7.2a). The DHA level for flaxseed-fed hens increased in a linear ($P < 0.0001$) and quadratic ($P < 0.05$) manner ($R^2 = 0.89$, Tables 7.6, Figure 7.2c) in the egg yolk. In the plasma, a linear ($P < 0.05$, $R^2 = 0.27$) accumulation in DHA levels for the flaxseed oil-fed group was observed (Table 7.7, Figure 7.2c). For DHA-fed hens, the DHA levels in egg yolk showed a linear ($P < 0.0001$) and quadratic ($P < 0.0001$) response ($R^2 = 0.95$, Table 7.6, Figure 7.2c). Similar results were also observed in plasma, with significant linear ($P < 0.001$) and quadratic ($P < 0.05$) responses ($R^2 = 0.95$, Table 7.7, Figure 7.2c) as a function of algal DHA levels in the diet of hens. Similarly, a significant ($P < 0.0001$) diet effect was observed in the deposition of EPA

Table 7.6: Fatty acid composition of egg yolk lipid (mg/g yolk, week 6 data) as a function of increasing levels of total omega-3 fatty acids using flaxseed oil or algal DHA in diets of hens fed from 36 to 42 weeks of age[1]

| | Total omega-3 in diet (%, by wt) | | | | | | | | | Contrast (P-values) | | |
| | Control | Flaxseed oil (FSO) | | | Algal DHA (DHA) | | | | | FSO vs. | DHA vs. | FSO vs. |
	0.03	0.20	0.40	0.60	0.20	0.40	0.60	SE	P-values	Control	Control	DHA
18:2n-6 (LA)	28.2	28.4	27.8	28.8	27.3	27.1	25.3	0.85	0.075	0.94	0.11	<0.05
18:3n-6 (GLA)	0.32[a]	0.27[ab]	0.26[b]	0.24[b]	0.22[bc]	0.18[cd]	0.15[d]	0.012	<.0001	<.0001	<.0001	<.0001
20:4n-6 (ARA)	4.69[a]	3.95[b]	3.26[cd]	2.97[de]	3.32[c]	2.79[e]	2.44[f]	0.08	<.0001	<.0001	<.0001	<.0001
18:3n-3 (ALA)[2,3]	0.61[e]	1.94[c]	3.12[b]	5.71[a]	0.73[de]	0.84[de]	0.89[d]	0.062	<.0001	<.0001	<0.01	<.0001
20:5n-3 (EPA)	0.014[f]	0.056[e]	0.129[d]	0.188[c]	0.130[d]	0.254[b]	0.403[a]	0.008	<.0001	<.0001	<.0001	<.0001
22:5n-3 (DPA)	0.13[e]	0.22[c]	0.33[b]	0.39[a]	0.14[de]	0.19[cd]	0.28[b]	0.012	<.0001	<.0001	<.0001	<.0001
22:6n-3 (DHA)[2,3]	1.46[f]	2.85[e]	3.48[de]	4.07[d]	5.63[c]	7.87[b]	10.5[a]	0.15	<.0001	<.0001	<.0001	<.0001
Total n-6[4]	33.2[a]	32.6[a]	31.3[ab]	32.0[a]	30.8[ab]	30.0[ab]	27.8[b]	0.78	<0.01	0.25	<0.01	<0.01
Total n-3[5]	2.22[f]	5.09[e]	7.36[d]	10.3[b]	6.65[d]	9.18[c]	11.6[a]	0.22	<.0001	<.0001	<.0001	<.0001
Ratio n-6/n-3	15.2[a]	6.41[b]	4.26[c]	3.21[d]	4.64[c]	3.27[d]	2.35[e]	0.10	<.0001	<.0001	<.0001	<.0001

[1]Data represents least square means (LSM) ± standard error (SE), n = 9 per treatment. [a-f]Means with different superscripts within a row are significantly different at $P < 0.05$.

[2]Flaxseed oil containing diets: ALA: linear, $P < 0.0001$, $R^2 = 0.96$; DHA: linear, $P < 0.0001$ and quadratic $P < 0.05$, $R^2 = 0.89$.

[3]Algal DHA containing diets: DHA: linear, $P < 0.0001$ and quadratic $P < 0.0001$, $R^2 = 0.95$.

[4]Total n-6 fatty acids: linoleic acid (LA), gamma-linolenic acid (GLA) and arachidonic acid (ARA).

[5]Total n-3 fatty acids: alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA).

Table 7.7: Fatty acid composition of plasma lipid (mg/ml plasma, week 6 data) as a function of increasing levels of total omega-3fatty acids using flaxseed oil or algal DHA in diets of hens fed from 36 to 42 weeks of age[1]

	Control	Flaxseed oil (FSO)			Algal DHA (DHA)					FSO vs.	DHA vs.	FSO vs.
	0.03	0.20	0.40	0.60	0.20	0.40	0.60	SE	P-values	Control	Control	DHA
18:2n-6 (LA)	2.13	2.55	2.48	2.50	2.65	2.11	1.63	0.26	0.09	0.27	0.99	0.071
18:3n-6 (GLA)	0.028^a	0.021^{ab}	0.021^{ab}	0.020^{ab}	0.021^{ab}	0.014^{bc}	0.009^c	0.003	<0.01	<0.05	<0.001	<0.01
20:4n-6 (ARA)	0.37^{ab}	0.40^a	0.33^{ab}	0.29^{abc}	0.33^{ab}	0.25^{bc}	0.17^c	0.032	<0.001	0.40	<0.01	<0.01
18:3n-3 (ALA)[2,3]	0.079^d	0.162^c	0.258^b	0.338^a	0.073^d	0.064^d	0.055^d	0.017	<0.0001	<0.0001	0.44	<0.0001
20:5n-3 (EPA)	0.000^d	0.003^{cd}	0.007^{cd}	0.013^{bc}	0.009^{cd}	0.022^{ab}	0.027^a	0.003	<0.0001	<0.05	<0.0001	<0.0001
22:5n-3 (DPA)	0.011^b	0.012^b	0.0234^a	0.024^a	0.008^b	0.011^b	0.015^{ab}	0.002	<0.0001	<0.01	0.79	<0.0001
22:6n-3 (DHA)[2,3]	0.18^c	0.29^c	0.34^c	0.37^{bc}	0.57^{ab}	0.72^a	0.78^a	0.05	<0.0001	<0.05	<0.0001	<0.0001
Total n-6[4]	2.53	2.97	2.83	2.81	3.00	2.37	1.81	0.29	0.062	0.38	0.74	<0.05
Total n-3[5]	0.20^c	0.47^{bc}	0.66^{ab}	0.85^a	0.66^{ab}	0.76^{ab}	0.88^a	0.08	<0.0001	0.0001	<0.0001	0.10
Ratio n-6/n-3	15.0^a	6.27^b	4.30^c	3.34^d	4.51^c	2.82^e	2.09^f	0.10	<0.0001	<0.0001	<0.0001	<.0001

[1]Data represents least square means (LSM) ± standard error (SE), n = 9 per treatment. [a-f]Different superscripts within a treatment group within a row are significantly different at $P < 0.05$.

[2]Flaxseed oil containing diets: ALA: linear, $P < 0.05$, $R^2 = 0.74$; DHA: linear, $P < 0.05$, $R^2 = 0.27$.

[3]Algal DHA containing diets: DHA: linear, $P < 0.001$ and quadratic $P < 0.05$, $R^2 = 0.56$.

[4]Total n-6 fatty acids: linoleic acid (LA), gamma-linolenic acid (GLA) and arachidonic acid (ARA).

[5]Total n-3 fatty acids: alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA).

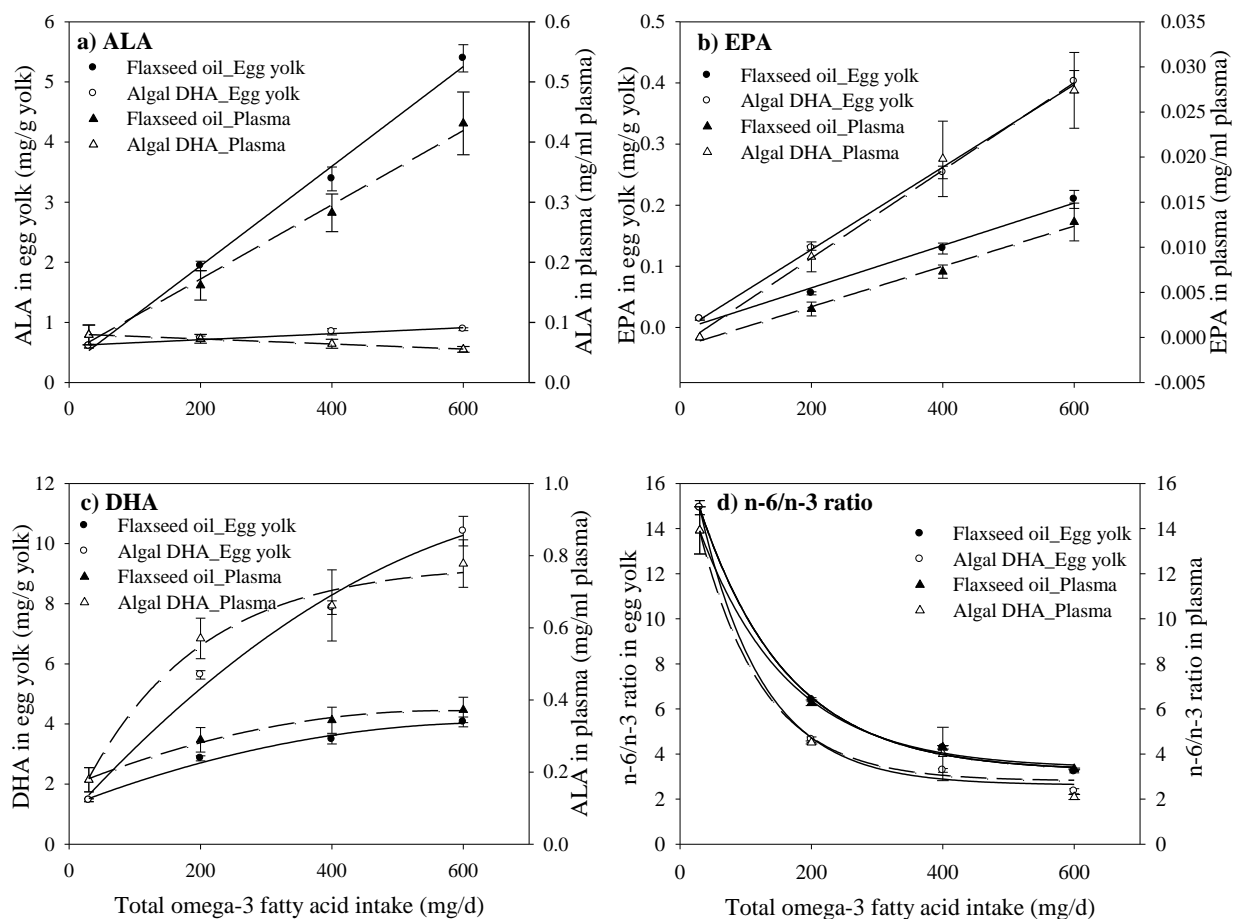**Figure 7.2.** Trends in the levels of a) alpha-linolenic acid, ALA; b) eicosapentaenoic acid, EPA; c) docosahexaenoic acid, DHA d) n-6/n-3 PUFA ratio in total lipids of egg yolk obtained from flaxseed oil or algal DHA treatments versus plasma derived from flaxseed oil or algal DHA treatments fed to hens from 36 to 42 weeks of age (week 6 data).
Each data point are group means ± SE (n = 9 per treatment).

in the egg yolk in both treatment groups (Tables 7.4 and 7.5), indicating a significant linear increase of this fatty acid in the egg of the ALA- ($P < 0.01$, $R^2 = 0.86$) as well as the DHA-fed groups (Table 7.6). The latter trends closely resembled those observed in the plasma (Figure 7.2b) as a function of ALA intake. Although levels of DPA in egg yolk and plasma (Tables 7.6 and 7.7) also increased in a manner similar to that of EPA in response to both flaxseed oil and algal DHA treatments, for the flaxseed oil treatment, a linear ($P < 0.001$, $R^2 = 0.81$; data not presented) increase was noted as a function of ALA intake. The accumulation trend of both fatty acids (EPA and DPA), were reflective of their biosynthesis from ALA.

As dietary omega-3 PUFA levels increased, the proportion of arachidonic acid (ARA), and therefore, the ratio of omega-6 to omega-3 PUFA (n-6/n-3) was reduced in the egg yolk ($P < 0.0001$) for both the flaxseed oil and algal DHA treatment groups compared to the control (Tables 7.4 and 7.5, respectively). Although there was no treatment effect on total omega-6 PUFA content of the eggs for flaxseed oil-fed hens (Table 7.4), a significant effect ($P < 0.0001$) was noted for the algal DHA treatment group (Table 7.5), decreasing the level at higher inclusion level compared to control. These effects were highly pronounced in the DHA-fed, as compared to the ALA-fed hens, in both the egg yolk (Table 7.6) and plasma (Table 7.7). Although the DHA-fed hens had significantly ($P < 0.05$; Table 7.5) reduced linoleic acid (LA) content in the yolk when algal DHA was included at highest level compared to the control or lower level of inclusion of algal DHA, the addition of flaxseed oil to the diets of hens had no effect on the level of LA in the egg yolk compared with the control (Table 7.4). However, by contrast ALA-fed hens had significantly greater amount of LA in the egg compared to the eggs obtained from the DHA-fed hens (Table 7.6); but this difference was not reflected in the plasma (Table 7.7). Gamma-linolenic acid (GLA) levels in both the flaxseed oil (particularly at higher levels of

intake) and the algal DHA treatment groups were significantly lowered compared to the control in both the egg yolk (mg/g yolk; $P < 0.0001$, Table 7.6) and the plasma (mg/ml plasma; $P < 0.05$, Table 7.7).

7.4.3   *Egg yolk and plasma lipids (total lipid, cholesterol and triacylglycerol, TAG)*

Total lipid extracts from eggs and plasma were not statistically different when hens were fed either flaxseed oil or algal DHA as a function of increasing either ALA or DHA contents (0.03, 0.20, 0.40 and 0.60% total omega-3 fatty acid) in their diets (Table 7.8). However, there was a diet × week interaction effect on the amount of egg yolk total lipid analysed over the 6-week period (data not presented). For ALA-fed hens, egg yolk total lipid increased from week 2 to 4 with no difference thereafter (32.6, 33.5 and 34.2 ± 0.39 g/100g yolk, $P < 0.05$; for weeks 2, 4 and 6; respectively). With respect to DHA-fed hens, the level in week 6 was higher compared to that in weeks 2 and 4, the total lipid content in the latter two weeks were similar (32.4, 33.0, 34.6 ± 0.34 g/100g yolk, $P < 0.0001$; for weeks 2, 4 and 6; respectively). The same analysis could not be determined for plasma (only week 6 samples were obtained). There was no significant difference due to treatment in the amount of egg yolk total cholesterol. No statistical changes were observed in plasma total cholesterol or TAG concentrations during the study, however, a slight reduced in TAG concentrations (tendency, $P = 0.05$) was evident at higher levels of inclusion of preformed DHA compared to precursor ALA (Table 7.8).

Table 7.8: Lipid and cholesterol contents in egg yolk and plasma; and triacylglycerol (TAG) levels of plasma as a function of increasing total omega-3fatty acids (derived from flaxseed oil or algal DHA) in diets of hens fed from 36 to 42 weeks of age (week-6 data)

| | Total omega-3 in diet (%, by wt) | | | | | | | | | Contrast (P-values) | | |
| | Control | Flaxseed oil (FSO) | | | Algal DHA (DHA) | | | | | FSO vs. | DHA vs. | FSO vs. |
	0.03	0.20	0.40	0.60	0.20	0.40	0.60	SE	P-values	Control	Control	DHA
Egg yolk lipid (g/100g yolk)	34.7	34.6	33.7	34.0	34.8	34.1	34.9	0.76	0.91	0.52	0.93	0.42
Plasma lipid (mg/mL)	6.34	5.93	6.01	5.86	6.22	5.57	4.48	0.69	0.57	0.64	0.29	0.35
Total cholesterol in egg (mg/yolk)	209	200	259	203	241	197	201	25.1	0.48	0.68	0.89	0.72
Total cholesterol in plasma (mmol/L)	2.75	2.58	3.06	2.84	2.64	2.61	2.14	0.22	0.17	0.78	0.30	0.05
Plasma TAG (mmol/L)	27.8	26.2	27.5	25.0	25.0	23.9	20.0	3.04	0.60	0.67	0.19	0.19

[1]Data represents least square means (LSM) ± standard error (SE), n = 6 per treatment. Different superscripts within a treatment group in a row represent significant difference at $P < 0.05$.

7.5 DISCUSSION

The aim of this study was primarily to investigate the effect of adding graded levels of either a terrestrial source of precursor ALA (flaxseed oil) or preformed DHA (algal DHA) to basal diets of hens low in omega-3 fatty acids on the enrichment levels of omega-3 fatty acids in eggs. In addition, hen performance indices and the concentration of other lipids of egg yolk (cholesterol) and plasma (cholesterol and TAG) were assessed. No significant differences were observed between treatments with respect to hen performance, eggshell quality, total yolk and plasma cholesterol levels, and plasma TAG. However, compared to control hens, feeding hens with diets containing increasing level of total omega-3, provided as either the precursor ALA or preformed DHA, greatly ($P < 0.0001$) increased the levels of both the individual as well as the total omega-3 fatty acids, and lowered ($P < 0.0001$) the n-6/n-3 ratio in eggs and plasma. While the current data support previous approaches for efficiently increasing the total omega-3 PUFA content of eggs using preformed sources, this study clearly defines the nature (level of omega-3 fatty acids, ALA) of the background diet and creates the basis for the novelty of this study. Based on the distinct relationship developed between hepatic levels of ALA and EPA in our previous study (Neijat et al., 2016b), controlling the level of one factor (ALA, in the diets containing preformed source of LCPUFA), may aim to elucidate mechanistic views in the accumulation of omega-3 LCPUFA in eggs.

Lower levels of dietary ALA intake (0.03 or 0.20% total omega-3 fatty acids, supplied by the control or flaxseed oil containing diets, respectively) led to the accumulation of significantly less ALA, with no variation with period of feeding compared to when hens were fed higher levels of ALA (flaxseed oil). Generally, the accumulation of omega-3 PUFA in the egg is a gradual process. However, the lack of a diet × week interaction in defining the maximal

accumulation level of DHA in eggs endogenously synthesised from ALA, at all levels of flaxseed inclusion in the diets of hens, compared to hens supplied similar levels of total omega-3 fatty acids using algal DHA, is evident of the difference that source and level of dietary omega-3 PUFA has on the enrichment of eggs (Guenter et al., 1971; González-Esquerra and Leeson, 2001). The current results imply that, at a lower level of total omega-3 fatty acid supplementation, preformed DHA tended to reach maximum accumulation earlier than treatments with the primary precursor ALA. In part, this may relate to the differences in the rate of absorption. Absorption of dietary fat (primarily ingested in form of TAG) from the intestinal lumen to the enterocyte proceeds after hydrolysis by pancreatic lipase, which acts mainly on the sn-1 and sn-3 position of the TAG molecules, releasing 2-monoaclyglycerol and free fatty acids (Krogdahl, 1985). During absorption, fatty acids bind to fatty acid-binding proteins depending on the degree of saturation and chain length. Long chain unsaturated fatty acids are preferentially bound compared to short and medium chain fatty acids (Ockner et al., 1972). It is also possible that preformed DHA and biosynthesized DHA may reside in different pools in the hepatocyte, with the former more readily incorporated in VLDL and secreted as compared to the biosynthesized DHA pool (Mashek, 2013). In addition, in the liver, the major site of fatty acid synthesis in avian species (Leveille et al., 1975), the amounts of synthesized omega-3 LCPUFA as well as the availability of ALA for egg yolk deposition depends on dietary levels of ALA (Grobas et al., 2001).

The results of yolk fatty acids, when adjusted statistically for the changes in yolk weight and the possible confounding effect of feed intake (which tended to be significant between diets), showed a diet × week interaction for the ALA- (only the 0.40 and 0.60% contents of total omega-3 fatty acids) and DHA-fed hens. The outcomes reflected greater level of accumulation

of ALA and DHA, as well as total n-3 PUFA (in either case) in the egg yolk in week-2, dropping in week-4 and stabilized at the same level thereafter. Previously, Lemahieu et al. (2015b) observed a decrease in the content of egg yolk omega-3 LCPUFA between days 14 and 25 (end of supplementation period) after reaching a maximal level in 2 weeks, with the authors relating this to a decrease in egg yolk weight. In the current study, egg yolk weights significantly increased over time (weeks), a pattern that would be expected as hens′ age (Johnston and Gous, 2007). Similar to the changes in the egg yolk weights, the amount of total lipid in egg yolk also increases with age but remains constant in mature hens (Nielsen, 1998). This latter parameter was evident within the 4-weeks of the study period (~40 weeks of hen age), particularly, for the ALA-fed hens when the total lipid content in the egg yolk were similar in weeks 4 and 6 compared to week 2. However, reflecting on the observed pattern of the diet × week interaction in the accumulation of ALA, DHA, or total n-3 PUFA in the egg yolk, which instead decreased in week 4 before stabilizing thereafter, may, in part, relate to the rate of oviposition. Although rate of egg production between treatments was not significant, the lower rate of egg production in week 1 which reflects the rate of yolk material formation for subsequent egg that get laid in week 2, since it takes at least 7 days for yolk material deposition may have contributed to a greater accumulation of yolk material during the first 2 weeks of egg production. In laying hens, Hargis et al. (1991) suggested a period of at least 2 weeks of feeding to attain a steady state of alteration in the fatty acids content of eggs. In previous studies, a steady state accumulation of omega-3 PUFA in the egg yolk was achieved in 2 weeks when using microalgae up to 4.8% (Herber and Van Elswyk, 1996) and 8.6% (~0.25% of diet total omega-3 fatty acids) (Lemahieu et al., 2015b); others, indicated 3 weeks using flaxseed (10-15% of the diet, ~5% ALA) (Scheideler et al., 2003) and 8 weeks utilizing 30% of diet hempseed (~1.2% of diet ALA)

(Neijat et al., 2014). Although difference may arise due to various factors including hen maturity (Herber and Van Elswyk, 1996; Scheideler et al., 1998), oxidative stability (Ao et al., 2015) and the form in which the substrate is fed (oil vs. seed or biomass) (Lemahieu et al., 2015a), the current results showed that the stability of enrichment of the egg yolk omega-3 PUFA utilizing up to 0.60% of diet total omega-3 fatty acids in form of either ALA (flaxseed oil, 1.19% of diet) or DHA (DHAgold[TM] S17-B, 3.36% of diet), was achievable within 4 weeks of feeding. For EPA, its accumulation in yolk was shown to attain a steady state within 1 week of feeding hens menhaden oil (3% of diet) compared to DHA or ALA which required 2 to 3 week of feeding (Hargis et al., 1991); this may explain the lack of treatment $\times$ week interaction effect for EPA in either treatment groups in the current study analyzed after 2 weeks of feeding the treatment diets.

In the current study, the amount of omega-3 PUFA, particularly the LCPUFA deposition through the inclusion of either precursor ALA (flaxseed oil) or preformed DHA (algal DHA) was significantly improved in the egg over that of the control. It has been shown that hens fed up to 15% flaxseed (~1.68% ALA in the diet), produced eggs containing ~250 mg/egg of ALA (Ferrier et al., 1995; Scheideler and Froning, 1996; Lewis et al., 2000). In the current study, at the highest levels of supplementation, 0.60% of dietary total omega-3 fatty acids, predominantly ALA (flaxseed oil) produced eggs containing 99.9 ± 2.33 and 68.6 ± 1.61 mg/egg of ALA and DHA, respectively; while the algal DHA-containing diet (predominantly DHA), produced eggs containing 185 ± 3.03 mg/egg of DHA. The lower efficiency (~60% less) of enrichment of DHA observed in eggs from the ALA-fed hens compared to DHA-fed hens is based on the inefficient metabolic conversion of ALA to DHA in the former group of hens (Cherian and Sim, 1991; Gakhar et al., 2012; Neijat et al., 2016a; b). Whereas DHA can be incorporated into the egg yolk without any conversion steps with a direct source of dietary omega-3 LCPUFA enabling more

efficient deposition of DHA (Herber and Van Elswyk, 1996; Cachaldora et al., 2005; Lawlor et al., 2010). Furthermore, based on the levels of supplementation, raising the level from 0.20% to 0.60% for the ALA- vs. DHA-treatment decreased the efficiency of incorporation of DHA in eggs from 25.3 to 12.1 vs. 50.3 to $32.1 \pm 1.53\%$, respectively. Hence, further raising the level of dietary omega-3 fatty acids, particularly for the ALA-fed hens, will have little further beneficial affect on DHA enrichment of egg yolk. Although a linear incorporation of ALA in the egg yolk occurs as a function of increasing flaxseed/oil (Scheideler and Froning, 1996), the non-linearity in the accumulation of its long chain metabolite (particularly, DHA), in part, relates to the influence of level of intake (mainly from primary precursors, ALA) on liver enzymes (hepatic regulation) and therefore *de novo* fatty acid synthesis of the LCPUFA metabolites, a process that is known to be similar to that in humans (Brenna et al., 2009).

With respect to the utilizing of preformed LCPUFA in diets of laying hens, in this study with algal DHA (DHAgold[TM] S17-B, 17% of biomass DHA content), similar results were also obtained by Sefer et al. (2011) who showed that feeding hens with levels up to 1% of diet micro algae (such as *Schizochytrium* spp., 17% DHA content) resulted in ~2-fold increase in DHA (1.50 vs. 4.90 % of total fatty acids, for 0 vs. 1% diet inclusion, respectively). Previous work in our lab, using graded levels of microencapsulated fish oil of up to 0.06% of diet inclusion in a 21-day trial (Lawlor et al., 2010), observed up to 299 mg/yolk DHA enrichment. In these studies no evidence of a plateau was reached in the enrichment of eggs using a preformed source of LCPUFA. In the current study, changes in omega-3 PUFA in total lipid of egg yolk closely reflected levels in the plasma lipids, and both showed linear and quadratic effects in the accumulation of DHA in response to graded levels of algal DHA inclusion. Similarly, other studies (Herber and Van Elswyk, 1996; Bruneel et al., 2013; Lemahieu et al., 2013) showed that

marine algae promotes efficient yolk DHA deposition; however, doubling the amount of the algae in the diet did not result in doubled omega-3 LCPUFA deposition into the eggs. As demonstrated by Leeson and Caston (2004) with lutein enrichment of egg yolks, indicating a limit in the enrichment of eggs regardless of levels of dietary supplementation, hence, the plateauing of lipid-soluble substances in egg yolk. Taken together, the results indicate that optimal utilization of LCPUFA from marine algae in the deposition of DHA in the egg yolk is limited when higher level of preformed LCPUFA is used in laying hen diets. However, a significant accumulation of DHA is achieved with preformed sources compared to that endogenously synthesized from precursor ALA.

A greater accumulation of EPA in the egg yolk and plasma was observed in the present study with the algal DHA supplementation, compared to control or the flaxseed oil treatments, likely due to the EPA content of the algal DHA product. Previous studies have indicated the lack of influence of algal product supplementation on EPA levels of yolk (Van Elswyk et al., 1995; Sefer et al., 2011). However, in these studies, the predominance of DHA compared to the level of EPA in products used may be an important factor in this regulation. Other studies utilizing preformed LCPUFA in diets of hens composed of higher concentrations of EPA relative to DHA have resulted in greater enrichment of the latter than the former fatty acid (Herber and Van Elswyk, 1996; Lawlor et al., 2010). This may be due to the involvement of EPA in different metabolic pathways (Sprecher et al., 1995; Jump, 2008), impacting the ability to enrich egg yolk with this fatty acid (Yalcyn et al., 2007; Souza et al., 2008). In this study, although EPA was detected in the egg yolk when hens consumed lower dietary total omega-3 fatty acids (0.03% level), the lack of detection of this fatty acid in the plasma of the same group of hens may be related to its removal to yolk components (Heald and Badman, 1963), as this fatty acid was least

represented among the LCPUFA. Both omega-3 sources decreased the ratio of n-6/n-3 PUFA of eggs hence improving the nutritional value (primarily by increasing the ALA or the DHA contents when supplemented with either flaxseed oil or algal DHA, respectively) achieving a ratio of 2.4 to 3.2/1 in the eggs and 2.8 to 3.3/1 in the plasma at highest total omega-3 fatty acid level in the diet, within the recommended levels for human consumptions (4/1)(Kris-Etherton et al., 2000) to prevent certain diseases.

A short-term feeding regimes (as such, a 6-week period in the current study) may, in part, contribute to the contradicting hen performance outcomes based on omega-3 supplementation in laying hen diets mentioned in earlier studies (Fraeye et al., 2012). However, Herber and Van Elswyk (1996), utilizing marine algae (2.4 vs. 4.8%, equivalent to 200 vs. 400 mg DHA/day, respectively) in laying hen diets in a 4-week study period was able to note differences in egg production between the first 24 weeks of hen age to a latter phase in the egg production cycle (56-week old hens) with the higher level of supplementation but not at the lower level. These results may indicate that level of supplementation and hen age rather than the period of feeding may play a big role. In the current results, hen performance and egg quality indices were not influenced by source and/or level of omega-3 fatty acids. Given that the control diet was essentially low in omega-3 fatty acids (contained the least amount of either ALA and no preformed DHA), this may provide some insights into the essentiality of omega-3 fatty acids for hens. Previous studies by Machlin et al. (1962) and Miller et al. (1963) showed that hens fed an LA-deficient diet still contained relatively large amounts of the fatty acid after 12 and 40 weeks, respectively of depletion period, suggesting that in order to deplete hens of LA, pullets may require to be fed an LA-deficient diet from hatch. It is likely that a similar principle may apply for ALA. Both LA and ALA are considered to be essential fatty acids for chickens (NRC, 1994).

However, it may be reasoned that, mature birds will have a store of DHA/ALA in their body fat reserve. As such, their metabolic needs for these fatty acids may have been met by the fatty acids present in fat stores that are released over the course of the study. Based on defined LA recommendation for inclusion in poultry diets (1.0% of diet), sufficient for productive purposes (NRC, 1994), this is achievable within the inclusion level of ~2% corn oil (LA, 53% of total fatty acids) in diets of laying hens (House, 2014). However, requirement estimate for ALA in laying hens, particularly in young hens, is not yet defined and may therefore warrant investigation. With respect to SFA and MUFA of the egg, the current results, utilizing ALA (flaxseed oil) agree as initially indicated by Jiang et al. (1991) that the laying hen has limited ability to change the SFA as well as the MUFA of the egg. Although the preformed DHA-fed hens showed a significant decline in the level of MUFA, these results may have reflected that of the laying hen diets. It may also follow that the PUFA present in the diet greatly impacted MUFA levels (particularly oleic acid, 18:1) in eggs, in part due to the attenuation of $\Delta9$-desaturase enzyme by higher dietary levels of PUFA in the conversion of stearic to oleic acid (Brenner, 1974; Cherian and Sim, 1991).

Dietary supplementation of graded levels of either flaxseed oil or algal DHA had no effect on total lipid and cholesterol content of the egg yolk or plasma, as well as plasma TAG. In the current study, although ALA-fed hens had achieved constant levels of egg total lipid between weeks 4 and 6, unlike with the DHA-fed hens, no difference was noted between treatment groups in week 6, indicating stability of egg yolk total lipids. In this study, egg cholesterol level was between the range of 197 and 259 ± 25.1 mg/yolk in both treatment groups. Previously, diets containing various types and levels of lipids had on effect on egg cholesterol content (remaining in the range of 200 to 250 mg/yolk) when hen diets contained up to 30% level ground flaxseed

(Caston and Leeson, 1990), 3% flaxseed oil (Mazalli et al., 2004), 3% menhaden oil (Hargis et al., 1991) or when utilizing different omega-3 rich microalgae species (Lemahieu et al., 2013). Generally, egg cholesterol is ~200 mg/egg and it is known to be very resistant to change based on mechanisms involved in yolk formation (Hargis, 1988; Griffin, 1992). The latter results underscore the central role of the hens′ liver in regulating yolk lipid content within limits for physiological requirements of the developing chick. However, Elkin (2007) has indicated that feeding hens statins, inhibitors of the rate-limiting enzyme for cholesterol synthesis (3-hydroxy-3-methylglutaryl-coenzyme A reductase) can result in the reduction of egg cholesterol contents by as much as 50%. The egg is viewed by some as a vehicle for the hen to "excrete" cholesterol, thereby, not all of the cholesterol in the egg is necessary for chicks to hatch (Elkin and Yan, 1999) or the embryos to survive (Austic and Hsu, 2015).

In summary, although no differences in performance indices was evident, this study defined dietary levels and source of omega-3 fatty acids needed to optimize DHA levels in plasma and eggs. Yolks derived from either precursor ALA or preformed DHA decreased the n-6/n-3 ratio to similar levels (with the range of 3.3 to 1 ratio), depositing 3-fold more DHA in the egg yolk when laying hen diets contained 0.60% of total omega-3 fatty acids with algal DHA (3.36 g/100g diet) over that of flaxseed oil (1.19 g/100g diet), achieving a steady state of accumulation within 4 weeks of feeding. There is a significantly high nutritional level of enrichment of eggs (providing almost half the daily allowance for omega-3 LCPUFA for the general population i.e. ~ 400-500 mg/day), to actively reduce risk for cardiovascular disease (Harris et al., 2009); achieved by including a source of dietary preformed DHA (such microalgae) compared to precursor ALA (flaxseed oil) in the laying hen diets. However, further investigation is needed into the mechanism leading to the decline in the efficiency of DHA

enrichment with preformed DHA as well as synthesised DHA at higher level of inclusion of their primary sources of omega-3 fatty acid in laying hen diets.

CHAPTER 8 MANUSCRIPT V

Impact of dietary precursor ALA versus preformed DHA on fatty acid profiles of eggs, liver and adipose tissue and expression of genes associated with hepatic lipid metabolism in laying hens

8.1 ABSTRACT

Dietary omega-3 polyunsaturated fatty acids (n-3 PUFA), including alpha-linolenic acid (ALA) and preformed longer chain PUFA (LCPUFA, particularly docosahexaenoic acid, DHA) differ in their egg LCPUFA enrichment efficiency. However, mechanisms leading to these differences are unclear. To this end, n-3 PUFA contents in different lipid classes, including triacylglycerol (TAG) and total phospholipid (PL) in yolk, liver and adipose, as well as the expression of key hepatic enzymes in lipid metabolism were evaluated in laying hens in response to changes in dietary supply. Seventy Lohmann hens (n=10/treatment) consumed either a control diet (0.03% total n-3 PUFA), or the control with supplementation (0.20, 0.40 and 0.60% total n-3 PUFA) from either flaxseed oil or algal product, as sources of ALA (precursor) or DHA (preformed), respectively. The study was arranged in a completely randomized design, and data were analyzed using the Proc Mixed procedure of SAS. ALA accumulated as a function of intake ($P < 0.0001$) in total and lipid classes of yolk, liver and adipose (TAG only) for ALA- and DHA-fed hens. Unlike flaxseed oil, preformed-DHA contributed to greater ($P < 0.0001$) accumulation of LCPUFA in yolk total PL and TAG pool, as well as adipose TAG. This may relate to elevated ($P < 0.0001$) mRNA expression of acyl-CoA synthetase (ACSL1). No difference in hepatic EPA level in total lipids was noted between both treatment groups; $EPA_{liver} = 2.1493x - 0.0064$; $R^2 = 0.70$, $P < 0.0001$ (x=dietary n-3 PUFA). The latter result may highlight the role of hepatic EPA in the regulation of LCPUFA metabolism in laying hens.

Key words: egg yolk and tissues; n-3 fatty acids; flaxseed oil and algal DHA; gene expression

8.2 INTRODUCTION

The link between omega-3 long chain polyunsaturated fatty acids (n-3 LCPUFA), particularly eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), and their role in the reduction and/or prevention of human diseases are well established (Yashodhara et al., 2009; Gogus and Smith, 2010). The scientific basis for the health benefits of LCPUFA, primarily relates to their ability to incorporate into or be used directly as substrates for biosynthesis of cellular phospholipid (PL) (Sprecher, 2000; Goyens et al., 2006). However, extensive studies in humans (Burdge, 2004; Plourde and Cunnane, 2007) and in animals (Blank et al., 2002; Fraeye et al., 2012) have demonstrated that there is limited ability to increase tissue levels of n-3 LCPUFA by increasing the level of precursor alpha-linolenic acid (ALA, 18:3n-3) in the diet. As such, adequate intakes of preformed n-3 LCPUFA to maintain optimum tissue function are recommended (Burdge, 2004; Brenna et al., 2009).

In laying hens, plant ingredients rich in the n-3 polyunsaturated fatty acids (PUFA), primarily ALA (e. g. flaxseeds, canola), have been used to enrich eggs with these fatty acids, and it is mainly the ALA, and to some extent the DHA, that is enriched, but not EPA. This differs from the situation where preformed LCPUFA such as marine oils (Cachaldora et al., 2006; Lawlor et al., 2010) and algae (Herber and Van Elswyk, 1996; Lemahieu et al., 2013; Ao et al., 2015) are included, as these have been shown to provide a significant enrichment of eggs with LCPUFA including EPA (Sefer et al., 2011). Moreover, the latter sources lead to the preferentially deposition of higher levels of DHA than EPA in the egg (González-Esquerra and Leeson, 2001; Cachaldora et al., 2006; Lawlor et al., 2010). Generally, the cause for the low incorporation of EPA in egg yolk and into body tissues e.g. thigh muscle (Nitsan et al., 1999) is still unclear.

Dietary fatty acids undergo several metabolic fates in order to maintain lipid homeostasis in the body (Jump, 2008). These include the partitioning of fatty acids into various lipid classes (Nguyen et al., 2008), their re-direction to other tissues, their use in other physiological processes such as being a source of energy (Burdge, 2004), their function as precursors for subsequent metabolites or their involvement in β-oxidation (Sprecher, 2000; Jump et al., 2013). However, the enrichment of LCPUFA in chicken eggs through the use of dietary sources high in ALA vs. preformed LCPUFA (EPA and DHA) requires further investigation, particularly in relation to the respective efficacy of the dietary sources in leading to egg yolk enrichment of the n-3 PUFA in different lipid classes and different tissues.

Hence, the purpose of the present study was to clarify the differential effects of supplementing laying hen diets with either precursor ALA (flaxseed oil) or preformed DHA (algal product) on the enrichment of chicken eggs with LCPUFA. The study aims to identify potential control points leading to differences in the enrichment efficiency of LCPUFA in eggs as a function of the total n-3 fatty acid intake in laying hens. The primary endpoints for this study will include the n-3 fatty acid profiles (level and distribution) in total lipid and the major lipid classes of egg yolk and key regulatory tissues (liver and adipose). In addition, the expression of key hepatic enzymes involved in lipid metabolism was also assessed.

8.3 MATERIALS AND METHODS

8.3.1 Experimental birds, housing, and dietary treatments

Seventy, 34 weeks old hens (Lohmann LSL-Classic) were individually housed in cages (width, 38 cm; length, 52 cm; and height, 51cm (front) and 46cm (rear)) in a three-deck battery facility, providing floor area/space of 1976 cm^2 and maintained under semi-controlled

environment conditions. Hens were adapted to the cages and diets over an initial 2-week period. During the first week of adaptation, hens were fed a commercial layer diet, and then transitioned to a 50:50 blend of the commercial and test diets in the second week. Following the adaptation period, the hens were weighed and assigned to 1 of 7 dietary treatments (n = 10 per group) consisting of corn-wheat-barley-soybean meal-based diets (Table 8.1). The diets were formulated to provide incremental levels of total n-3 PUFA of 0.20, 0.40 and 0.60% (by wt of diet) corresponding to supplementation of 0.36, 0.77 and 1.19 g per 100 g feed of flaxseed oil (Dyets, Inc., Bethlehem, PA, USA) or 1.00, 2.18 and 3.36 g per 100g feed of DHAgold[TM] S17-B (heterotrophic microalgae; DSM Nutritional Products, Parsippany, NJ, USA), respectively. The inclusion of either flaxseed oil or algal product in the experimental diets was made at the expense of almond oil, a source of oil low in omega-3 fatty acids. The latter oil was also used to formulate a control diet low in total omega-3 fatty acids (0.03% of total n-3 fatty acid). The algal product, although contained other fatty acids, it was predominantly DHA (Table 8.1) and regarded as a source of exogenous DHA, hence, herein was referred to as algal DHA or exogenous/preformed DHA. The diets contained similar levels of total fat (4%), energy (2800kcal/kg) and crude protein (16%), to meet the minimum recommendation of laying hens consuming 105 to 115 g of feed per day in accordance to the strain's management guide (LSL-Classic, Layer Management, 2004). The ingredients and chemical composition of diets are shown in Table 8.1. The experimental protocol for this study was reviewed and approved by the Animal Care Committee of the University of Manitoba (Winnipeg, MB, Canada) and the experimental hens were handled in accordance with the guidelines described by the Canadian Council on Animal Care (1993).

Table 8.1: Diet composition, macronutrient and major fatty acid composition of experimental diets for laying hens containing increasing levels of total omega-3 fatty acids[1]

	Control	Flaxseed oil diets			Algal DHA diets		
Total omega-3 fatty acids (% of diet)	0.03	0.20	0.40	0.60	0.20	0.40	0.60
Ingredient (%)							
Soybean meal	25.1	25.1	25.1	25.1	25.1	24.8	24.5
Wheat	2.10	2.10	2.10	2.11	0.00	0.00	0.00
Corn	56.9	56.9	56.9	56.9	58.4	58.3	58.3
Vitamin-mineral premix[2]	2.50	2.50	2.50	2.50	2.50	2.50	2.50
Sodium chloride	0.39	0.39	0.39	0.39	0.40	0.40	0.40
Limestone	9.70	9.70	9.70	9.70	9.59	9.14	8.70
Dicalcium phosphate	1.90	1.90	1.90	1.90	1.91	1.91	1.92
L-Lysine-HCl	0.038	0.038	0.038	0.038	0.041	0.049	0.057
DL-methionine	0.092	0.092	0.092	0.092	0.093	0.095	0.097
Flaxseed oil	0.00	0.36	0.77	1.19	0.00	0.00	0.00
DHAgold[TM] S17-B	0.00	0.00	0.00	0.00	1.00	2.18	3.36
Almond oil	1.35	0.99	0.58	0.16	0.97	0.55	0.14
Calculated macronutrients							
AMEn (Poultry; kcal/kg)	2850	2850	2850	2850	2850	2850	2850
Crude fat (%)	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Crude protein (%)	16.5	16.5	16.5	16.5	16.5	16.5	16.5
Analyzed nutrients (As-is basis)							
MEn (Poultry; Kcal/kg)	2821	2812	2808	2796	2813	2815	2840
Crude protein (%)	15.3	15.5	15.7	16.1	15.4	16.1	15.7
Crude fibre (%)	2.46	2.46	2.81	3.45	2.77	2.71	2.67
Crude fat (%)	4.04	4.18	3.93	3.74	3.68	3.59	3.75
Calcium (%)	4.39	4.22	4.39	4.27	4.30	4.11	3.82
Phosphorus (%)	0.70	0.72	0.69	0.70	0.71	0.70	0.74
Calculated fatty acids(%, by wt)							
18:2n-6 (LA)	1.30	1.29	1.28	1.27	1.25	1.17	1.10
18:3n-3(ALA)	0.03	0.20	0.40	0.60	0.03	0.03	0.03
22:6n-3 (DHA)	0.00	0.00	0.00	0.00	0.17	0.37	0.57
Total omega-3 fatty acids	0.03	0.20	0.40	0.60	0.20	0.40	0.60
Analyzed fatty acid profile (%,by wt)							
Total SFA[3]	0.34	0.36	0.38	0.39	0.43	0.55	0.67
Total MUFA[4]	1.00	0.96	0.83	0.70	0.83	0.67	0.49
18:2n-6 (LA)	1.28	1.34	1.36	1.32	1.15	1.15	1.04
18:3n-3 (ALA)	0.05	0.20	0.36	0.53	0.05	0.05	0.05
20:5n-3 (EPA)	0.00	0.00	0.00	0.00	0.006	0.012	0.017
22:5n-3 (DPA)	0.00	0.00	0.00	0.00	0.002	0.004	0.006
22:6n-3 (DHA)	0.00	0.00	0.00	0.00	0.15	0.33	0.50
Total omega-3 fatty acids[5]	0.05	0.20	0.36	0.53	0.21	0.40	0.57
Ratio n-6/n-3	25.6	6.7	3.8	2.5	5.8	3.0	1.9

[1]Major source of omega-3 fatty acid content (%, by wt) in almond oil, 0.20% ALA (18:3n-3); flaxseed oil, 53.2% ALA; DHAgold[TM] S17-B, $17.9 \pm 0.05\%$ DHA (22:6n-3) (total fat: $39.9 \pm 4.14\%$ by wt).

[2]Provided per kilogram of diet, vitamin-mineral premix contained: 11,000 IU of vitamin A; 3,000 IU of vitamin D_3, 150 IU of vitamin E, 3 mg of vitamin K_3 (as menadione), 0.02 mg of vitamin B_{12}, 0.2 mg of biotin, 6.5 mg of riboflavin, 4 mg of folic acid, 10 mg of calcium pantothenate, 39.9 mg of niacin, 2.2 mg of thiamine, 4.5 mg of pyridoxine, 1000 mg of choline chloride, 125mg antioxidant (ethoxyquin), 66 mg of manganese oxide, 70 mg of zinc oxide, 80 mg of ferrous sulfate, 10 mg of copper sulfate, 0.3 mg of sodium selenite, 0.4 mg of calcium iodate, 0.67 mg of sodium chloride.

[3]Saturated (SFA) = myristic (14:0), palmitic (16:0), stearic (18:0).

[4]Monounsaturated (MUFA) = palmitoleic (16:1), oleic (18:1).

[5]Total omega-3 fatty acids = sum of ALA, EPA, DPA and DHA.

8.3.2   Sample collection

Samples of diet (in duplicate per treatment) and tissue (egg yolk, liver and adipose, n=10 per treatment) were obtained during the last experimental feeding period (week-6). During that week, eggs were collected during the last 3 days of the experimental period, and 1 egg per hen (n=10) per treatment was cracked and the yolk separated from the white using an egg separator. The yolks were individually weighed and stored in plastic bags at -20°C until analyzed. Birds were euthanized by cervical dislocation, then samples of liver (~3 g for lipid extraction and another 2 g for total RNA extraction) and adipose (abdominal fat pad, ~3 g) were quickly removed, rinsed in cold saline and snap-frozen in liquid nitrogen for storage at -80°C until analyzed.

8.3.3   Extraction and analysis of fatty acids

Approximately 1 g each of ground feed, yolk, liver and adipose tissue (abdominal fat pad) were used for the extraction of total lipids using chloroform-methanol (2:1, by vol) according to Folch et al. (1957). The feed, egg yolk liver and adipose lipid extracts were first dissolved in hexane to obtain aliquots of a known volume (containing 40 - 50 mg per solvent) for either total or individual lipid class fatty acid analysis. The separation of the major lipid classes, triacylglycerol (TAG) and total PL and its fractions, phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) of egg yolk, liver and adipose, were conducted by thin layer chromatography (TLC) on silica gel plates (Analtech, Inc, Newark, DE, USA) according to procedures previously reported (Neijat et al., 2016). Prior to methylation, the TAG fraction was saponified according to the method previously described (Hartman, 1973). All lipid fractions as well as the total lipids of all tissues were dried under a stream of nitrogen gas then methylated using 3ml of 3N methanolic HCl at 80°C for two hours. For adipose tissue, 1 mL toluene was

first added before methylation. For both total and lipid classes, C17:1 (Nu-Chek Prep Inc.,

Elysian, MN) was included during the methylation process as the external standard and used for

the quantification of fatty acids. The resulting fatty acid methyl esters (FAME) were extracted

into iso-octane, and the fatty acid methyl esters for the lipid classes were further concentrated to

50 µL, except for PtdEtn (25 µL), under a stream of nitrogen gas. Samples were analyzed using

a Varian 450 gas chromatography with a flame ionization detector (GC-FID) and equipped with

a DB225MS column (30 m × 0.25 mm diameter and 0.25 µm film thickness; Agilent

Technologies Canada Inc., Mississauga, Ontario). The specific details of this procedure have

recently been published (Neijat et al., 2016a). Each fatty acid peak was identified by comparing

its retention time to authentic standard samples of known composition (Lipid standards; PUFA 1,

Cat # 1093, Matreya LLC, Lipid and Biochemicals, PA, USA). The fatty acid content of total

lipid was calculated as concentration (mg/g fat) = [(peak area of a given fatty acid ×

concentration of internal standard (mg/mL)/peak area of internal standard) x dilution factor of

extracted fat] divided by extracted fat weight from tissue sample (g). For lipid classes of yolk,

liver and adipose, the level of each fatty acid was calculated by summing up the total number of

moles derived using the molecular weight of the corresponding FAME for all identified fatty

acids (g fatty acid/100g total fatty acid), then expressed as mol%.

8.3.4   *RNA isolation, cDNA synthesis and qRT-PCR analysis*

Total RNA was extracted with TRIzol® reagent (Invitrogen, Canada) according to the

manufacturer's instructions, and homogenized using SPEX Sample Prep Geno /Grinder®.

Extracted RNA integrity was assessed via agarose gel electrophoresis, and the RNA

concentration and purity were determined using a NanoDrop (2000/2000c Thermo Fisher

Scientific Inc, 2009, Canada). Reverse transcription was carried out using a high capacity cDNA

Reverse Transcription kit (Applied Biosystems, Canada) according to the manufacturer's protocol. Briefly, 20 μL of each reaction mixture containing 2μL of 10× RT buffer, 0.8 μL of 25× dNTPs (100 mM), 2 μL of 10× RT random primers, 1 μL of MultiScribe^{TM} Reverse Transcriptase (50 U/μL), 4.2 μL nuclease-free water and 10 μL total RNA (1.5 μg) was incubated for 10 min at 25°C followed by 2 h at 37°C and finally for 5 min at 85°C using a Bio Rad T100^{TM} Thermal Cycler. The reverse transcription products (cDNA) were stored at −20°C for relative mRNA expression by real-time quantitative polymerase chain reaction (qRT-PCR). Pairs of primers for genes involved in fatty acid transport, activation, β-oxidation and synthesis were designed using sequences obtained from the National Centre for Biotechnology Information (NCBI) except for some fatty acid synthesis genes for which primer sequences were obtained from previously published literature (Gakhar et al., 2012). Pairs of primers and their sequences used for qRT-PCR are indicated in the supplementary data (Appendix II). Quantitative real-time RT-PCR using an OneStep Real-Time Detection System (Applied Biosystems, Canada) was done in duplicates with 1 μL of cDNA solution, including non-template controls, 5μL of SYBR Green as a detector, 2 μL of nuclease-free water and 1 μL each of forward and reverse primer for a final reaction volume of 10 μL. The qRT-PCR cycle parameters involved a holding stage of 95°C for 3 min; and 40 cycles of denaturation at 95°C for 10 sec and combined annealing/extension at 61°C for 30 sec, and a one 2-segment cycle of product melting stage of 95°C for 10 sec and 60°C for 5 sec. Data were generated using ΔΔCt method by normalizing the expression of the target gene to a housekeeping gene, actin beta (β-actin) and the values were reported as mean fold changes of the expression of the target genes in the experimental groups compared with the control (as a calibrator) which is assigned a value of one.

8.3.5   *Statistical analysis*

The effects of source and level of inclusion of n-3 PUFA in the basal diet were analysed in a completely randomised design as fixed effects using the MIXED procedure of SAS (SAS Institute Inc., Cary, NC) accounting for the random effect of hens. Least squares means (LSM), adjusted using Tukey's significant difference test, were compared for significant difference ($P <$ 0.05). Orthogonal contrasts were used to test the effects of the different dietary sources of total n-3 fatty acids using flaxseed oil (Control vs. FSO), algal DHA (Control vs. DHA) and between both treatment groups (FSO vs. DHA) as the main effects. Using the same statistical package, Proc Reg and Proc Corr procedures were used to predict and compare accumulation levels of omega-3 fatty acids in the tissues.

8.4 RESULTS

8.4.1   Total lipid and fatty acid content of experimental diets

Although the total n-3 fatty acid of the control diet (mainly ALA) was formulated to be 0.03%, the analyzed value was slightly higher (0.05%; Table 8.1). However, the ALA levels in the basal ingredient for algal DHA-containing diets were equally influenced. Therefore, as designed, the background diet may serve to remove any confounding effects of ALA in the algal DHA supplemented group, and make the latter comparable to the control diet-fed hens. Overall, the analyzed total n-3 fatty acid contents of the experimental diets were well within the range of calculated values. All dietary treatments were formulated to contain similar level of linoleic acid (LA, 18:2n-6) in the range of 1.23 ± 0.12 (SD) % of diet. At highest level of inclusion, the algal DHA containing group had higher saturated fatty acid (SFA, 0.67%) and lower monounsaturated fatty acid (MUFA, 0.49%) levels compared with the control diet (0.34 and 1.0% of diet; respectively) as it replaced almond oil in the basal diet (Table 8.1).

8.4.2 Amount of total lipid in egg yolk, liver and adipose

Dietary treatment (level and source of omega-3 fatty acids) had no effect on the total lipid content of egg yolk (34.7, 34.6, 33.7, 34.0, 34.8, 34.1, and 34.9 ± 0.79 (SE); $P = 0.91$), liver (8.49, 7.50, 7.70, 6.63, 8.19, 8.64 and 9.37 ± 0.69; $P = 0.15$) and adipose tissues (92.0, 91.7, 91.6, 90.6, 91.9, 91.8 and 92.0 ± 0.95; $P = 0.94$) % by wt of fresh sample, respectively in the order: the control, the 3 levels of flaxseed oil, followed by the 3 levels of algal DHA containing group.

8.4.3 Fatty acid profile of total lipid in egg yolk, liver and adipose

The SFA and MUFA composition of the diets were reflected in the fatty acid composition of the egg yolk, liver and adipose tissues. Although no differences were observed in the total SFA level in the total lipids in egg yolk between treatments (Table 8.2), the levels of these fatty acids were greatly increased ($P < 0.05$) in the liver (Table 8.3) and adipose (Table 8.4) total lipids in the preformed DHA-fed group when compared to the flaxseed oil-fed hens. This was mainly due to a highly significant ($P < 0.0001$) increase (2-fold more) in the amount of myristic acid (14:0) in both tissues in the former than the latter group of hens (data not shown). Although there were no main effect differences due to treatment in the total MUFA level in all tissues (egg yolk, liver and adipose), by contrast, the level of total MUFA content in the liver was observed to be reduced ($P < 0.05$; Table 8.3) in the DHA- (preformed LCPUFA) compared to the control-fed hens but not the flaxseed oil-fed hens. The n-6 PUFA concentration was reduced in egg yolk ($P < 0.05$; Table 8.2) and in the liver ($P < 0\,001$; Table 8.3) with algal DHA containing diets compared to flaxseed oil or the control, particularly at higher level of inclusion. However, differences between treatments were not observed in the n-6 PUFA concentrations in the total lipid fraction of hen adipose tissue (Table 8.4).

Table 8.2: Egg yolk total lipid fatty acid profile (mg/g fat) as a function of feeding hens diets containing total omega-3 fatty acids sourced from either flaxseed oil or algal DHA[1]

| | Total omega-3 in diet (%, by wt) | | | | | | | | | Contrasts (P-values)[2] | | |
| | Control | Flaxseed oil (FSO) diets | | | Algal DHA (DHA) diets | | | | | FSO vs. | DHA vs. | FSO vs. |
	0.03	0.20	0.40	0.60	0.20	0.40	0.60	SE	P-values	Control	Control	DHA
Total SFA[3]	234	230	230	232	237	242	250	8.42	0.59	0.72	0.38	0.079
Total MUFA[4]	290	285	268	277	279	264	267	12.0	0.68	0.35	0.16	0.48
Total PUFA[5]	101^b	110^{ab}	114^{ab}	120^a	108^{ab}	115^{ab}	115^{ab}	4.24	0.068	0.010	0.023	0.63
18:2n-6 (LA)	80.0	82.6	82.9	85.1	78.5	79.7	72.8	3.25	0.16	0.38	0.45	0.016
18:3n-6 (GLA)	0.92^a	0.79^{ab}	0.78^{ab}	0.69^{bc}	0.64^{bc}	0.53^{cd}	0.44^d	0.04	<0.0001	0.000	<0.0001	<0.0001
20:4n-6 (ARA)	13.6^a	11.4^b	9.68^c	8.77^{cd}	9.56^c	8.2^d	7.01^e	0.28	<0.0001	<0.0001	<0.0001	<0.0001
18:3n-3 (ALA)	1.74^d	5.64^c	9.65^b	16.9^a	2.09^d	2.48^d	2.56^d	0.29	<0.0001	<0.0001	0.057	<0.0001
18:4n-3 (SDA)	0.091	0.080	0.067	0.075	0.085	0.080	0.087	0.016	0.96	0.35	0.70	0.43
20:5n-3 (EPA)	0.04^d	0.16^d	0.39^c	0.61^b	0.37^c	0.75^b	1.15^a	0.03	<0.0001	<0.0001	<0.0001	<0.0001
22:5n-3 (DPA)	0.37^e	0.63^d	0.98^b	1.14^a	0.39^{ef}	0.55^{de}	0.81^c	0.04	<0.0001	<0.0001	<0.0001	<0.0001
22:6n-3 (DHA)	4.23^f	8.26^e	10.3^{de}	11.7^d	16.2^c	23.1^b	29.9^a	0.66	<0.0001	<0.0001	<0.0001	<0.0001
Total n-6	94.2^{ab}	94.8^a	93.4^{ab}	94.5^a	88.7^{ab}	88.4^{ab}	80.3^b	3.40	0.028	0.99	0.048	0.003
Total n-3	6.41^e	14.8^d	21.9^c	29.8^b	19.2^c	27.0^b	34.5^a	0.88	<0.0001	<0.0001	<0.0001	<0.0001
Ratio n-6/n-3	15.2^a	6.41^b	4.26^c	3.17^d	4.64^c	3.27^d	2.35^e	0.10	<0.0001	<0.0001	<0.0001	<0.0001

[1]Data are presented as least square means (LSM) ± Pooled SE; n=8.
[2]Contrasts between treatment groups are significant at $P < 0.05$.
[3]Saturated fatty acids = myristic (14:0), palmitic (16:0), stearic (18:0).
[4]Monounsaturated fatty acids = palmitoleic (16:1), oleic (18:1).
[5]Polyunsaturated fatty acids = LA (18:2n-6), GLA (18:3n-6), ARA (20:4n-6), ALA (18:3n-3), SDA (18:4n-3), EPA (20:5n-3), DPA (22:5n-3) and DHA (22:6n-3).
[a-f]Different superscripts between treatments within a row are significantly different at $P < 0.05$.

Table 8.3: Liver total lipid fatty acid profile (mg/g fat) as a function of feeding hens diets containing total omega-3 fatty acids sourced from either flaxseed oil or algal DHA[1]

| | Total Omega-3 in diet (%, by wt) | | | | | | | | | Contrasts (P-values)[2] | | |
| | Control | Flaxseed oil (FSO) diets | | | Algal DHA (DHA) diets | | | | | FSO vs. | DHA vs. | FSO vs. |
	0.03	0.20	0.40	0.60	0.20	0.40	0.60	SE	P-values	Control	Control	DHA
Total SFA	284^{ab}	275^{ab}	271^{b}	274^{ab}	276^{ab}	282^{ab}	293^{a}	4.75	< 0.05	0.066	0.98	< 0.05
Total MUFA	322	322	297	284	287	275	285	15.2	0.17	0.23	< 0.05	0.14
Total PUFA	121^{b}	133^{ab}	139^{ab}	153^{a}	135^{ab}	130^{ab}	129^{ab}	6.40	< 0.05	< 0.01	0.17	0.054
18:2n-6 (LA)	87.6^{ab}	88.3^{ab}	88.3^{ab}	93.6^{a}	88.0^{ab}	77.9^{b}	77.6^{b}	3.16	0.005	0.51	0.080	0.001
18:3n-6 (GLA)	1.07^{a}	0.93^{ab}	0.86^{ab}	0.85^{ab}	0.69^{bc}	0.49^{c}	0.49^{c}	0.08	<0.0001	< 0.05	<0.0001	<0.0001
20:4n-6 (ARA)	24.6^{a}	25.9^{a}	21.6^{ab}	22.8^{ab}	19.6^{abc}	15.7^{bc}	12.7^{c}	1.84	<0.0001	0.57	< 0.001	<0.0001
18:3n-3 (ALA)	1.78^{d}	5.60^{c}	9.95^{b}	15.4^{a}	1.99^{d}	2.03^{d}	2.28^{d}	0.27	<0.0001	<0.0001	0.29	<0.0001
18:4n-3 (SDA)	0.170^{a}	0.152^{ab}	0.111^{bc}	0.084^{c}	0.142^{ab}	0.113^{bc}	0.098^{c}	0.009	<0.0001	<0.0001	<0.0001	0.82
20:5n-3 (EPA)	0.06^{e}	0.29^{de}	0.71^{bc}	1.24^{a}	0.47^{cd}	0.93^{ab}	1.21^{a}	0.08	<0.0001	<0.0001	<0.0001	0.058
22:5n-3 (DPA)	0.28^{d}	0.63^{c}	1.12^{b}	1.48^{a}	0.29^{d}	0.45^{cd}	0.70^{c}	0.08	<0.0001	<0.0001	< 0.05	<0.0001
22:6n-3 (DHA)	5.58^{d}	13.2^{cd}	17.9^{bc}	20.7^{bc}	23.6^{b}	32.2^{a}	34.9^{a}	1.96	<0.0001	<0.0001	<0.0001	<0.0001
Total n-6	113^{a}	114^{a}	111^{ab}	117^{a}	108^{abc}	94.1^{bc}	90.8^{c}	4.47	< 0.001	0.90	0.004	<0.0001
Total n-3	7.85^{d}	19.9^{c}	30.3^{ab}	39.5^{a}	26.5^{bc}	35.8^{ab}	38.9^{a}	2.22	<0.0001	<0.0001	<0.0001	< 0.05
Ratio n-6/n-3	15.1^{a}	6.07^{b}	3.99^{c}	2.97^{d}	4.18^{c}	2.69^{d}	2.21^{d}	0.24	<0.0001	<0.0001	<0.0001	<0.0001

[1]Data are presented as least square means (LSM) ± Pooled SE; n=8.
[2]Contrasts between treatment groups are significant at $P < 0.05$.
[3]Saturated fatty acids = myristic (14:0), palmitic (16:0), stearic (18:0).
[4]Monounsaturated fatty acids = palmitoleic (16:1), oleic (18:1).
[5]Polyunsaturated fatty acids = LA (18:2n-6), GLA (18:3n-6), ARA (20:4n-6), ALA (18:3n-3), SDA (18:4n-3), EPA (20:5n-3), DPA (22:5n-3) and DHA (22:6n-3).
[a-e]Different superscripts between treatments within a row are significantly different at $P < 0.05$.

Table 8.4: Adipose tissue (abdominal fat pad) total lipid fatty acid profile (mg/g fat) as a function of feeding hens diets containing total omega-3 fatty acids sourced from either flaxseed oil or algal DHA[1]

	Control	Flaxseed oil (FSO) diets			Algal DHA (DHA) diets			SE	P-values	FSO vs. Control	DHA vs. Control	FSO vs. DHA
	0.03	0.20	0.40	0.60	0.20	0.40	0.60					
Total SFA	241^{ab}	239^{ab}	236^{b}	235^{b}	233^{b}	243^{ab}	261^{a}	5.04	0.015	0.41	0.50	0.047
Total MUFA	411	422	431	427	417	442	409	11.2	0.40	0.23	0.37	0.67
Total PUFA	196	209	209	210	195	187	199	6.86	0.16	0.091	0.80	0.008
18:2n-6 (LA)	186	192	186	178	186	175	183	6.08	0.56	0.96	0.54	0.43
18:3n-6 (GLA)	1.04	1.17	1.16	1.21	1.18	1.02	1.06	0.12	0.88	0.37	0.76	0.38
20:4n-6 (ARA)	0.44^{bc}	0.45^{bc}	0.29^{c}	0.26^{c}	0.43^{bc}	0.65^{ab}	0.87^{a}	0.05	<0.0001	0.083	0.001	<0.0001
18:3n-3 (ALA)	8.10^{d}	15.5^{c}	21.4^{b}	28.4^{a}	7.90^{d}	8.02^{d}	9.24^{d}	0.78	<0.0001	<0.0001	0.75	<0.0001
18:4n-3 (SDA)	0.266	0.209	0.217	0.165	0.259	0.233	0.176	0.027	0.092	0.031	0.16	0.25
20:5n-3 (EPA)	ND	ND	ND	ND	0.078^{b}	0.266^{b}	0.489^{a}	0.056	<0.001	-	-	-
22:5n-3 (DPA)	ND	ND	ND	ND	ND	0.077	0.130	0.021	0.13	-	-	-
22:6n-3 (DHA)	ND	0.078^{d}	0.078^{d}	0.083^{d}	0.87^{c}	1.88^{b}	3.98^{a}	0.064	<0.0001	-	-	-
Total n-6	187	194	187	180	187	177	185	6.13	0.56	0.95	0.55	0.46
Total n-3	8.45^{e}	16.0^{c}	21.8^{b}	30.2^{a}	9.13^{e}	10.6^{de}	14.3^{cd}	0.99	<0.0001	<0.0001	0.017	<0.0001
Ratio n-6/n-3	22.2^{a}	12.2^{c}	8.51^{d}	6.04^{e}	20.9^{a}	16.8^{b}	13.1^{c}	0.50	<0.0001	<0.0001	<0.0001	<0.0001

The top spanning headers read: "Total omega-3 in diet (%, by wt)" over Control through Algal DHA columns, and "Contrasts (P-values)[2]" over the last three columns.

[1]Data are presented as least square means (LSM) ± Pooled SE; n=8.
[2]Contrasts between treatment groups are significant at $P < 0.05$.
[3]Saturated fatty acids = myristic (14:0), palmitic (16:0), stearic (18:0).
[4]Monounsaturated fatty acids = palmitoleic (16:1), oleic (18:1).
[5]Polyunsaturated fatty acids = LA (18:2n-6), GLA (18:3n-6), ARA (20:4n-6), ALA (18:3n-3), SDA (18:4n-3), EPA (20:5n-3), DPA (22:5n-3) and DHA (22:6n-3).
[a-e]Different superscripts between treatments within a row are significantly different at $P < 0.05$. ND, not detected/only traces found.

Graded levels of total n-3 fatty acids in the diets of hens sourced from either flaxseed oil or algal DHA significantly ($P < 0.0001$) increased the levels of the n-3 PUFA (ALA, EPA, DPA and DHA, except for stearidonic acid (SDA, 18:4n-3)) in the total lipid of the egg yolk (Table 8.2), liver (Table 8.3) and adipose (Table 8.4) as compared to the control group. The ALA content in the total lipids of egg yolk, liver and adipose significantly ($P < 0.0001$) increased as a function of intake (Tables 8.2 – 8.4). The ALA deficient diets containing algal DHA also incorporated similar amounts of ALA (expressed as in mg/g fat) in the total lipids of the egg yolk, liver and adipose as that obtained from the control diet-fed hens; reflecting the ALA composition of the background diet (Tables 8.2 – 8.4). In both the ALA- and the DHA-fed hens, the levels of ALA (mg/g fat) in the egg yolk, liver and adipose tissue were incorporated in a similar proportion as a function of intake; however, the latter tissue, accumulated 3 to 4-fold more ($P < 0.0001$) ALA than that observed in the egg yolk or liver at all levels of total n-3 fatty acid intake (Figures 8.1a and b).

Although the amount of SDA in the total lipid of egg yolk or adipose tissue was not influenced by levels of dietary n-3 fatty acids from either flaxseed oil or preformed DHA source in the laying hen diets (Tables 8.2 and 8.4, respectively), the hens deposited ~60% less ($P < 0.0001$) SDA in the total lipid of the yolk compared to that in the adipose tissue (on average, mg/g fat: 0.078 vs. 0.21 ± 0.008 for flaxseed oil-fed hens and 0.085 vs. 0.23 ± 0.009 for DHA-fed hens, respectively; data not presented). In the liver total lipid, a similar level of SDA accumulated ($P = 0.82$) in both the ALA- and the DHA-fed hens, however, the amount of this fatty acid significantly ($P < 0.0001$) declined as a function of total n-3 fatty acid intake (Table 8.3). Although the magnitude of the decline may be small, it accounted for ~50% reduction of the fatty acid at the highest level of intake of either sources of n-3 supplementation compared to
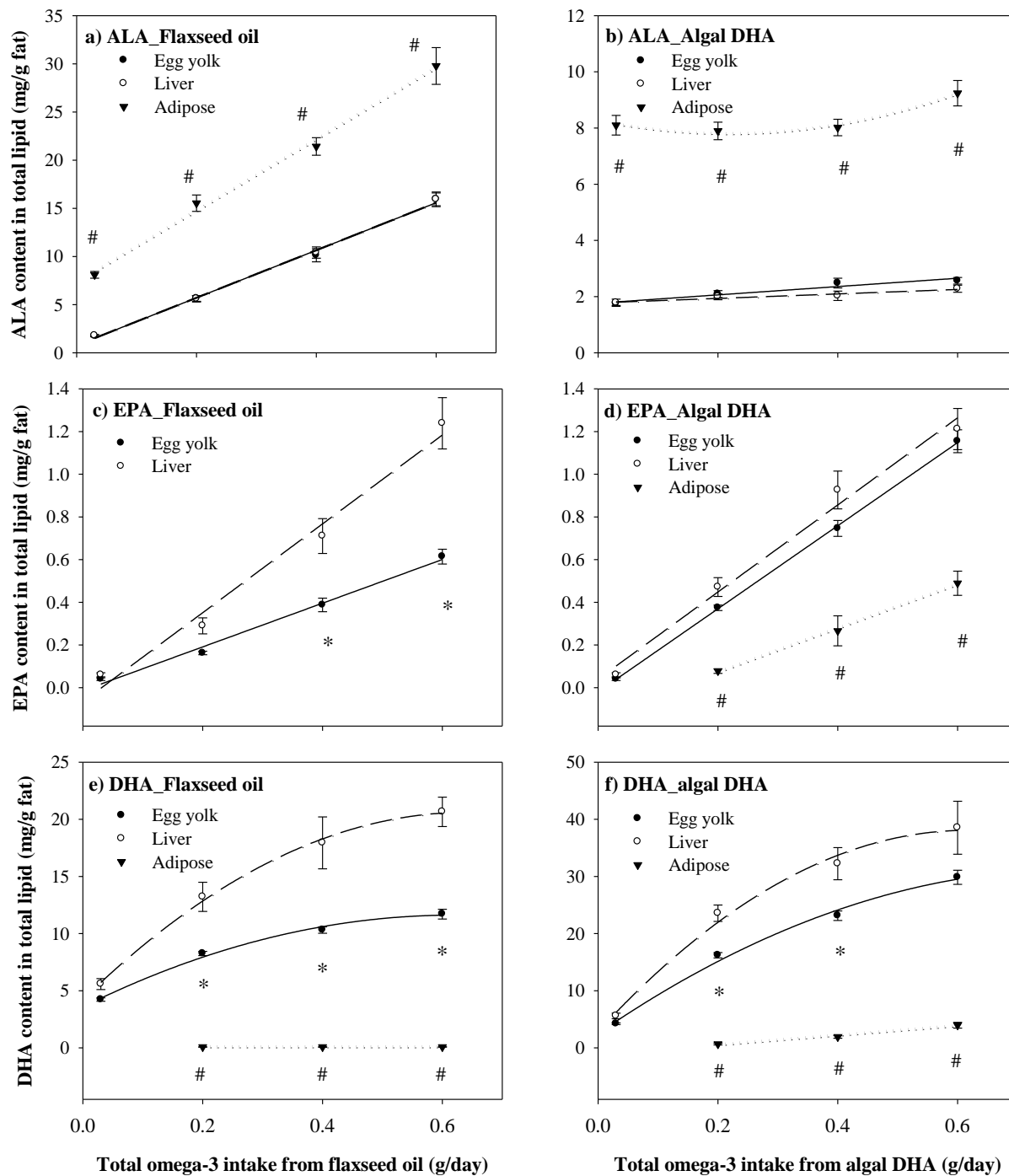
Figure 8.1. Comparison in the trends of accumulation of (a-b) alpha-linolenic acid, ALA; (c-d) eicosapentaenoic acid, EPA; and (e-f) docosahexaenoic acid, DHA in total lipid of egg yolk, liver and adipose tissue in hens fed diets containing either flaxseed oil or algal DHA.
Each data point (n = 8) are treatment means ± SE. Symbols within each plot are significantly different at $P < 0.05$; #, fatty acid in the liver and egg yolk differ from adipose;*, fatty acid in the liver differ from egg yolk.

the control (Table 8.3). In addition, as a function of total n-3 intake, a high inverse correlation was observed between SDA and the n-3 LCPUFA level in the liver total lipid, particularly with EPA (r = -0.76; $P < 0.0001$) and DHA (r = -0.49; $P < 0.0001$) derived from combined data sets for both flaxseed oil and algal DHA inclusions (Appendix III).

Despite the gradual decline in the SDA level in the liver total lipid, the amount of EPA significantly increased ($P < 0.0001$) and accumulated a similar ($P = 0.058$) level of the fatty acid in the liver total lipid as a function of dietary intake of either the flaxseed oil or the algal DHA by the laying hens (Table 8.3). Regression equations derived for estimates of prediction values (95% confidence interval) in the accumulation of SDA and EPA in the liver total lipid (developed using the combined data sets for both the flaxseed oil and algal DHA treatment groups, mg/g fat) can be expressed as: $SDA_{liver} = 0.1733 - 0.1482x$; $R^2 = 0.52$, $P < 0.0001$; $EPA_{liver} = 2.1493x - 0.0064$; $R^2 = 0.70$, $P < 0.0001$, where x is the of hen's intake level of total n-3 fatty acids for flaxseed oil and algal DHA containing diets. Although the levels of EPA that accumulated in the total lipid of the liver for ALA- and DHA-fed hens were not different (Table 8.3), 2-fold more ($P < 0.0001$) EPA was deposited in the egg yolk of the latter treatment group compared to the former (Table 8.2) which closely paralleled the accumulation trend in the liver (Figure 8.1c and d); preformed EPA was contained in the algal product (0.38 ± 0.11 (SD) % EPA by wt of algal product) and was considered in the formulation (Table 8.1). In the adipose tissue, EPA was observed to accumulate with algal DHA treatment, greatly increased ($P < 0.001$) at highest level of intake (6 times more between the highest vs. the lowest level of preformed DHA supplementation) but was not detected in the flaxseed oil- or the control diet-fed hens (Table 8.4).

The accumulation of docosapentaenoic (DPA, 22:5n-3), in the egg yolk and liver mostly reflected the level of ALA in the diets. The fatty acid increased ($P < 0.0001$) by 4- and 7-fold in the egg yolk and liver, respectively in the ALA- compared to the control diet-fed hens, at highest level of flaxseed oil intake (Tables 8.2 and 8.3). Although the background diet contained a similar level of ALA, the algal DHA treatment, at higher inclusion levels, was observed to accumulate more ($P < 0.0001$) DPA in the egg yolk and liver than the control diet-fed hens (Tables 8.2 and 8.3). In addition, the amounts of DPA in the total lipid of the adipose tissue did not significantly differ as a function of total n-3 PUFA intake of either flaxseed oil or algal DHA (Table 8.4). Preformed DPA was also contained in the algal DHA product (0.11 ± 0.03 (SD) % DPA by wt of algal product).

The content of DHA in the total lipid of egg yolk and liver increased ($P < 0.0001$) by ~3-fold with flaxseed oil and by 7-fold with algal DHA supplementations, at the highest level of dietary inclusion, over that in the control (Tables 8.2 and 8.3). In the adipose tissue, both treatment groups deposited DHA but lower ($P < 0.0001$) than the levels observed in the egg yolk or liver (Figure 8.1e and f). As a function of intake, the DHA-fed hens accumulated increasing ($P < 0.0001$, Table 8.4) amounts of DHA in the adipose tissue (~5-fold greater at the highest compared to the least supplementation level of algal DHA). No treatment effect was observed in the level of DHA in the total lipid of adipose tissue for flaxseed oil treatment group and no detectable levels were note in the control group (Table 8.4).

8.4.4 *Fatty acid profile of lipid classes in egg yolk, liver and adipose*

In the major lipid classes (TAG, Table 8.5 and total PL, Table 8.6), ALA was highly contained in the TAG than the total PL of both the egg yolk and liver; its level in the TAG increased as a function of total n-3 fatty acid intake, and reflected a parallel pattern of

Table 8.5: Fatty acid levels (mol%) in triacylglycerol (TAG) of egg yolk, liver and adipose tissue as a function of feeding hens diets containing total omega-3 fatty acids sourced from either flaxseed oil or algal DHA[1]

| | Total omega-3 in diet (%, by wt) | | | | | | | | | Contrasts (P-values) | | |
| | Control | Flaxseed oil (FSO) diets | | | Algal DHA (DHA) diets | | | | | FSO vs. | DHA vs. | FSO vs. |
	0.03	0.20	0.40	0.60	0.20	0.40	0.60	SE	P-values	Control	Control	DHA
Egg yolk												
18:3n-3 (ALA)	0.30^e	1.01^c	1.83^b	2.66^a	0.39^{de}	0.47^{de}	0.51^d	0.042	<0.0001	<0.0001	0.003	<0.0001
18:4n-3 (SDA)	0.040^{cd}	0.051^{bc}	0.063^{ab}	0.067^a	0.037^{cd}	0.035^d	0.037^{cd}	0.003	<0.0001	<0.0001	0.29	<0.0001
20:5n-3 (EPA)	0.002^e	0.012^{de}	0.026^{cd}	0.039^c	0.028^{cd}	0.063^b	0.11^a	0.004	<0.0001	<0.0001	<0.0001	<0.0001
22:5n-3 (DPA)	0.018^e	0.041^d	0.065^{bc}	0.084^{ab}	0.032^{de}	0.049^{cd}	0.089^a	0.005	<0.0001	<0.0001	<0.0001	0.12
22:6n-3 (DHA)	0.056^e	0.12^{de}	0.16^d	0.18^d	0.32^c	0.64^b	1.07^a	0.021	<0.0001	0.0003	<0.0001	<0.0001
Total n-6	11.7	12.2	12.5	12.4	11.9	12.7	11.8	0.40	0.54	0.18	0.41	0.47
Total n-3	0.41^e	1.23^c	2.13^b	3.15^a	0.80^d	1.24^c	1.83^b	0.081	<0.0001	<0.0001	<0.0001	<0.0001
Total SFA	36.0^{cd}	35.0^d	35.3^d	35.6^{cd}	37.0^{bc}	38.0^{ab}	39.1^a	0.34	<0.0001	0.10	<0.0001	<0.0001
Total MUFA	50.3^a	50.1^a	48.6^{ab}	47.3^{bc}	48.9^{ab}	46.6^{bc}	45.8^c	0.60	<0.0001	0.030	0.0001	0.004
Liver												
18:3n-3 (ALA)	0.25^d	0.90^c	1.72^b	2.24^a	0.28^d	0.27^d	0.32^d	0.081	<0.0001	<0.0001	0.66	<0.0001
18:4n-3 (SDA)	0.044^{bc}	0.055^{abc}	0.066^{ab}	0.076^a	0.040^c	0.037^c	0.041^c	0.005	<0.0001	0.002	0.51	<0.0001
20:5n-3 (EPA)	ND	0.004^c	0.005^c	0.011^{bc}	0.014^{bc}	0.024^b	0.041^a	0.004	<0.0001	-	-	-
22:5n-3 (DPA)	0.016	0.030	0.054	0.046	0.005	0.012	0.015	0.011	0.057	0.041	0.668	0.004
22:6n-3 (DHA)	0.26^b	0.27b	0.26^b	0.24^b	0.36^b	0.49^b	0.85^a	0.072	<0.0001	0.97	0.001	<0.0001
Total n-6	9.89	11.3	11.1	11.3	10.2	10.0	9.64	0.53	0.13	0.035	0.91	0.006
Total n-3	0.57^d	1.26^c	2.11^b	2.85^a	0.85^{cd}	0.89^{cd}	1.26^c	0.14	<0.0001	<0.0001	0.011	<0.0001
Total SFA	38.9	36.8	37.0	37.4	38.7	38.5	39.7	0.73	0.082	0.036	0.94	0.004
Total MUFA	47.9	47.7	47.0	45.7	47.6	47.1	45.4	0.94	0.39	0.34	0.28	0.87
Adipose												
18:3n-3 (ALA)	0.82^d	1.55^c	2.16^b	3.67^a	0.81^d	0.81^d	0.92^d	0.072	<0.0001	<0.0001	0.77	<0.0001
18:4n-3 (SDA)	0.064^b	0.073^b	0.080^{ab}	0.096^a	0.068^b	0.074^b	0.082^{ab}	0.005	0.002	0.003	0.076	0.045
20:5n-3 (EPA)	ND	ND	ND	0.002^b	0.010^b	0.030^a	0.039^a	0.006	0.002	-	-	-
22:5n-3 (DPA)	ND	ND	ND	0.001^b	0.002^b	0.005^b	0.011^a	0.001	0.0002	-	-	-
22:6n-3 (DHA)	0.039^d	0.033^d	0.037^d	0.048^d	0.089^c	0.16^b	0.34^a	0.007	<0.0001	0.99	<0.0001	<0.0001
Total n-6	19.5	19.9	19.3	20.2	19.7	18.7	18.7	0.51	0.27	0.67	0.39	0.076
Total n-3	0.92^d	1.66^c	2.28^b	3.42^a	0.98^d	1.10^d	1.40^{cd}	0.12	<0.0001	<0.0001	0.095	<0.0001
Total SFA	29.3	28.5	28.2	28.2	28.6	28.9	30.4	0.51	0.063	0.10	0.97	0.020
Total MUFA	46.8	46.5	46.7	45.1	47.4	47.5	45.8	0.72	0.21	0.39	0.89	0.16

[1]Data are presented as least square means (LSM) ± Pooled SE; n=5. [a-f]Different superscripts between treatments within a row of a fatty acid for each tissue type are significantly different at $P < 0.05$. Contrasts between treatment groups are significant at $P < 0.05$. ND, not detected/only traces found.

Table 8.6: Fatty acid levels (mol%) in total phospholipids (PL) of egg yolk, liver and adipose tissue as a function of feeding hens diets containing total omega-3 fatty acids sourced from either flaxseed oil or algal DHA[1]

| | Control | Flaxseed oil (FSO) diets | | | Algal DHA (DHA) diets | | | | | Contrasts (P-values) | | |
| | | | | | | | | | | FSO vs. | DHA vs. | FSO vs. |
	0.03	0.20	0.40	0.60	0.20	0.40	0.60	SE	P-values	Control	Control	DHA
Egg yolk												
18:3n-3 (ALA)	0.095^d	0.305^c	0.43^b	0.57^a	0.083^d	0.073^d	0.070^d	0.011	<0.0001	<0.0001	0.126	<0.0001
18:4n-3 (SDA)	0.042	0.049	0.044	0.044	0.036	0.038	0.027	0.005	0.077	0.45	0.14	0.004
20:5n-3 (EPA)	0.013^e	0.064^d	0.11^c	0.19^b	0.11^c	0.21^{ab}	0.24^a	0.010	<0.0001	<0.0001	<0.0001	<0.0001
22:5n-3 (DPA)	0.10^e	0.20^c	0.28^b	0.33^a	0.10^e	0.12^{de}	0.16^d	0.009	<0.0001	<0.0001	0.037	<0.0001
22:6n-3 (DHA)	1.82^f	3.86^e	4.55^{de}	5.01^d	7.03^c	9.50^b	11.7^a	0.22	<0.0001	<0.0001	<0.0001	<0.0001
Total n-6	19.7^a	18.8^a	17.5^b	17.0^{bc}	16.4^c	14.7^d	12.8^e	0.24	<0.0001	<0.0001	<0.0001	<0.0001
Total n-3	2.07^f	4.48^e	5.41^{de}	6.15^d	7.36^c	9.94^b	12.2^a	0.22	<0.0001	<0.0001	<0.0001	<0.0001
Total SFA	47.8^d	47.8^d	48.0^{cd}	48.6^{cd}	48.8^{bc}	49.5^{ab}	50.2^a	0.20	<0.0001	0.15	<0.0001	<0.0001
Total MUFA	27.4^a	27.2^{ab}	27.4^a	26.8^{ab}	25.4^b	23.5^c	21.7^d	0.41	<0.0001	0.60	<0.0001	<0.0001
Liver												
18:3n-3 (ALA)	0.067^d	0.18^c	0.28^b	0.35^a	0.072^d	0.038^d	0.051^d	0.010	<0.0001	<0.0001	0.25	<0.0001
18:4n-3 (SDA)	0.039	0.047	0.046	0.044	0.044	0.047	0.036	0.005	0.56	0.20	0.51	0.39
20:5n-3 (EPA)	0.032^d	0.12^{cd}	0.23^{bc}	0.42^a	0.15^{cd}	0.25^{bc}	0.36^{ab}	0.034	<0.0001	<0.0001	<0.0001	0.96
22:5n-3 (DPA)	0.092^c	0.16^{bc}	0.28^a	0.35^a	0.077^c	0.11^{bc}	0.19^b	0.019	<0.0001	<0.0001	0.15	<0.0001
22:6n-3 (DHA)	2.00^f	4.33^e	5.44^{de}	5.80^d	7.59^c	9.20^b	10.9^a	0.28	<0.0001	<0.0001	<0.0001	<0.0001
Total n-6	22.7^a	22.1^{ab}	20.8^{bc}	20.2^c	19.7^c	18.1^d	15.7^e	0.33	<0.0001	<0.0001	<0.0001	<0.0001
Total n-3	2.23^f	4.83^e	6.27^d	6.99^{cd}	7.93^c	9.64^b	11.5^a	0.27	<0.0001	<0.0001	<0.0001	<0.0001
Total SFA	46.6	46.4	46.1	46.8	46.6	47.0	47.3	0.25	0.082	0.51	0.25	0.019
Total MUFA	22.8^{ab}	22.6^{ab}	23.1^a	22.3^{ab}	21.7^{ab}	21.0^{bc}	19.5^c	0.42	<0.0001	0.82	0.0002	<0.0001
Adipose												
16:0 (palmitic)	37.4	35.5	35.8	37.2	38.1	36.1	38.3	2.03	0.94	0.58	0.98	0.43
16:1 (palmitoleic)	0.64	0.51	0.56	0.54	0.69	0.73	0.33	0.14	0.53	0.55	0.73	0.71
18:0 (stearic)	39.1	36.4	34.6	37.1	32.5	34.6	38.2	3.16	0.78	0.40	0.28	0.72
18:1 (oleic)	12.4	13.2	14.0	11.0	13.7	15.2	11.8	1.19	0.23	0.78	0.39	0.41
18:2n-6 (LA)	6.89	8.61	8.31	6.36	6.52	8.21	7.90	0.93	0.43	0.44	0.57	0.78

[1]Data are presented as least square means (LSM) ± Pooled SE; n=5. [a-f]Different superscripts between treatments within a row of a fatty acid for each tissue type are significantly different at $P < 0.05$. Contrasts between treatment groups are significant at $P < 0.05$.

accumulation between the two tissues, particularly for the flaxseed oil-fed hens (Figures 8.2a and b). The level of ALA in the total PL increased in both the egg yolk and the liver as a function of ALA intake for the flaxseed oil-fed hens (supplied the ALA-rich diets, Table 8.6); however, the rate of accumulation of ALA (mol%) in the liver (regression equation, $ALA_{liver\_PL} = 0.0663 + 0.4914x$; $R^2 = 0.97$, $P < 0.01$) was lower ($P < 0.0001$, Figure 8.3a) compared to that in the egg yolk (regression equation, $ALA_{yolk\_PL} = 0.1015 + 0.8079x$; $R^2 = 0.97$, $P < 0.05$). Similar accumulation trends of ALA in the egg yolk versus liver were observed in the PtdCho and PtdEtn fractions (Figures 8.4a - b and 8.5a - b, respectively). In the adipose tissue, as a function of total n-3 fatty acid intakes with either flaxseed oil- or algal DHA supplementation, the n-3 PUFA were only observed to accumulate in the TAG and not in the total PL or the subclasses PtdCho and PtdEtn (Tables 8.5 – 8.7; Figures 8.3 – 8.5). In the adipose tissue, where the content of ALA was greater than that in the egg yolk or liver, ALA accumulated as a function of intake and paralleled the pattern of accumulation of this fatty acid in the other two tissues for both the flaxseed oil- and DHA-fed hens (Figures 8.2a and b).

The LCPUFA (EPA, DPA and DHA) dominated the total PL pool of both tissues (egg yolk and liver) in the flaxseed oil- as well as the algal DHA-treatment group. As a function of total n-3 fatty acid intake, the EPA content in the egg was greatly increased in all lipid classes (TAG, total PL and PtdCho; $P < 0.0001$) except in the PtdEtn ($P = 0.97$) with the dietary supplementation of algal DHA compared to flaxseed oil (Tables 8.5 – 8.7). The greatest proportional increase ($P < 0.0001$) in the level of EPA in the yolks obtained from the DHA-fed hens over that of the flaxseed oil-fed hens was noted in the TAG (61%) than the total PL (33%) fraction (Table 8.5), with the overall averages being, for TAG, 0.056 vs. 0.022 ± 0.002 and, for
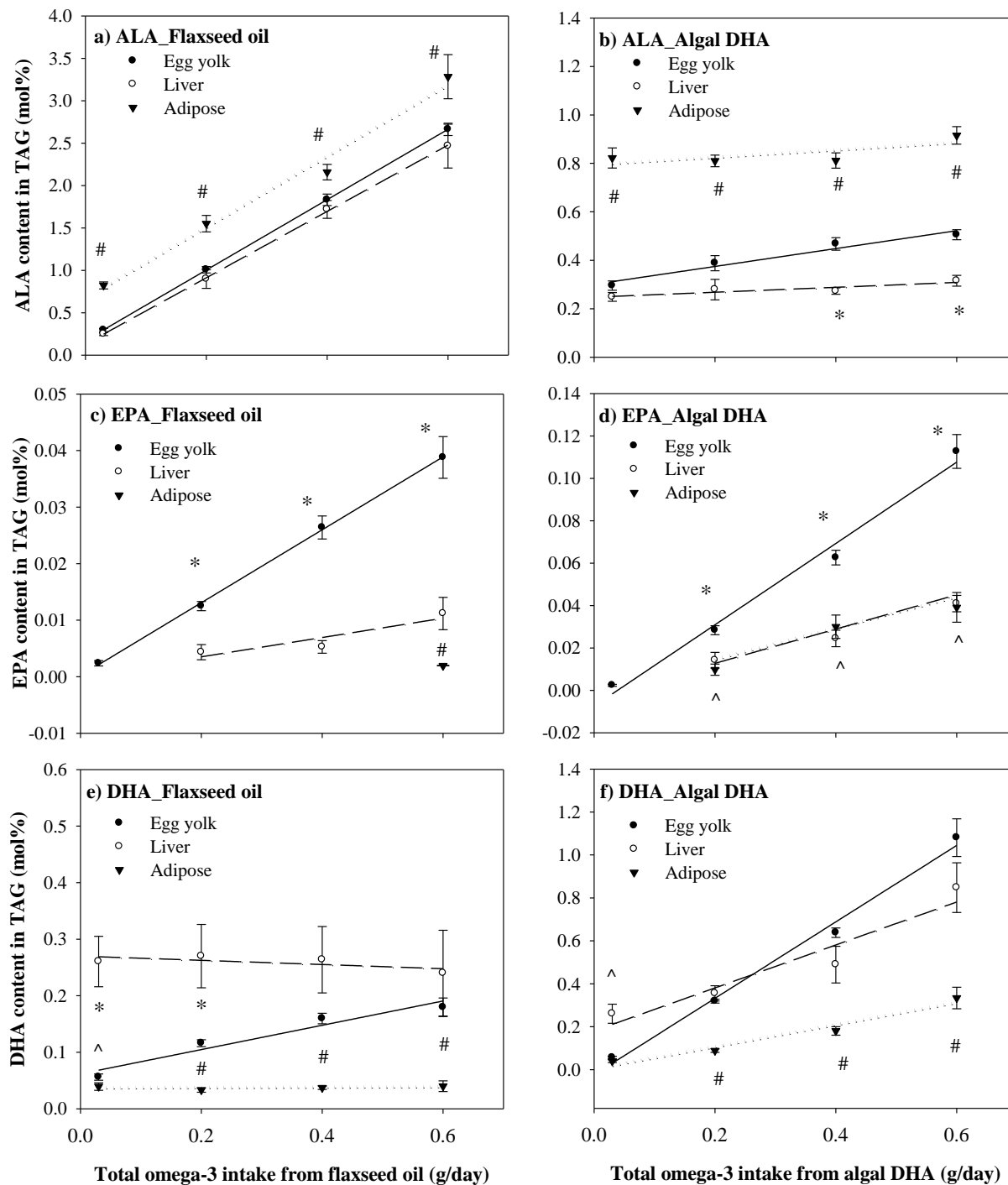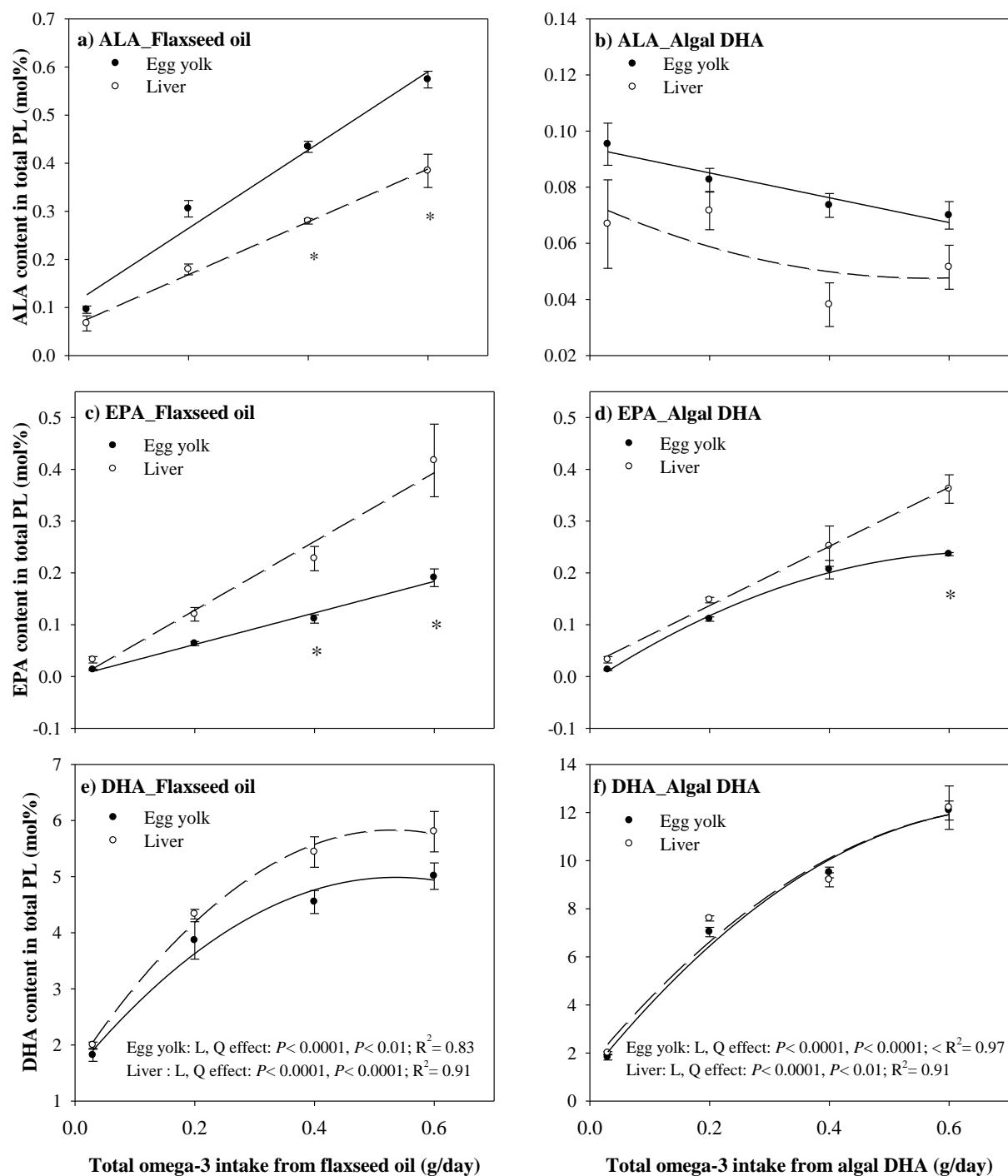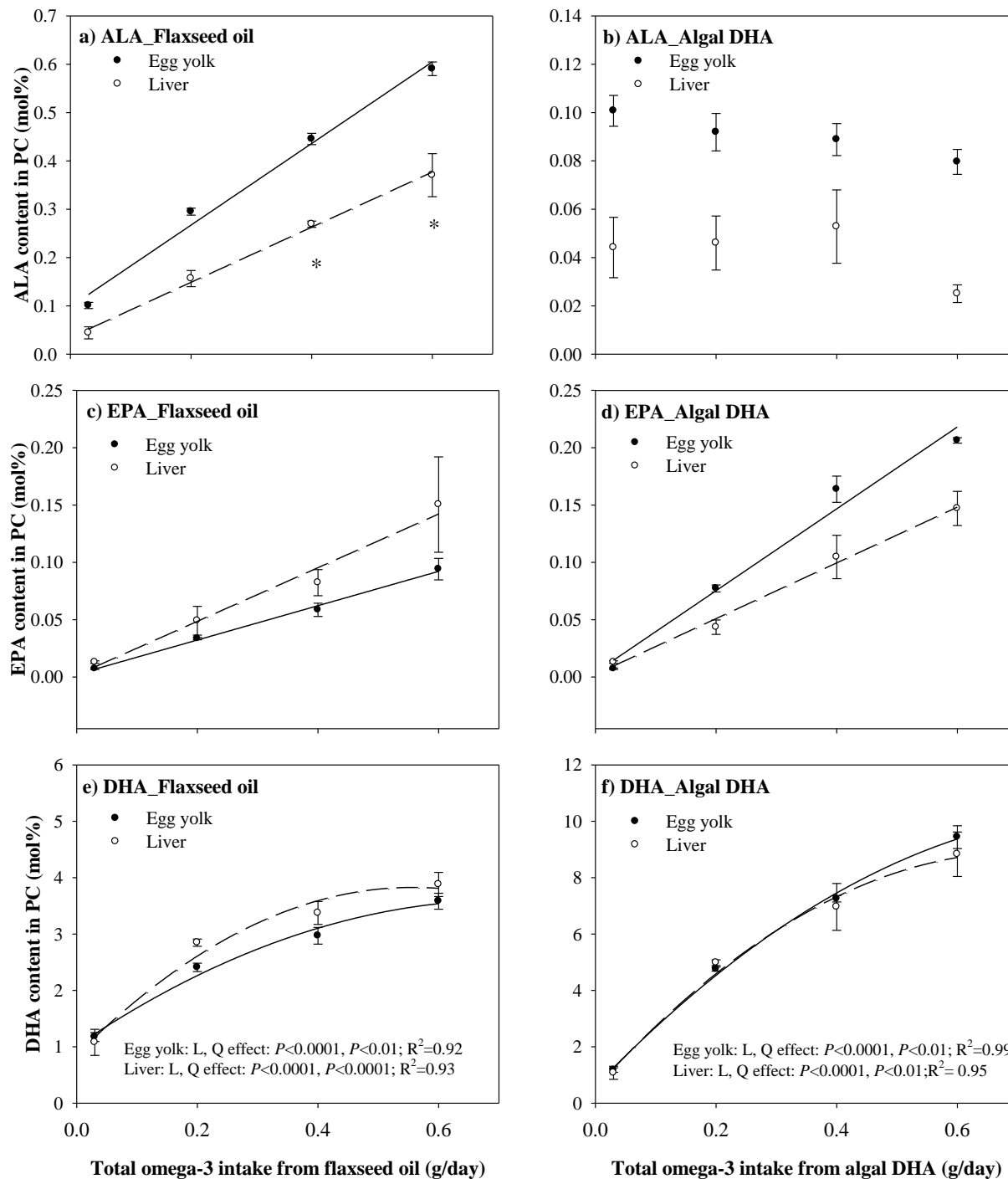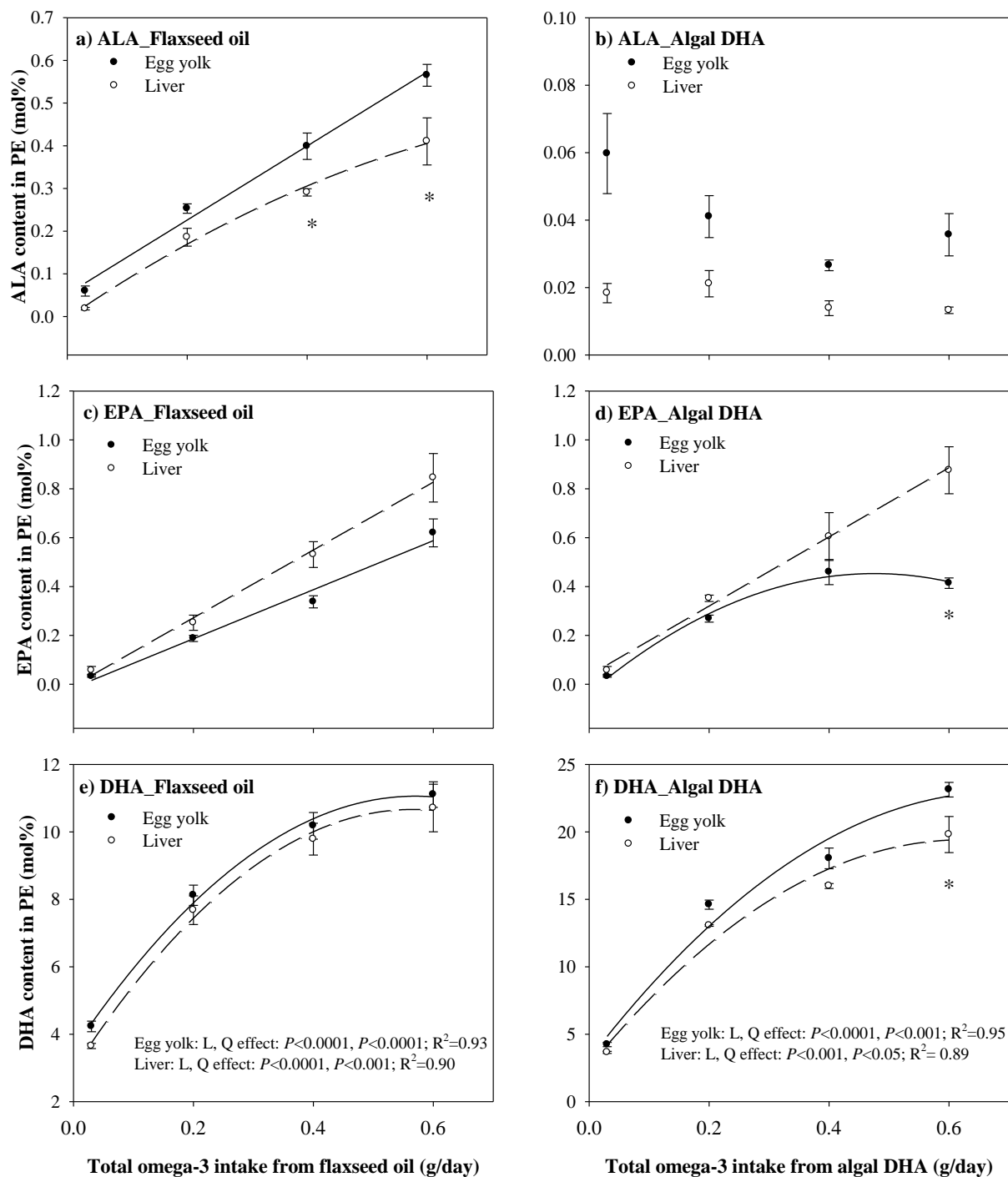
Figure 8.2. Effect of either flaxseed oil or algal DHA sources of total omega-3 fatty acids in diets of hens on the accumulations pattern of (a-b) alpha-linolenic acid, ALA; (c-d) eicosapentaenoic acid, EPA; and (e-f) docosahexaenoic acid, DHA levels in the triacylglycerol (TAG) of egg yolk, liver and adipose tissue. Each data point (n = 5) are treatment means ± SE. Symbols within each plot are significantly different at $P < 0.05$; #, fatty acid in the liver and egg yolk differ from adipose;*, fatty acid in the liver differ from egg yolk; ^ liver or egg yolk differ from adipose.

Figure 8.3. Effect of either flaxseed oil or algal DHA sources of total omega-3 fatty acids in diets of hens on the accumulations pattern of (a-b) alpha-linolenic acid, ALA; (c-d) eicosapentaenoic acid, EPA; and (e-f) docosahexaenoic acid, DHA levels in the total phospholipids (PL) of egg yolk and liver.

Each data point (n = 5) are treatment means ± SE. Symbols within each plot are significantly different at $P < 0.05$; *, fatty acid in the liver differ from egg yolk. L, Q denote linear and quadratic, respectively.

Figure 8.4. Effect of either flaxseed oil or algal DHA sources of total omega-3 fatty acids in diets of hens on the accumulations pattern of (a-b) alpha-linolenic acid, ALA; (c-d) eicosapentaenoic acid, EPA; and (e-f) docosahexaenoic acid, DHA levels in the phosphatidylcholine (PtdCho) of egg yolk and liver.
Each data point (n = 5) are treatment means ± SE. Symbols within each plot are significantly different at $P < 0.05$; *, fatty acid in the liver differ from egg yolk. L, Q denote linear and quadratic, respectively.

Figure 8.5. Effect of either flaxseed oil or algal DHA sources of total omega-3 fatty acids in diets of hens on the accumulations pattern of (a-b) alpha-linolenic acid, ALA; (c-d) eicosapentaenoic acid, EPA; and (e-f) docosahexaenoic acid, DHA levels in the phosphatidylethanolamine (PtdEtn) of egg yolk and liver. Each data point (n = 5) are treatment means ± SE. Symbols within each plot are significantly different at $P < 0.05$; *, fatty acid in the liver differ from egg yolk. L, Q denote linear and quadratic, respectively.

Table 8.7: Omega-3 fatty acid levels (mol%) of phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) in egg yolk and liver as a function of feeding hens diets containing total omega-3 fatty acids sourced from either flaxseed oil or algal DHA[1]

	Control	Flaxseed oil (FSO) diets			Algal DHA (DHA) diets			SE	P-values	FSO vs. Control	DHA vs. Control	FSO vs. DHA
	0.03	0.20	0.40	0.60	0.20	0.40	0.60					
Phosphatidylcholine												
Egg yolk												
18:3n-3 (ALA)	0.10^d	0.30^c	0.45^b	0.59^a	0.092^d	0.089^d	0.080^d	0.009	<0.0001	<0.0001	0.19	<0.0001
18:4n-3 (SDA)	0.043	0.052	0.054	0.052	0.045	0.048	0.042	0.003	0.10	0.023	0.68	0.010
20:5n-3 (EPA)	0.007^f	0.034^{ef}	0.059^{de}	0.094^c	0.077^{cd}	0.16^b	0.21^a	0.007	<0.0001	<0.0001	<0.0001	<0.0001
22:5n-3 (DPA)	0.054^e	0.11^c	0.17^b	0.21^a	0.069^{de}	0.088^{cd}	0.12^c	0.007	<0.0001	<0.0001	<0.0001	<0.0001
22:6n-3 (DHA)	1.17^g	2.41^f	2.97^e	3.58^d	4.76^c	7.25^b	9.05^a	0.12	<0.0001	<0.0001	<0.0001	<0.0001
Liver												
18:3n-3 (ALA)	0.044^d	0.16^c	0.27^b	0.33^a	0.046^d	0.053^d	0.025^d	0.012	<0.0001	<0.0001	0.83	<0.0001
18:4n-3 (SDA)	0.039	0.039	0.043	0.057	0.042	0.036	0.037	0.007	0.45	0.40	0.91	0.20
20:5n-3 (EPA)	0.013^c	0.049^{bc}	0.082^{abc}	0.12^{ab}	0.044^{bc}	0.11^{abc}	0.15^a	0.018	0.0005	0.019	0.005	0.29
22:5n-3 (DPA)	0.053^c	0.084^{bc}	0.14^{ab}	0.18^a	0.038^c	0.060^c	0.087^{bc}	0.014	<0.0001	<0.0001	0.61	<0.0001
22:6n-3 (DHA)	1.31^e	2.85^d	3.37^d	3.88^{cd}	4.98^{bc}	6.17^b	9.41^a	0.29	<0.0001	<0.0001	<0.0001	<0.0001
Phosphatidylethanolamine												
Egg yolk												
18:3n-3 (ALA)	0.060^d	0.25^c	0.40^b	0.57^a	0.041^d	0.027^d	0.036^d	0.017	<0.0001	<0.0001	0.21	<0.0001
18:4n-3 (SDA)	ND	ND	ND	ND	ND	ND	ND	-	-	-	-	-
20:5n-3 (EPA)	0.032^e	0.19^{de}	0.34^{bcd}	0.62^a	0.27^{cd}	0.46^b	0.41^{bc}	0.036	<0.0001	<0.0001	<0.0001	0.97
22:5n-3 (DPA)	0.25^d	0.44^c	0.66^b	0.77^a	0.22^d	0.24^d	0.28^d	0.022	<0.0001	<0.0001	0.999	<0.0001
22:6n-3 (DHA)	4.23^f	8.12^e	10.2^d	11.1^d	14.6^c	18.0^b	23.1^a	0.45	<0.0001	<0.0001	<0.0001	<0.0001
Liver												
18:3n-3 (ALA)	0.018^d	0.19^c	0.29^b	0.34^a	0.021^d	0.014^d	0.013^d	0.006	<0.0001	<0.0001	0.71	<0.0001
18:4n-3 (SDA)	0.039	0.038	0.041	0.048	0.041	0.029	0.033	0.007	0.47	0.70	0.49	0.15
20:5n-3 (EPA)	0.057^d	0.25^{cd}	0.53^{bc}	0.98^a	0.35^{cd}	0.61^{bc}	0.88^{ab}	0.081	<0.0001	<0.0001	<0.0001	0.72
22:5n-3 (DPA)	0.18^{bc}	0.29^b	0.44^a	0.51^a	0.11^c	0.14^c	0.19^{bc}	0.026	<0.0001	<0.0001	0.32	<0.0001
22:6n-3 (DHA)	3.65^f	7.68^e	9.79^d	10.7^d	13.1^c	16.0^b	18.7^a	0.46	<0.0001	<0.0001	<0.0001	<0.0001

[1]Data are presented as least square means (LSM) ± Pooled SE; n=5. [a-g]Different superscripts between treatments within a row of each fatty acid for a tissue type are significantly different at $P < 0.05$. Contrasts between treatment groups are significant at $P < 0.05$. ND, not detected/only traces found.

total PL, 0.152 vs. 0.102 ± 0.005 % total fatty acids, respectively (data not presented). In both

treatment groups, lower levels of EPA accumulated in the TAG of the liver than that observed in

the egg yolk (Figure 8.2c and d). In addition, although no marked differences were observed in

the level of EPA in the liver TAG as a function of flaxseed oil intake, the fatty acid accumulated

in a linear manner ($P < 0.01$, $R^2 = 0.88$) in the egg yolk. Similarly, in the DHA-fed hens, linear

accumulation of EPA was also observed in the egg TAG ($P < 0.05$, $R^2 = 0.94$). No detectable

level of EPA was noted in the liver TAG of the control diet-fed hens (Table 8.5; Figure 8.2c and

d). In the total PL pool, EPA was deposited in the egg yolk at lower rate (lower regression

coefficient, $P < 0.0001$) ($EPA_{yolk\_PL} = 0.3273x + 0.0010$; $R^2 = 0.98$, $P < 0.01$) compared to that

in the liver ($EPA_{liver\_PL} = 0.7135x - 0.0051$; $R^2 = 0.96$, $P < 0.05$) for flaxseed oil-fed hens (Figure

8.3c); similar trends were observed in the subclasses (PtdCho and PtdEtn, Figures 8.4 and 8.5,

respectively), reflecting that in the total lipids of both tissues (Figure 1a). As such, EPA in the

total PL was observed to accumulate in a linear trend for flaxseed oil-fed hens ($P < 0.05$, $R^2 =$

0.89), however, for the DHA-fed hens, both linear ($P < 0.0001$) and quadratic ($P < 0.001$), $R^2 =$

0.95 responses were observed (Table 8.6, Figures 8.3d). In addition, a lower rate of

accumulation (lower regression coefficient) of ALA in the PL pool in the liver ($ALA_{liver\_PL} =$

$0.4914x + 0.0663$; $R^2 = 0.97$, $P < 0.01$, Figure 8.3a) compared to the level of EPA accumulating

in the liver of the same hens ($EPA_{liver\_PL} = 0.7135x - 0.0051$; $R^2 = 0.96$, $P < 0.05$; Figure 8.3c),

was observed for the flaxseed oil-fed hens. This relationship was highly significant ($P < 0.0001$)

in the liver PtdEtn fraction. Furthermore, the similarity ($P = 0.058$) in the concentration of EPA

in the liver total lipid, aforementioned, between the preformed DHA- vs. flaxseed oil-fed the

hens (Table 8.3), was more evident in the liver PtdEtn fraction ($P = 0.72$, Table 8.7) of the

flaxseed-fed hens ($EPA_{liver\_PtdEtn} = 1.4573x - 0.0052$; $R^2 = 0.99$, $P < 0.01$; Figure 8.5c) vs. the

DHA-fed hens (EPA$_{liver\_PtdEtn}$ = 1.4590x + 0.0429; R^2 = 0.99, P < 0.01; Figure 8.5d). By

contrast, there was a lack of difference (P = 0.97, Table 8.7) in the amount of EPA deposited in

the egg yolk PtdEtn pool between the flaxseed oil- and DHA-fed hens, particularly at the highest

total n-3 fatty acid intake (0.60% by wt of diet). In the adipose TAG, although EPA was only

detected at the highest level of flaxseed oil inclusion in the diets of hens, it was incorporated in a

similar trend to that in the liver for the DHA-fed hens. Three-fold more EPA accumulated in the

adipose TAG at the highest compared to the least level of total n-3 PUFA intake (0.60 vs. 0.20 %

by wt of diet) when algal DHA was supplemented.

The SDA level in TAG of egg yolk (P < 0.0001), liver (P < 0.01) or adipose tissue (P <

0.01) increased as a function of flaxseed oil intake, particularly at higher levels, compared to that

in the control or DHA-fed hens (Table 8.5). No treatment effects where observed in the level of

SDA in the total PL (Table 8.6) and PtdCho fraction (Table 8.7) in both the egg yolk and liver of

either the flaxseed oil- or DHA-fed hens; in addition, this fatty acid was not detected in the liver

PE of both treatment groups (Table 8.7). Docosahexaenoic acid, endogenously synthesized

(from flaxseed oil) and preformed (from algal DHA) was observed to accumulate in the TAG

fraction of all tissues (egg yolk, liver and adipose tissues, Tables 8.5). In all tissues (egg yolk,

liver and adipose), DHA content in the TAG increased (P < 0.0001) by ~4 to 10-fold more with

algal DHA compared to the flaxseed oil supplementation in the laying hen diets (Tables 8.5).

Although the level DHA in the TAG of the liver and adipose tissue was not influenced by dietary

level of ALA (flaxseed oil supplementation) used in this study (Table 8.5), a significant linear

increase in the content of this fatty acid was observed in the yolk TAG for flaxseed oil-fed hens

(P < 0.001, R^2 = 0.82) and DHA-fed hens (P < 0.01, R^2 = 0.95). Both treatment groups

deposited proportionally less DHA in the adipose compared to levels in either the egg yolk or the

liver (Figure 8.2e and f). In all the PL fractions (total PL, PtdCho and PtdEtn), DHA was observed to accumulate in a linear ($P < 0.001$) and quadratic ($P < 0.05$) manner ($0.83 < R^2 < 0.83$) in the egg yolk and liver in both treatment groups (Figure 8.3 – 8.5e and f). Despite the plateau as a function of total n-3 PUFA intake by the hens in both treatment groups, greater (2-fold more, $P < 0.0001$) DHA accumulated in all the PL fractions in both tissues of the DHA-fed hens over that of flaxseed oil-fed hens (Tables 8.6 and 8.7). Except in the PtdEtn of the DHA-fed hens, levels of DHA in the egg yolk closely reflected those in the liver (Figure 8.5f). Docosapentaenoic acid levels in the TAG of the egg yolk obtained from the flaxseed oil-fed hens followed similar trends as those for EPA (Table 8.5). The level of DPA in the TAG of the liver was not influenced by diet ($P < 0.057$; Table 8.5). However, comparing the least vs. the highest levels of the DHA supplementations, the TAG pool of the adipose tissue preferentially deposited DPA which increased by 10-fold compared to a 4-fold increase in the incorporation of DHA in the same group of hens (Table 8.5). The accumulation of DPA in the PL fractions (Tables 8.6 and 8.7) was noted to be more dependent on the levels of dietary ALA in this study, as the algal DHA product only contained traces of the preformed DPA.

While the level of total SFA in the egg yolk TAG and PL was increase ($P < 0.0001$) with the preformed DHA treatment compared to either the control or the flaxseed oil-fed hens, the MUFA content significantly decrease in both the TAG ($P < 0.05$) and the PL ($P < 0.0001$) in the egg of the former than in the latter groups of hens (Table 8.5 and 8.6, respectively). The level of LA in the liver PL, closely reflected the dietary levels of the fatty acid (included in the range of 1.23 ± 0.12 (SD) % of diet), and it appeared to be slightly lower for the highest algal DHA containing diet compared to the control or the flaxseed containing diets (Table 8.1). Although there was no significant difference in the content of LA in liver PL between the flaxseed oil- and

the control diet-fed groups, the accumulation of lower level of this fatty in the liver PL of the DHA-fed hens ($P < 0.0001$), particularly at the highest level of inclusion (0.60% by wt of diet), was reflected in a similar trend of reduction ($P < 0.0001$) in the arachidonic acid (ARA, 20:4n-6) level in the same pool of both the liver and the egg yolk (Appendix IV). However, the level of ARA was significantly increased in the TAG of the egg yolk and the adipose of the algal DHA supplemented hens but not for the flaxseed oil-fed hens (Appendix V). Both n-3 fatty acid sources reduced ($P < 0.0001$) the total n-6 PUFA content of the egg yolk and liver total PL fraction (Table 8.6); however, the levels in the TAG in both tissues were not affected by the source or level of dietary treatment (Table 8.5).

8.4.5   *Expression of key hepatic genes involved in lipid metabolism*

The feeding regimens did not influence the hepatic expression of the fatty acid binding protein gene 1 (FABP1; Table 8.8), but all other investigated genes were differentially expressed in response to the diets. Overall, one upregulation and seventeen downregulations were observed (Table 8.8). A 167% upregulation was observed for the ACSL1 gene (acyl-CoA synthetase long-chain family member 1) after the 0.60% algal-DHA feeding. In contrast, ACSL1 was downregulated throughout all levels of flaxseed oil (-0.49% at 0.20% FSO; -0.37% at 0.40% FSO; -0.59% at 0.60% FSO). Peroxisome proliferator-activated receptor alpha (PPARα) was downregulated by 52 and 69% after 0.60% flaxseed oil and algal DHA feeding, respectively but not at lower levels of either source. The mRNA expressions for carnitine palmitoyltransferase 1A (CPT1A)*,* acyl-CoA oxidase 1 (ACOX1)*,* and fatty acid desaturase 2 (FADS2) were downregulated by 68%, 54% and 67%, respectively, in response to the 0.60% algal-DHA feeding, a phenomenon not observed for the lower algal-DHA or all flaxseed oil diets.

Table 8.8: Differential expression of transcripts of hepatic genes involved in fatty acid transport/activation, β-oxidation and synthesis as a function of hen diets[1]

		Total omega-3 in diet (%, by wt)								Contrast (P-values)		
	Control	Flaxseed oil (FSO) diets			Algal DHA (DHA) diets					FSO vs.	DHA vs.	FSO vs.
	0.03	0.20	0.40	0.60	0.20	0.40	0.60	SE	P-values	Control	Control	DHA
Fatty acid transport/activation[2]												
ACSL1	1.00[bc]	0.51[cd]	0.63[cd]	0.41[d]	0.81[bcd]	1.25[b]	2.67[a]	0.14	<0.0001	<0.01	0.001	<0.0001
FABP	1.00	0.72	0.76	0.62	0.67	0.72	0.93	0.11	0.13	<0.05	0.066	0.41
Fatty acid β-oxidation[3]												
PPARα	1.00[a]	0.83[ab]	0.56[abc]	0.48[bc]	0.69[abc]	0.70[abc]	0.31[c]	0.11	0.001	<0.01	0.001	0.53
CPT1A	1.00[ab]	0.52[b]	1.50[a]	0.70[ab]	1.06[ab]	1.16[ab]	0.32[b]	0.21	<0.01	0.68	0.52	0.75
ACOX1	1.00[a]	0.94[ab]	0.96[ab]	0.60[ab]	0.76[ab]	0.63[ab]	0.46[b]	0.13	<0.05	0.23	0.010	0.053
Fatty acid synthesis[4]												
FADS1	1.00[a]	0.78[ab]	0.86[ab]	0.58[bc]	0.74[ab]	0.75[ab]	0.27[c]	0.090	<0.0001	<0.05	0.0001	<0.05
FADS2	1.00[a]	0.69[ab]	0.80[a]	0.74[ab]	0.77[a]	1.12[a]	0.33[b]	0.10	<0.0001	<0.05	<0.05	0.95
ELOVL2	1.00[a]	0.53[bc]	0.63[b]	0.43[bc]	0.57[bc]	0.61[bc]	0.27[c]	0.080	<0.0001	<0.0001	<0.0001	0.53
ELOVL5	1.00[a]	0.70[abc]	0.63[abc]	0.60[bc]	0.84[ab]	0.78[abc]	0.43[c]	0.092	<0.01	0.001	<0.01	0.61

[1]Target genes in either treatment groups were normalized to the housekeeping gene (β-actin) and expression levels were reported as a fold change of the expression of a target gene for hens fed diets containing 0.20, 0.40 and 0.60 % total omega-3 FA compared to hens fed the control diet (as a calibrator), which is assigned a value of one. Values are means ± SE, n = 10. Different superscripts within a row are significantly different, $P < 0.05$. Contrasts between treatment groups are significant at $P < 0.05$.
[2]ACSL1 = acyl-CoA synthetase long-chain family member 1; FABP = fatty acid binding protein 1.
[3]PPARα = peroxisome proliferator-activated receptor alpha; CPT1A = carnitine palmitoyltransferase 1A; and ACOX1 = acyl-CoA oxidase 1.
[4]FADS1 = fatty acid desaturase 1; FADS2 = fatty acid desaturase 2; ELOVL2 = ELOVL fatty acid elongase 2; and ELOVL5 = ELOVL fatty acid elongase 5.

Decreased expression of fatty acid desaturase 1 (FADS1) were observed at 0.60% flaxseed oil (-42%) and 0.60% algal-DHA (-73%). Similarly, ELOVL5 expression was decreased at 0.60% flaxseed oil (-40%) and 0.60% algal-DHA (-57%). Differential gene expression was not observed for other dietary groups. Consistent downregulation for all feeding regimens were observed for ELOVL2 (-0.47% at 0.20% flaxseed oil; -0.37% at 0.40% flaxseed oil; -0.57% at 0.60 % flaxseed oil; -0.43% at 0.20% algal-DHA; -0.39% at 0.40% algal-DHA; -0.73% at 0.60% algal-DHA).

8.5 DISCUSSION

The aim of this study was to investigate the effect of dietary sources of n-3 PUFA, supplied as either the primary precursor ALA (flaxseed oil) or as preformed DHA (algal DHA) on the fatty acid composition of total lipid and major lipid classes in the egg yolk, liver and adipose tissue. Furthermore, the impact of dietary n-3 source and levels on the gene expression of key hepatic enzymes involved in lipid metabolism was investigated, in order to understand the mechanism(s) regulating n-3 LCPUFA deposition in chicken eggs. The results indicated that the accumulation of DHA in egg yolk is influenced by the source of dietary n-3 fatty acids. As such, the potential control points and the subsequent overall impacts derived from the results are discussed in subsections below.

8.5.1   *Maintaining levels of SDA and EPA in the liver*

The conversion of dietary ALA into EPA and DHA is limited (Brenna, 2002; Blank et al., 2002; Burdge, 2004; Goyens et al., 2006; Fraeye et al., 2012). In human studies, the efficiency of conversion of ALA to EPA to DPA and then to DHA was observed to be ~0.20%, 3.65% and 37%, respectively (Pawlosky et al., 2001; Plourde and Cunnane, 2007), indicating a tighter

regulation in the first phase of the metabolic pathway of the LCPUFA. This is consistent with reports from other studies (Yamazaki et al., 1992; Brenna, 2002; Burdge, 2004), which suggest that the initial Δ6-desaturase (FADS2) reaction involved in the conversion of ALA to SDA is the rate-limiting step. Stearidonic acid is primarily derived from ALA. However, in this study, the trend toward the similarity in the level of SDA in the liver lipid as a function of total n-3 fatty acid intake observed between the flaxseed oil- and DHA-fed hens, was surprising given the low level of ALA (basal diet) used in the latter treatment group. In this regard, the conversion of ALA to SDA, may not be the limiting step, confirming previous suggestions (Goyens et al., 2005), since the low level of ALA (in the algal DHA containing diets) could be converted to SDA at a similar rate as the ALA-rich containing diet (with flaxseed oil) and being highest with the control. This result may point out a separate limiting factor, possibly aimed at maintaining certain levels of SDA in the liver. As such, it was noted that the level of SDA in the liver lipid, in both the flaxseed oil- and DHA-fed groups, was closely associated and possibly regulated by the prevailing concentration of LCPUFA in the same pool, particularly EPA (r = -0.76; $P < 0.0001$) and to some extent DHA (r = -0.49; $P < 0.0001$) (Suppl. data, Figure S1). These results are in agreement with previous studies (Raz et al., 1998; Emken et al., 1999; Vermunt et al., 2000) indicating inhibitory effects of EPA and DHA on the conversion efficiency of the short chain omega-3 PUFA to subsequent longer chain metabolites. This would potentially indicate a putative control point in maintaining the concentration of SDA and therefore regulating the concentrations of LCPUFA in the tissues of the hen.

With regards to EPA levels, as a function of total dietary n-3 fatty acid intake supplied in the hens' diets utilising either flaxseed oil or algal DHA (which also contained EPA), the hens incorporated similar but increasing levels of EPA in the liver lipid (reflective of the trend in the

total PL, particularly the PtdEtn fraction). Although the regulation of LCPUFA metabolism is poorly understood, dietary PUFA (ALA and preformed LCPUFA) undergoes several metabolic fates in order to maintain lipid homeostasis in the body (Jump, 2008). Therefore, to maintain these dynamics (levels of SDA and EPA), the liver, as a regulatory tissue, can influence the incorporation of fatty acids in other tissues (Kinsella et al., 1990) and partitioning them into different lipid pools (Nguyen et al., 2008).

8.5.1.1 Shunting of fatty acids to the adipose tissue

In the current study, the adipose tissue, a potential route of storage of dietary ALA (Tang et al., 1993), acting as a disposal of an overflow of ALA in the egg yolk, accumulated 3 to 4-fold more ALA than either the egg yolk (confirming the observations of others; Elkin et al., 2015) or liver in both the flaxseed oil and the algal DHA supplemented groups. This may present a potential step in regulating the flux of dietary ALA, resulting in a low level of ALA entering the LCPUFA biosynthetic pathway with ultimate conversion to SDA and then to EPA, confirming earlier suggestions (Burdge, 2004). This in itself may potentially be beneficial, whereby the endogenously synthesised LCPUFA like its preformed counterpart, possibly attains physiological levels or serves to support membrane turnover (Burdge, 2004; Barceló-Coblijn and Murphy, 2009). Taken together, these results may support suggestions that the biological activity of ALA and/or SDA may closely relate to their conversion to EPA (Calder, 2012).

In the current study, it was also observed that the preformed (using algal DHA) rather than the endogenously synthesised (using precursor ALA) LCPUFA (EPA, DPA and DHA) were greatly deposited into the adipose tissue of the hens. This was particularly noted at higher levels of algal DHA supplementation in the hen diets. This may be a potential control point aimed at reducing excessive flux of the preformed LCPUFA from accumulating in the liver and

transporting them to different tissues including the adipose tissue (Ellis et al., 2011). Although

DPA (preformed in the algal DHA product) was supplemented at a comparative low level, it was

highly incorporated into the adipose tissue of the algal DHA-fed hens, accounting for 2 to 3-fold

more DPA than the amount of DHA or EPA, respectively incorporated in the same tissue. This

may support the important intermediary role for DPA (Tapiero et al., 2002), not only as a storage

form of the LCPUFA metabolites (EPA and DHA) but also as an efficient control point, possibly

in reducing EPA level, thus regulating the levels of these fatty acids.

8.5.1.2 Partitioning fatty acids into different lipid pools

As a control measure, it also seems likely that the liver not only has the ability of shifting

excess LCPUFA (EPA, DPA, and DHA) to the adipose tissue but is also involved in the

partitioning of these fatty acids into different lipid classes (Nguyen et al., 2008) as a means of

regulating fatty acid levels in the body (Sprecher, 2000). The use of preformed LCPUFA (algal

DHA) in the hen diets showed a greater (2 to 6-fold more) capacity to enhance the incorporation

of these fatty acids (EPA and DHA) in the TAG pool of the egg yolk compared to those observed

with the precursor ALA-fed hens. Furthermore, in the DHA-fed hens, EPA, DPA and DHA

were readily incorporated in the TAG of adipose tissue (TAG-rich tissue) as a function of total n-

3 fatty acid intake. Interestingly, the accumulation of these LCPUFA in the TAG rather than the

PL pool of the adipose tissue, as would be the case in the liver and egg yolk, observed in the

current study and previous studies (Jiang et al., 1991; Neijat et al., 2016a), may indicate that their

role is principally as an energy source. Eicosapentaenoic acid (as well as DPA) unlike DHA

derived from flaxseed oil is poorly incorporated into the TAG pool not only in the adipose tissue

but also in the liver, particularly at lower levels of the ALA containing diets. The latter

observation is in accordance with our previous findings (Neijat et al., 2016b) which showed low

incorporation of EPA in the hepatic TAG fraction at low level of dietary ALA intake from hempseed/hempseed oil. The reason for this poor acylation of EPA into the hepatic TAG is because it is regarded as a poor substrate for the enzyme (diacylglycerol acyltransferase) involved in the esterification of diacylglycerol in the synthesis of TAG (Berge et al., 1999). However, from the current results, it may follow that synthesised EPA (derived from ALA) in the liver is not sufficient to saturate the PL pool, indicating that the shift to store LCPUFA in the TAG fraction arises as a means to alleviate the burden of these fatty acids overloading the PL pool, based on the fact that the PUFA content in the sn-2 position of the PL cannot exceed a certain level of accumulation (Schreiner et al., 2004).

Polyunsaturated fatty acids synthesized in the liver are metabolised into various lipid classes including PL and TAG (Nguyen et al., 2008), initially requiring the activation of fatty acids to their acyl-coenzyme A (acyl-CoA) derivatives (Digel et al., 2009). Hence, the enzymes (ACSL isoforms) activating these fatty acids do not only have the capacity to synthesize these complex glycerolipids (TAG and PL) but also control the flux of acyl-CoAs that enter different pathways (Wendel et al., 2009), including fatty acid partitioning to different lipid classes, regulating long chain acyl-CoA use for β-oxidation (Coleman and Lee, 2004) and to be transported to different tissues, such as the adipose tissue (Ellis et al., 2011).. The enzyme ACSL1 catalyzes the synthesis of acyl-CoA from long-chain fatty acids (Ellis et al., 2011). Hence, in the current study, the upregulation of liver ACSL1, assayed based on its involvement in the synthesis of TAG (Marszalek et al., 2005), appear to be responsible for the enhancement of the LCPUFA in the TAG fraction of all tissues, particularly at the highest level (0.60%) of algal DHA supplementation.

8.5.2   *Egg PL pool limiting at higher LCPUFA enrichment (preformed)*

The algal DHA-fed hens deposited a greater (~60% more) amount of DHA in the egg lipid compared to ALA-fed hens, 29.9 vs. 11.7 ± 0.66 mg/g fat of yolk, equivalent to 173 vs. 66.9 ± 2.25 mg/yolk, respectively (~6 g fat per yolk), at the highest level of total n-3 fatty acid intake. A similar pattern of enrichment of egg yolk lipid with DHA was reported in earlier studies utilizing up to 15% flaxseed (~1.68% ALA in the diet) with eggs containing ~90 mg/yolk (Aymond and Van Elswyk, 1995). On the other hand, when heterotrophic microalgae were utilized in laying hen diets, up to 200 mg/yolk of DHA have been reported (Fraeye et al., 2012).

The current study showed that the levels of DHA as well as EPA in the egg yolk were significantly enriched by the inclusion of algal DHA over that of flaxseed oil (precursor ALA), not only through the enhancement of the total PL, but also the TAG pool of the egg yolk. However, egg enrichment of the total PL pool was more specific to the PtdEtn than the PtdCho fraction. In the former fraction, although the accumulation of DHA was more with the preformed DHA- than the ALA-fed hens, this pool was limited to both the synthesised as well as the preformed LCPUFA, indicating a quadratic effect of accumulation, reflecting trends in the egg lipid and total PL. With respect to EPA, utilizing precursor ALA from flaxseed oil supplementation resulted in linear accumulation of EPA in the TAG and total PL (as well as PtdCho and PtdEtn fractions) of the egg yolk in a similar trend to that observed in the liver. Algal DHA supplementations, based on a low ALA background diet, the level of EPA (primarily, contained in the algal DHA product, therefore preformed) in the total PL pool of the egg yolk (similar to the trends in the liver) plateaued at higher levels of total n-3 intake. These results may imply that egg PL pool, particularly the PtdEtn fraction, is a limiting pool when higher levels of preformed source of LCPUFA are used.

Moreover, based on the proportion of contribution of yolk lipid components, the incorporation of DHA in eggs, 6-fold (in the TAG) and 2-fold (in the total PL pool) more from the preformed source (algal DHA) compared to that synthesised from ALA, is an indication of the limited egg PL pool for enrichment with the former treatment. As mentioned earlier, this shift to store LCPUFA in the TAG fraction relates to the limited sn-2 position of the PL for more acylation of the LCPUFA (Schreiner et al., 2004). This indicates that the egg TAG pool has a potential for enrichment, preferentially with preformed sources of LCPUFA.

8.5.3   *Role of ALA in the regulation of DHA levels in the liver*

A linear relationship between levels of dietary ALA and tissue EPA using human cell lines (Portolesi et al., 2007) and liver PL pool in laying hens (Neijat et al., 2016b), relate to suggestions that tissue EPA concentration increases in a predictable manner as a function of dietary ALA intake (Mantzioris et al., 1995). Based on these relationships it is noteworthy that in the current study, the level of ALA in the basal diets containing the algal DHA remained similar between graded levels of algal product inclusion. Furthermore, in the flaxseed oil-fed hens, a lower rate of accumulation (lower regression coefficient) of ALA was observed in the liver PL, particularly in the PtdEtn, a pool where the biosynthesis of LCPUFA take place (Sprecher, 2000), compared to the level of EPA accumulating in the same lipid pool. Hence, in both cases, the data support a "scarcity" of ALA in hepatic PL (Sprecher, 2000; Goyens et al., 2006), which relative to EPA level, may limit further endogenous synthesis/accumulation of DHA. These outcomes relate to earlier suggestions by Neijat et al. (2016b), that when ALA is used as a source of omega-3 PUFA in hen diets, there is a strong inverse correlation between the ratio of ALA to EPA and the amount of DHA in hepatic PL pool, linking low availability ALA in the liver and its limited conversion into DHA. Similarly, the availability of ALA, when

preformed LCPUFA are used as substrates in diets of hens, may also play an important role,

possibly, maintaining SDA levels. Furthermore, while ALA serves as a substrate for the

synthesis of its longer chain metabolites (Sprecher et al., 1995; Sprecher, 2000), the presence of

dietary ALA may be vital in overcoming enzymatic competition at the expense of n-6 PUFA

(Herber and Van Elswyk, 1996), considering that the 18-carbon PUFA (and more possibly,

ALA) are preferred substrates for the Δ6-desaturase than the 24-carbon PUFA (Blasbalg et al.,

2011; Gregory et al., 2011). Hence, it is possible that high quantities of n-6 fatty acids can limit

further incorporation of EPA and DHA (De Schrijver and Privett, 1982; Blank et al., 2002;

Goldberg et al., 2013), when preformed sources of LCPUFA devoid of ALA are used in laying

hen diets.

8.5.4 *Differential regulation of EPA and DHA levels in the liver*

In previous studies, utilizing preformed sources of EPA in laying hen diets, such as

menhaden oil (Herber and Van Elswyk, 1996), microencapsulated fish oil (Lawlor et al., 2010)

or microalgae species (such as *Nannochloropsis oculata*; Lemahieu et al., 2013), predominantly

rich in EPA, have reported preferential deposition of DHA and not EPA in the egg yolk.

Moreover, Nitsan et al. (1999) who supplemented laying hen diets with microalgae

Nannochloropsis sp. (containing high concentration of EPA and devoid of other n-3 fatty acids

including ALA) also observed an accumulation of only DHA and not EPA in both the liver and

egg yolk. Based on the results from the current study, since the accumulation of EPA in the liver

may possibly be aimed at maintaining specific levels, then as a means to counteract increased

levels of EPA (from preformed source) in the liver (possibly relative to amounts of ALA and/or

SDA levels, as suggested), EPA may have to be readily converted to its longer chain metabolites

(DPA and DHA) and principally deposited as DHA in the egg yolk.

Furthermore, while DHA is principally derived from either a preformed source or precursor metabolites, EPA can additionally be derived from a retro-conversion of DHA (Sprecher, 2000), further indicating the central role of EPA in the regulation of hepatic levels of LCPUFA. The retro-conversion process involves the regulation of a transcription factor, peroxisome proliferator receptors alpha (PPARα) (Jump et al., 2008). In the current study, a downregulation of PPARα at higher levels of either flaxseed oil or the algal DHA treatment group may indicate the absence of retro-converted EPA from DHA. Similarly, a downregulation of the genes involved in β-oxidation (CPT1A and ACOX1) and that encoding the conversion of ALA to SDA (FADS2) in response to the 0.60% algal DHA supplementation in the hen diets, coincides with the potential control measure regulating EPA, derived by feeding the algal product (0.60%), being greatly incorporated into the TAG pool and being transported to the adipose tissue. Although this phenomenon was not observed for all levels of flaxseed oil supplementation, the hepatic level of EPA (derived from ALA) may have been potentially controlled by the shift of ALA to the adipose tissue (as earlier described) subsequently influencing DHA levels in the liver and egg yolk.

In summary, the current study provided evidence that as a function of total omega-3 fatty acid intake by laying hens, both endogenously synthesised (from precursor ALA) as well as exogenously derived n-3 LCPUFA (from preformed source) lead to non-proportional increases in the enrichment of these fatty acids in the egg, particularly in the PL pools, a trend similarly reflected in the liver PL. The hepatic PE pool, possibly aimed at maintaining specific level of EPA, may be potentially regulating the biosynthesis of omega-3 LCPUFA. Such measures include controlled flux of ALA (and SDA) or the preformed LCPUFA (EPA, DPA and DHA) by being deposited into adipose tissue and their preferential incorporation into the TAG pool,

mainly through the activation of ACSL1 by algal DHA. While the egg TAG pool has the

potential for enrichment with omega-3 LCPUFA, with the preformed source of LCPUFA (algal

DHA), a greater enrichment of this pool with these fatty acids may lead to sensory issues.

Overall, the current study provided novel evidence of potential regulatory points in the

enrichment of endogenous as well as exogenous origins of LCPUFA in eggs, hence, facilitate

opportunities for flexibility in the guidelines permitting content claim for omega-3 PUFA in

eggs.

CHAPTER 9 GENERAL DISCUSSION

The overarching objective of this thesis was to identify factors regulating the enrichment of omega-3 LCPUFA (in particular, DHA) in chicken eggs, while concurrently evaluating potential alternative source(s) of plant (terrestrial) derived omega-3 PUFA (ALA) for use in laying hen diets. This information is relevant because omega-3 enriched eggs are an economical source for boosting population consumption of these fatty acids recognised as beneficial to human health (Simopoulos, 1991; Griffiths and Morse, 2006). Furthermore, based on considerable interests in the putative health effects of these fatty acids, Canada has now permitted the use of a health claim, related to blood triacylglycerol (TAG) lowering effect, for the omega-3 fatty acids, but only for DHA and EPA. In order to achieve this claim, a serving of a particular food would need to contain 500 mg of DHA and EPA, while maintaining sensory attributes. Achieving this level of enrichment in eggs may be out of reach for current egg producers. At the current level of omega-3 LCPUFA enrichment in eggs derived from hens consuming ALA-rich source of omega-3 PUFA, an average size egg (56-60 g/egg) can provide approximately 25% (Scheideler and Froning, 1996; Morris, 2003) of the estimated intake levels of 250 to 500 mg/day for EPA and DHA (Harris et al., 2009). This raises concern for the egg industry (producers) because omega-3 PUFA supplementation in hens' diets is predominantly based on ALA-rich seeds or oils, a substrate that is extensively documented to be limited in its conversion to the longer chain metabolites, both in humans (Nakamura and Nara, 2004; Plourde and Cunnane, 2007) and poultry (Hargis and Van Elswyk, 1993; Aymond and Van Elswyk, 1995; Gakhar et al., 2012). Hence, an understanding of the factors regulating endogenously synthesised omega-3 LCPUFA and their deposition into the egg may provide valuable insights

on how the levels of these fatty acids in eggs may further be enhanced. In this chapter, major highlights, limitations and considerations taken into account are discussed.

In the first experiment of this research (Chapters 4 - 6), the safety and efficacy of utilizing hempseed (HS) and hempseed oil (HO) as potential dietary sources of ALA in laying hen diets was assessed. Hempseed and hempseed oil contain 7 and 18% ALA, respectively (Parker et al., 2003), compared to the flaxseed oil (containing 53% ALA; Morris, 2007) used in the second experiment (Chapters 7 and 8), and resulted in a conversion efficiency of ALA into DHA and total omega-3 PUFA in the egg yolk of ~3.5 and 12.5%, respectively for both HS and HO compared to that of flaxseed oil of 11 and 29%, respectively, at the highest inclusion levels of total omega-3 PUFA in the diets of hens (Table 9.1). Hence, based on the level of ALA, while hempseed products represented a weak model, employing flaxseed oil, in Experiment 2 (Chapters 7 and 8), provided a more aggressive model, as the basis of the substrates to study the factors regulating endogenously synthesised omega-3 LCPUFA. Although the two models were employed as two separate experiments, Experiment 1 and Experiment 2, tissue samples were collected for lipid profile assessment at 34 and 42 weeks of hen age, respectively. These periods were within the first phase of the production cycle (50% egg lay to 45 weeks of age; Lohmann LSL-Classic, 2004). As expected by the strain of the hens (Lohmann LSL-Classic, 2004), in both experiments, regardless of treatments, the hens consumed ~105 g/hen/day feed, laid ~98% of eggs and produced eggs weighing ~60 g/egg. In addition, the inclusion of a preformed source of LCPUFA (algal DHA) in diets of hens, alongside the supplementation of flaxseed oil had the greatest efficiency of incorporation of total omega-3 PUFA and DHA in the egg yolk compared to the latter treatment, confirming previous literature (Fraeye et al., 2012). Furthermore, because

Table 9.1: Sources of omega-3 fatty acids, egg enrichment and efficiency of conversion

Dietary omega-3 fatty acid sources	Total omega-3 fatty acids in diet (mg/100g of diet)	Amount deposited in eggs (mg/yolk)		Efficiency of conversion into eggs (%)	
		Total omega-3 fatty acids	DHA	Total omega-3 fatty acids	DHA
Corn oil-based (control)	96	13.2	14.1	13	14
Hempseed	1220	155	41.7	13	3.4
Hempseed oil	1280	156	45.2	12	3.5
Almond oil-based (control)	50	37.6	24.7	75	49
Flaxseed oil	600	174	66.9	29	11
Algal DHA	600	201	173	31	29

supplementation of algal DHA, particularly, at higher levels of dietary inclusion, also indicated

similar trends in the distributions of LCPUFA in the PL fractions of egg yolk and liver in

comparison to that of endogenous origin, suggests a possible similarity in the mechanism

controlling the biosynthetic pathway of LCPUFA for both sources of omega-3 PUFA.

The results outlining the potential control points (Chapter 6 and 8) in the regulation of

endogenous synthesis of omega-3 LCPUFA in the chicken, are summarized in Figure 9.1 and

points majorly towards ALA and EPA levels; possibly aimed at maintaining a physiological

level of the latter in the liver. The model (Figure 9.1), provides a putative hepatic PL regulation

of endogenously versus exogenously derived DHA (DHA-endo versus DHA-exo, respectively)

for egg enrichment, indicating possible explanation why less DHA is incorporated into the egg

yolk by hens fed ALA (or SDA) versus preformed DHA. Regardless the source of omega-3

PUFA, a specific level of EPA was maintained in the liver PE, presumably to regulate the flux of

omega-3 LCPUFA in the liver. However, preformed DHA (from algal DHA), compared to the

endogenously synthesised DHA, is efficiently incorporated into the egg because the former

metabolite enters/exits the pathway, downstream post-EPA, therefore bypassing the major

control point in the synthesis of LCPUFA. Likewise, the use of preformed sources high in EPA

laying hen diets (Lawlor et al., 2010; Sefer et al., 2011) and the lack of EPA in egg yolk or low

levels in liver suggest that it is rapidly metabolized to DPA and DHA, suggestive of the need to

maintain only "certain" levels of EPA in liver. In addition, the efficiency of regulation of

preformed LCPUFA, either for the conversion of EPA to DHA or the overall levels of the

LCPUFA compared to endogenously synthesised DHA may relate to the efficient activation (into

its derivative acyl-CoA), reflected in an up-regulation of ACSL1 observed when utilizing

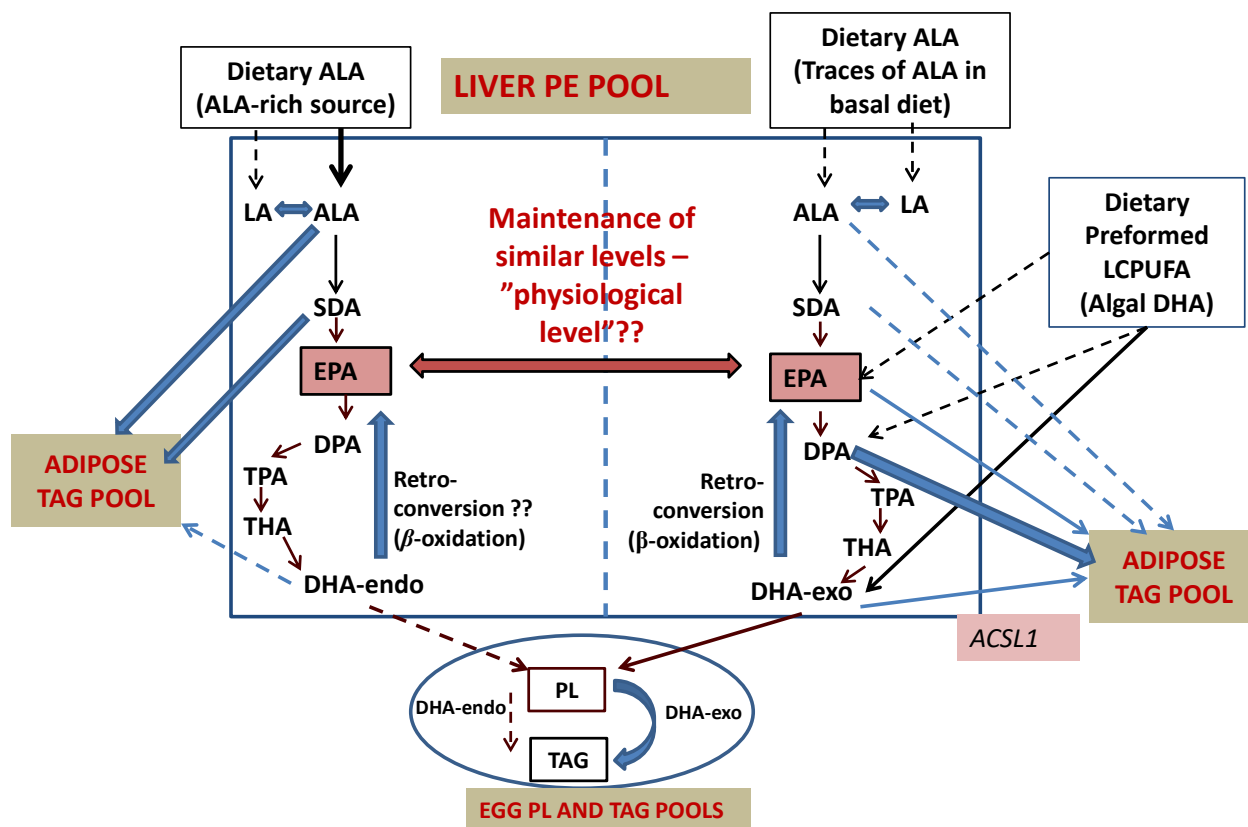preformed LCPUFA (Chapter 8) as well as directing the enrichment of the TAG fraction

Figure 9.1. Model summarizing a putative hepatic PL regulation of omega-3 LCPUFA. Incorporation of DHA into the egg by hens fed ALA or algal DHA (predominantly DHA but also containing traces of EPA and DPA) were compared. Based on a similar total omega-3 PUFA intake, a "certain level, possibly a physiological level" of EPA was achieved in the liver PL (PE). Excess ALA and SDA (of endogenous source) were shunted to the adipose tissue. The competitive action (for enzymes) of LA against ALA may support a significant control point/regulator of the flux of ALA. Excess preformed EPA, DHA, and greatly DPA, were incorporated into the TAG and shunted to the adipose tissue. Because algal DHA contained preformed EPA and possibly sufficient to maintain the "required level" of EPA in the liver, hence, trace levels of ALA and SDA from the preformed treatment group were also shunted to adipose tissue. Traces of endogenously synthesised DHA (DHA-endo) accumulated in the adipose tissue. However, the incorporation of preformed LCPUFA into the adipose tissue coincides with an upregulation of mRNA expression of ACSL1, hence greatly enhancing the activation and transfer of these fatty acids to the adipose tissue. Presuming, EPA as a focal point in the regulation of LCPUFA, it is not clear whether DHA-endo is robust enough to retro-convert to EPA as may be the case for exogenously derived DHA (DHA-exo). Finally, although DHA-exo is more readily incorporated into the egg PL and enriching the TAG pool (possibly an overflow from the PL pool, being a limiting pool for further enrichment of DHA), DHA-endo is not sufficient or robust enough to saturate the egg PL pool, hence, reflecting a limited enrichment of DHA-endo in the TAG pool of the egg.

and directing fatty acids to other tissues (Wendel et al., 2009; Ellis et al., 2011).

The putative hepatic PL regulation of omega-3 LCPUFA may also support the theory that ALA is a preferred substrate for Δ6-desaturase (Gregory et al., 2011), presumably to efficiently convert ALA to EPA. This step may be of great significance because as an indicator of efficiency of conversion of ALA to EPA, and confirming others (Barlow et al., 1990), there are difference between sources of omega-3 fatty acids, particularly among plant-derived ALA sources, in part, based on the differences in the biological efficiencies of the substrate's metabolism. Utilizing datasets from both ALA-containing models, the trends/relationships presented in Figure 9.2a, shows that in the liver PE, the level of EPA was greatly increased relative to the levels of ALA in the same pool for hens fed flaxseed oil compared to those fed hempseed/oil. As a result, more DHA accumulates in the liver and egg yolk for flaxseed oil compared to hempseed/oil treatments.

Hence, the novel findings of this study, firstly, relates to the attenuation of similar levels of EPA in the PE fraction of the liver in hens as a function of total dietary omega-3 PUFA, fed either flaxseed oil (ALA-rich) or algal-DHA (DHA-rich, a preformed source) as a central point of regulation. Regardless of source of dietary omega-3 PUFA, positioned as the central regulator in the metabolism of other omega-3 LCPUFA, EPA level in liver PE, may be limiting ALA entry into the same pool, possibly because sufficient/required level of EPA in the liver PL pool (particularly the PE; Figure 9.1) is achieve, therefore, limiting further endogenous conversion to DHA. Limited accumulation of ALA in the liver PL fraction was noted in the current study and mentioned in previous studies (Goyens et al., 2005). Hence, endogenously synthesised DHA is not sufficient or robust enough to enrich the egg.

Another novel finding in the current study is that, as a function of dietary ALA intake, the levels of ALA that accumulate in the liver PE of the flaxseed oil- as well as the hempseed products-fed hens falls within a similar 95% confidence limits of the upper and lower predicated values (Figure 9.1b). The trend of accumulation of ALA in the liver PE with both precursor ALA models may be predicted with a regression equation as $\text{ALA}_{\text{liver-PE (mol\%)}} = 0.1564 + [0.2587 \times \text{ALA intake (g/hen/day)}]$, $R^2 = 0.96$, $P < 0.0001$. Hence, these results indicate that, regardless of the sources of ALA (which differ in biosynthesis/enrichment efficiencies of metabolites in the egg) the level of ALA accumulating in the PE fraction is within a similar limit. This outcome, may explain suggestion by Grobas et al. (2001) that moderate amounts of ALA (less than 1% by wt of diet) in laying hen diet is optimal for inclusion.

In the current study, hepatic genes involved in fatty acid β-oxidation were differentially expressed, indicating a downregulation at higher levels of either treatment groups compared to the control. The effects PUFA in regulating transcription factors and/or modulating gene expression involved in hepatic lipid metabolism (Sampath and Ntambi, 2006; Jump, 2008) relates to the overall energy balance and insulin sensitivity (Price et al., 2000). In this regard, the chicken would represent a unique model. Firstly, in chickens, circulating concentrations of glucose has been reported to be 220 to 234 mg/dL (equivalent to 13 ± 0.7 mM; Scanes, 2008), confirmed in the current study, in Chapter 4, utilizing HS and HO in the laying hen diets, glucose level in hens in plasma was in range of 13.2 to 13.6 ± 0.19 mmol/L). Hence, poultry species can be considered hyperglycemic (Hazelwood and Lorenz, 1959; Scanes, 2008). Secondly, they may respond to insulin with a depression in circulating concentrations of glucose but are generally insensitive to insulin (Hazelwood and Lorenz, 1959; Edwards et al., 1999). Hence, the results may, in part, explain the reasons why the assessment of fatty acid profiles may not translate in a
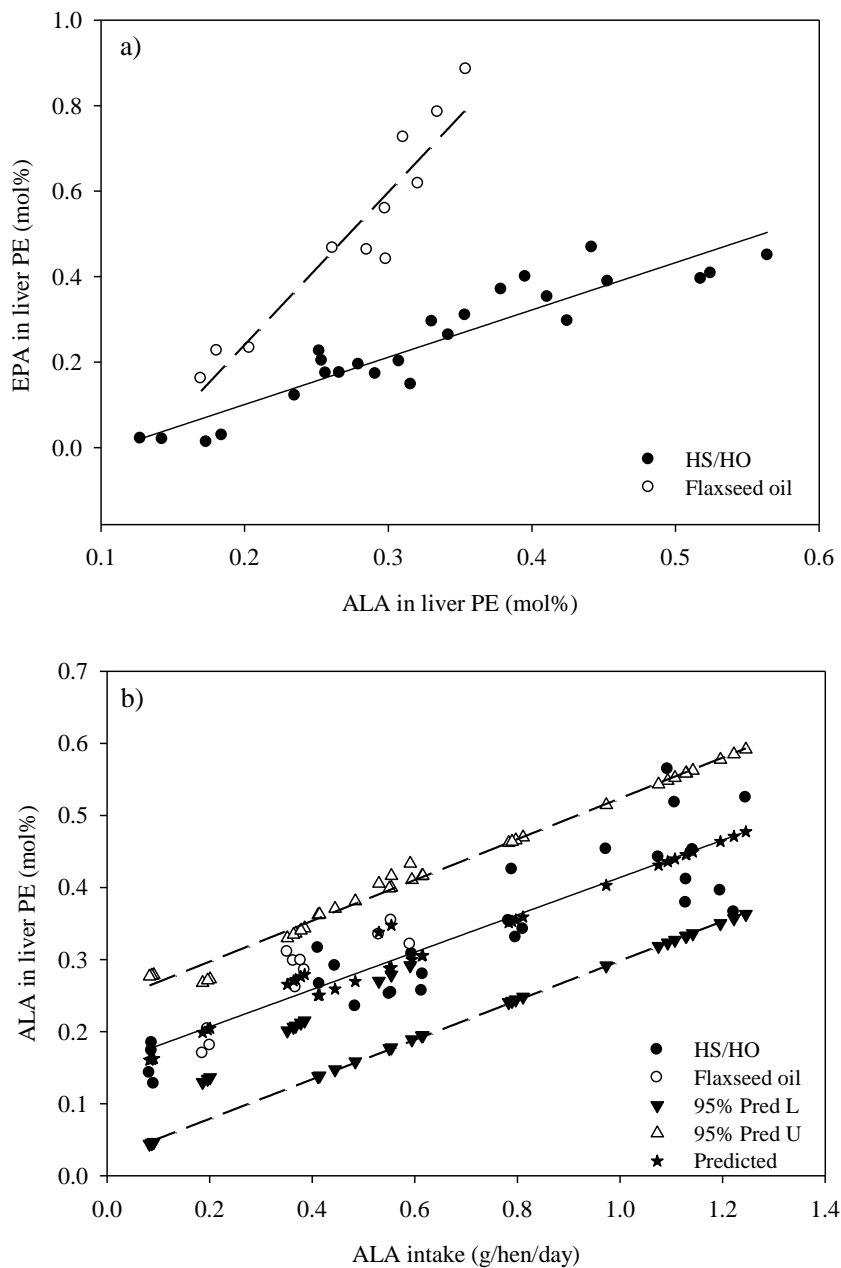
Figure 9.2. Relationships between the contents (mol%) of a) EPA and ALA in liver-PE b) ALA in liver-PE as a function of ALA intake in laying hens consuming either hempseed products (hempseed (HS) and hempseed oil (HO)) or flaxseed oil.

Regression equation: $ALA_{liver-PE}$ (mol%) = 0.1564 + [0.2587 × ALA intake (g/hen/day)], $R^2 = 0.96$, $P < 0.0001$. Individual data points are presented along with line of best fit (predicted, solid line) plus predicated upper (pred U) and predicated lower (pred L) 95% confidence limits (dashed lines).

similar trend in the mRNA expressions of key genes in lipid metabolism as observed in other studies (Cheng et al., 2006; Tu et al., 2010; Elkin et al., 2016). Hence, taken together, these results support the first hypothesis that the assessment of amount and distribution of omega-3 PUFA in the different lipid classes of tissues provide the basis in the regulation of their metabolism.

The major route for the incorporation of LCPUFA into PLs is mediated by the deacylation/reacylation cycle described as the Land's cycle/ pathway (Lands et al., 1982), which requires the activation of fatty acids to acyl-CoAs. Subsequently, an upregulation of the genes involved in the activation of the fatty acids results, with preference for the preformed LCPUFA than the precursor ALA. In accordance with previous suggestions (Mashek, 2013; Elkin et al., 2016), preformed DHA rather than biosynthesized DHA is known to exist in a pool that enables the former to be more readily incorporated in VLDL and deposited into the egg or other tissues as compared to the biosynthesized DHA. The current study provides evidence that preformed DHA rather than endogenously synthesised DHA is more readily incorporated in the TAG pool of the liver enabling the former to be more readily incorporated in VLDL and into the egg (in addition to the adipose tissue). Hence, ACSL1, involved in the synthesis of TAG (Marszalek et al., 2005), relates to greater enrichment of DHA in the egg TAG with the algal DHA than flaxseed oil supplementation, concomitant with a decreased efficiency for enrichment of EPA and DHA in the egg PL at higher levels of total n-3 PUFA intake. These results support the second hypothesis that: Egg PL pool, particularly the PE fraction, is a limiting pool when higher levels of preformed LCPUFA are used in laying hen diets; reflecting a potential enrichment of the TAG pool in eggs.

A limitation in the current study may include the nature/composition of the control diets. The control diet used in the first experiment 1 (Chapters 4-6), a corn oil-based diet, relates to a commercial cereal-based diet for laying hens. However, in the experiment 2 (Chapters 7 and 8), almond oil was included in the control diet, an oil practically devoid of omega-3 fatty acids, formulated to contain 0.03% total omega-3 fatty acid (analysed value, 0.05%). As explained in Chapter 7, the purpose of using this oil was to remove the confounding effect of ALA on the preformed DHA containing diets. Almond oil contained high levels of oleic acid (65.6%, Table 7.2). Principally, the concentrations of MUFA in the body has been shown to be much more dependent on its (the MUFA) content in the diet rather than on *de novo* formation (Ratnayake et al., 1989). This may raise a concern (limitation) in the metabolism of omega-3 PUFA such ALA, which is also greatly esterified in the same pool (the TAG) as is the case for oleic acid, major components of neutral lipids (TAG and CE) fraction (Mennicken et al., 2005) as well as being greatly deposited in the adipose tissue. Similarly, in animals, ALA as well as LA are readily stored in adipose TAG (Matthews et al., 2000). This was confirmed in the current work in which ALA was observed to increase in total lipid of the adipose tissue as a function of ALA intake in the hens (Figure 8.1). In the current analysis (Table 9.1), the control diet containing almond oil exhibited a greater efficiency of conversion of ALA into total omega-3 PUFA and DHA (75 and 49%, respectively) compared to the corn oil containing control diet (13 and 14%, respectively). It is possible that with corn oil, the high level of LA compared to ALA interferes with the conversion of ALA to LCPUFA due to competition for the same desaturase enzymes (Burdge, 2004). It has also been demonstrated in our lab (Goldberg et al., 2013) that the ratio of SFA:LA+oleic acid has a great impact on the levels of omega-3 PUFA in eggs, higher ratio favouring enrichment levels.

CHAPTER 10   SUMMARY AND CONCLUSION

The characterization of polyunsaturated fatty acids (PUFA) profiles (amount and distribution) in different lipid pools of different related tissues of hens is an important criterion in elucidating aspects regulating the endogenous synthesis of omega-3 long chain PUFA (LCPUFA), hence the enrichment of these fatty acids in chicken eggs. The hepatic phospholipid (PL), particularly the phosphatidylethanolamine (PtdEtn or PE) pool in relation to the level of eicosapentaenoic acid (EPA) is a potential regulatory point in the biosynthesis of omega-3 LCPUFA.

Based on the results obtained from this study, it can be concluded that:

1. Specific level of EPA was maintained in the liver PE, presumably to regulate the flux of omega-3 PUFA.

2. At the levels of total omega-3 PUFA supplementations of laying hen diets tested, the flux of omega-3 PUFA in the liver was potentially controlled by shunting fatty acids to the adipose tissue and partitioning fatty acids into different lipid pools, particularly the triacylglycerol (TAG) pool (mobile pool). The latter was initiated by the activation of fatty acids involving long chain acyl-CoA synthetases 1 (ACSL1).

3. Although egg enrichment with docosahexaenoic acid (DHA) is more efficient when preformed sources of LCPUFA are used rather than that endogenously synthesised from precursor ALA, both supplementation strategies are influenced by the same regulatory mechanism.

4. Egg TAG pool has the potential for greater enrichment with preformed omega-3 LCPUFA supplementation, unlike with precursor ALA. This is an indicator of the

limiting PL pool of the egg, particularly to preformed sources of omega-3 LCPUFA. However, greater enrichment of the TAG fraction of egg yolk or other tissues may be responsible for sensory issue.

5. Novel ingredients like hempseed and hempseed oil can be used in diets of laying hens as alternative sources of ALA for enrichment of omega-3 PUFA in eggs.

6. Attenuation of stability of enrichment of omega-3 PUFA in egg yolk with hempseed products takes longer than that achieved with flaxseed oil (8 compared to 4 weeks, respectively). However, the level of DHA in the TAG fraction of egg yolk for the former source of ALA were not affected by the levels of inclusion (up to 1.2% of diet ALA from either hempseed or hempseed oil) compared to the flaxseed oil treatment that increased at 0.40 and 0.60% of diet ALA feeding compared to the control. Hence, considering differences between the two ALA models in the levels of enrichment of omega-3 LCPUFA of the PL pool, the hempseed products are more likely not to result in sensory issues compared to flaxseed oil.

7. Based on the enrichment efficiencies of DHA into the egg yolk, recommended levels for inclusion of the ALA sources are at 0.50% (10% of diet hempseed), 0.66% (4.5% of diet hempseed oil) and 0.40% (0.77% of diet flaxseed oil) providing DHA enrichment levels of 42.9, 40.5 and 59.9 mg/yolk, respectively. Therefore, regardless of the source, the level of precursor ALA recommended for inclusion in laying hen diets would be within the range of 0.53 ± 0.13 (SD) % of diet. This provides the point at which a plateau in the enrichment of DHA attenuates and possibly with no impact on sensory aspects (no or little enrichment of egg TAG fraction for hempseed products and flaxseed oil,

respectively). This further support the novel finding in this study that the level of ALA accumulating in the PE fraction is within a similar limit.

8. Algal DHA supplementation in the hen diet indicated decreasing efficiencies of incorporation of DHA in the egg from 49.7 to 32.5 to 28.8% for 0.20 to 0.40 to 0.60% of total omega-3 fatty acid in the diet, respectively. An inclusion level of 0.40% of total omega-3 fatty acid in the diet (2.18g per 100g diet of algal DHA), which leads to 130mg/yolk DHA enrichment, was determined to be most optimal for use in laying hen diet. At this level no EPA is lost to the adipose tissue and egg TAG enrichment is at levels to ensure minimal sensory problems.

CHAPTER 11   FUTURE RESEARCH DIRECTIONS

This study provided novel evidence of potential regulatory points limiting the conversion of dietary ALA to its longer chain metabolite and its subsequent accumulation in the chicken egg. However, several other points in the metabolism of omega-3 PUFA may be involved in the regulation of egg yolk levels of these fatty acids. These will be mentioned as specific topics, as follows:

1.   Regulation of exogenously synthesised DHA

The current study demonstrated that exogenously synthesised DHA, at higher levels of inclusion of algal DHA, was observed to exhibit a non-proportional increase in the PL fraction of egg yolk, plasma and liver. In previous studies (Herber and Van Elswyk, 1996; Bruneel et al., 2013), a limited DHA enrichment of eggs with marine oils/algae at higher levels of inclusion was also identified. Reflecting on the ALA levels of hepatic PL, being the limiting substrate and pool for endogenous synthesis of the longer chain metabolites, the same limitation may also apply for exogenous synthesis/accumulation of DHA in the liver or egg yolk. In the present study, similar levels of ALA were used in the background diets, however, it would also be interesting to demonstrate the impact of preformed LCPUFA (such as algal DHA) supplementation against a basal diet that also contains gradually increasing levels of ALA in laying hen diets. This may serve to explain the role of ALA (in this setting), not as a major contributor to the accumulation of DHA into the egg yolk or liver, but as a vital substrate in maintaining the levels of other PUFA such as SDA and the balance between the omega-3 and omega-6 PUFA series. Presumably, this kind of study may further elucidate the contribution of ALA, when used in combination with exogenous sources and may provide further understanding in the regulation of endogenously synthesised DHA (derived solely from precursor ALA).

2.   Immune status and essentiality of ALA in laying hens

The level of ALA used in control diets in the current research may be adequate to support hen performance indices (Chapter 4 and 7), possibly through the ability to maintain tissue function as described in Chapter 8 and as was mentioned in previous studies (Barceló-Coblijn and Murphy, 2009). Because the immune response of chickens can be influenced by nutritional status of PUFA (Fritsche and Cassity, 1992), the lack of significant differences between dietary source and amount of omega-3 fatty acids on hen performance and eggshell quality in the current study (Chapter 4 and 7) and in previous work (Silversides and Lefrançois, 2005; Gakhar et al., 2012), may reflect the normal health status of the hens during the experimental period. Studies with different levels and sources of omega-3 fatty acids in relation to omega-6 PUFA produce different eicosanoids (Calder, 2008). Hence, dose-dependent responses to incremental intake of ALA in laying hen diets under immune-challenged situation, for example, by subjecting hens to intravenous injection of 8 mg/kg of BW of *Escherichia coli* lipopolysaccharide (Shini et al., 2008), may be used to test the essentiality of ALA (with respect to hen performance indices and subsequent impact on the level of enrichment of omega-3 fatty acids in eggs) in relation to immune responses in laying hens.

3.   Targeted approach of analysis

The positional distribution of fatty acids on the glycerol molecule, using a targeted approach of analysis may provide a more detailed assessment of the distribution of omega-3 PUFA in different lipid pools of the egg yolk in relation to levels in the plasma and liver. This may possibly highlight the regulation of omega-3 LCPUFA enrichment of the egg yolk particularly based on their (the LCPUFA) ability to significantly enrich of the TAG fraction of

the egg when preformed sources of omega-3 PUFA are used in the hen diets as observed in the current study and others (Mashek, 2013).

4.   Variants in the genes that encode rate limiting steps

Studies suggest that the efficiency in the biosynthesis of LCPUFA is highly impacted by variants in the genes that encode for the enzymatic steps, particularly the rate limiting steps, the FADS genes (Khang et al., 2007; Chilton et al., 2014). Many of these studies are conducted on humans. Although there is high protein sequence identity between the chicken and humans FADS1 and FADS2 genes (NCBI, 2012), there is still need for more research in chickens.

CHAPTER 12   REFERENCES

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APPENDICES
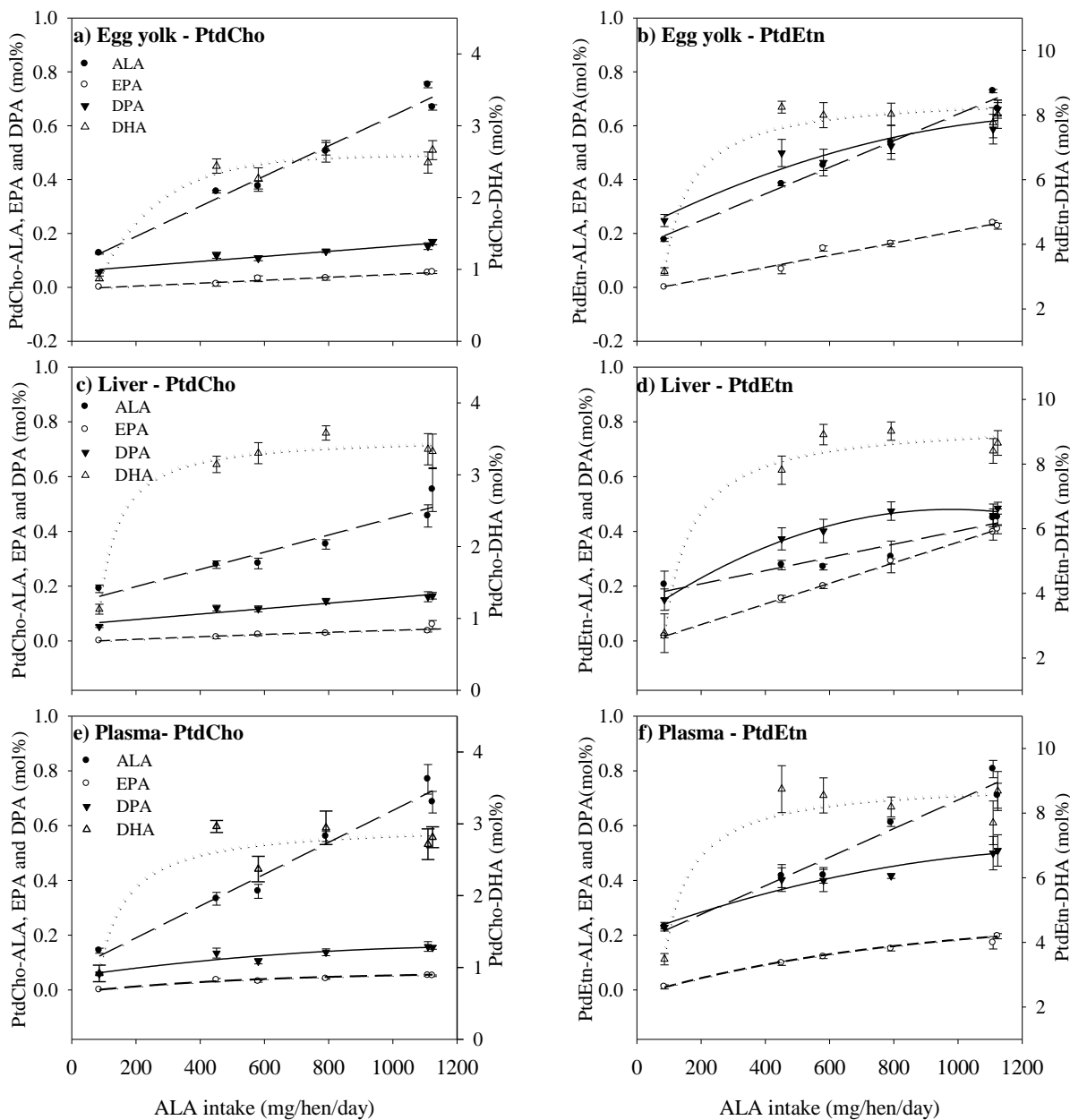
Appendix I: Trend analysis of phosphatidylcholine (PtdCho, left hand side of figure) and phosphatidylethanolamine (PtdEtn, right hand side of figure) n-3 fatty acids: alpha-linolenic, ALA; eicosapentaenoic, EPA; docosapentaenoic, DPA; and docosahexaenoic, DHA in a – b) egg yolk, c – d) liver and e – f) plasma of hens fed diets containing increasing levels of ALA derived from hempseed products.
Each data points are group means ± SE (n=7, egg yolk; n=5, liver; n=4, plasma).

Appendix II: Avian (*Gallus gallus*) - Specific primer sequences for genes involved in fatty acid transport/activation, synthesis and β-oxidation used for the quantitative real-time PCR[1]

Gene name	Accession No.	Amplicon length (bp)	Primer sequence (5'– 3')	Annealing temp (°C)	Reference
Fatty acid transport or activation					
ACSL1	NM_001012578.1	299	F: GGTTTCGGACAGAGCAGAGT R: CGATGGTGCTGAGGATTGGA	60	This study
FABP1	NM_204192.3	102	F: ACCGAACTCAACGGAGACAC R: TGATAAGTGCATGCAGGGTC	60	This study
Fatty acid β-oxidation					
PPARα	NM_001001464.1	233	F: TTGGACGAATGCCAAGGTCT R: TGCCATGCACAAGGTATCCA	60	This study
CPT1A	NM_001012898.1	151	F: GGGTTGCCCTTATCGTCACA R: TACAACATGGGCTTCCGTCC	60	This study
ACOX1	NM_001006205.1	110	F: TTGACCTTGTGCGAGCATCT R: CTCAGTCAAGACAGCGCAGA	60	This study
Fatty acid Synthesis[2]					
FADS1	XM_004941487.1 (XM_421052)	200	F: CTTGGCGAACAAAAGAAGAAAT R: CCCAGTAAGGGCAGGTAGGT	59	Gakhar et al (2012)
FADS2	NM_001160428.2 (XM_421053)	126	F: AACCATCGTCACTTCCAACATC R: CTTCAGCTTCTTCTTGCCGTAC	60	Gakhar et al (2012)
ELOVL2	NM_001197308.1	148	F: GCCATGTGGGTTTCCCTTTG R: GACTTCTGTTGTGACGGGGG	60	This study
ELOVL5	NM_001199197.1 (XM_426204)	180	F: ATTGGGTGCCTTGTGGTCA R: AGCTGGTCTGGAAGATTGTCA	60	Gakhar et al (2012)
Housekeeping gene[2]					
β-actin	NM_205518.1 (X00182)	205	F: CAACACAGTGCTGTCTGGTGGTA R: ATCGTACTCCTGCTTGCTGATCC	62	Gakhar et al (2012)

[1]ACSL1 = acyl-CoA synthetase long-chain family member 1; FABP1 = fatty acid binding protein 1; PPARα = peroxisome proliferator-activated receptor alpha; CPT1A = carnitine palmitoyltransferase 1A; ACOX1 = acyl-CoA oxidase 1; FADS1 = fatty acid desaturase 1; FADS2 = fatty acid desaturase 2; ELOVL2 = ELOVL fatty acid elongase 2; ELOVL5 = ELOVL fatty acid elongase 5; β-actin = actin, beta.
[2]Accession numbers in brackets were as provided in previous study (Gakhar et al., 2012).
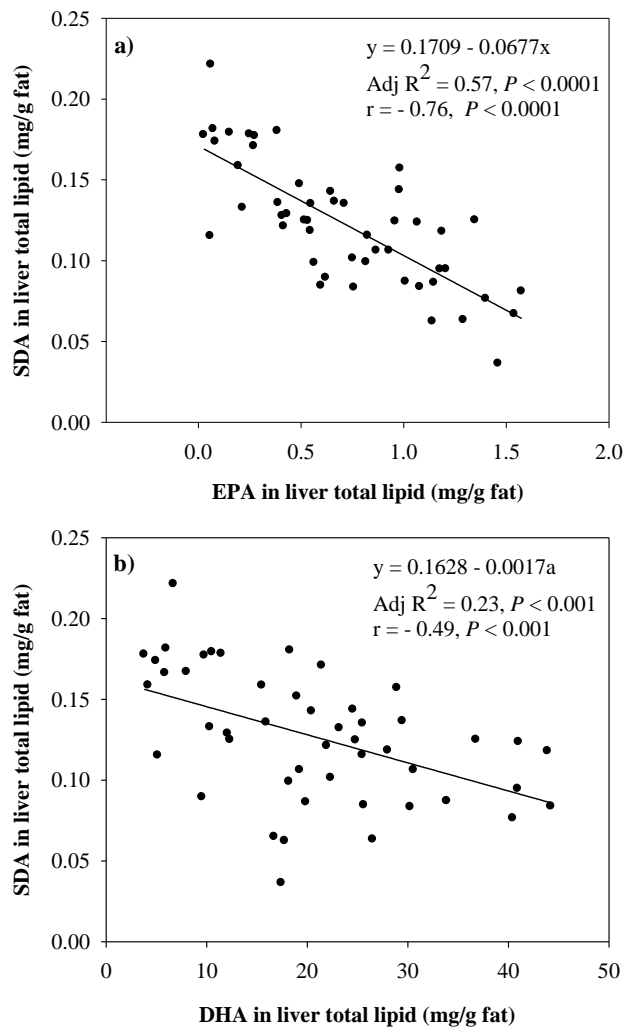
Appendix III: Relationships between SDA level in the liver as a function of a) EPA level in the liver total lipid and b) DHA content in the liver total lipid.
(n = 9 per treatment).

Appendix IV: Omega-6 fatty acid levels (mol%) in total phospholipids (PL) of egg yolk and liver as a function of feeding hens diets containing total omega-3 fatty acids sourced from either flaxseed oil or algal DHA[1]

| | Total omega-3 in diet (%, by wt) | | | | | | | | | Contrasts (P-values) | | |
| | Control | Flaxseed oil (FSO) diets | | | Algal DHA (DHA) diets | | | | | FSO vs. | DHA vs. | FSO vs. |
	0.03	0.20	0.40	0.60	0.20	0.40	0.60	SE	*P*-values	Control	Control	DHA
Egg yolk												
LA(18:2n-6)	13.3[a]	13.4[a]	12.8[ab]	12.9[ab]	12.1[b]	11.0[c]	10.0[d]	0.22	<0.0001	0.25	<0.0001	<0.0001
GLA(18:3n-6)	0.158[a]	0.155[a]	0.146[ab]	0.128[ab]	0.109[bc]	0.089[c]	0.071[c]	0.009	<0.0001	0.14	<0.0001	<0.0001
ARA(20:4n-6)	6.24[a]	5.24[b]	4.52[bc]	4.00[cd]	4.17[cd]	3.54[d]	2.74[e]	0.15	<0.0001	<0.0001	<0.0001	<0.0001
Liver												
LA(18:2n-6)	12.7[a]	12.6[a]	12.6[a]	12.6[a]	11.8[ab]	11.3[b]	10.2[c]	0.25	<0.0001	0.77	<0.0001	<0.0001
GLA(18:3n-6)	0.186[a]	0.164[ab]	0.155[ab]	0.154[ab]	0.104[bc]	0.102[bc]	0.076[c]	0.014	<0.0001	0.11	<0.0001	<0.0001
ARA(20:4n-6)	9.81[a]	9.29[a]	7.98[b]	7.37[bc]	7.75[b]	6.71[c]	5.44[d]	0.18	<0.0001	<0.0001	<0.0001	<0.0001

[1]Data are presented as least square means (LSM) ± Pooled SE; n=5. [a-e]Different superscripts between treatments within a row of a fatty acid for each tissue type are significantly different at $P < 0.05$. Contrasts between treatment groups are significant at $P < 0.05$.

Appendix V: Omega-6 fatty acid levels (mol%) in triacylglycerol (TAG) of egg yolk, liver and adipose tissue as a function of feeding hens diets containing total omega-3 fatty acids sourced from either flaxseed oil or algal DHA[1]

| | Total omega-3 in diet (%, by wt) | | | | | | | | | Contrasts (P-values) | | |
| | Control | Flaxseed oil (FSO) diets | | | Algal DHA (DHA) diets | | | | | FSO vs. | DHA vs. | FSO vs. |
	0.03	0.20	0.40	0.60	0.20	0.40	0.60	SE	*P*-values	Control	Control	DHA
Egg yolk												
LA(18:2n-6)	11.4	11.8	12.2	12.2	11.6	12.3	11.5	0.39	0.48	0.16	0.40	0.41
GLA(18:3n-6)	0.120^a	0.110^a	0.115^a	0.099^{ab}	0.090^{abc}	0.079^{bc}	0.064^c	0.007	<0.0001	0.15	<0.0001	<0.0001
ARA(20:4n-6)	0.256^{ab}	0.215^{abc}	0.198^{bc}	0.168^c	0.226^{abc}	0.243^{ab}	0.280^a	0.016	0.0007	0.003	0.74	0.0001
Liver												
LA(18:2n-6)	9.68	11.1	10.9	11.1	10.1	9.90	9.50	0.51	0.13	0.029	0.81	0.007
GLA(18:3n-6)	0.111^a	0.108^a	0.088^{ab}	0.084^{ab}	0.059^b	0.061^b	0.053^b	0.009	<0.0001	0.091	<0.0001	<0.0001
ARA(20:4n-6)	0.098	0.116	0.089	0.093	0.083	0.081	0.079	0.014	0.59	0.95	0.31	0.13
Adipose												
LA(18:2n-6)	19.4	19.8	19.1	20.0	19.6	18.5	18.5	0.51	0.25	0.63	0.39	0.068
GLA(18:3n-6)	0.143	0.122	0.120	0.139	0.121	0.113	0.113	0.018	0.85	0.46	0.20	0.44
ARA(20:4n-6)	0.035^{bc}	0.040^{bc}	0.031^c	0.023^c	0.043^{bc}	0.055^{ab}	0.071^a	0.005	<0.0001	0.59	0.002	<0.0001

[1]Data are presented as least square means (LSM) ± Pooled SE; n=5. [a-c]Different superscripts between treatments within a row of a fatty acid for each tissue type are significantly different at *P* < 0.05. Contrasts between treatment groups are significant at *P* < 0.05.