# University of Alberta

# "Improving the Effectiveness of Laying Hens for Use in Value-Added Egg Production"

By

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## DEDICATION

This thesis is dedicated to my father **Ramlal Nain,** and my mother **Anusuiya Nain** for their continuous moral support to finish my thesis. This dedication would not be complete if I do not mention my mentor **Dr. Robert Renema** for supporting and believing in me all the way through.

# ABSTRACT

A series of experiments were conducted to explore factors affecting transfer of value-added ingredients from the diet to table eggs, with the goal of contributing to improvements in the enrichment process.

Flaxseed-based ω-3 PUFA enrichment did not reduce lutein enrichment**.** The combine enrichment of lutein and ω-3 PUFA had decresed lipid oxidation potential. Also, when fed a ω-3 PUFA diet, birds scored as energetic Efficient had longer and wider villi, resulting in greater absorptive surface area/villi than Non-efficient hens. However, histomorphological differences did not affect transfer of ω-3 PUFA from diet to egg. Finally, birds fed graded levels of ω-3 PUFA to characterize change in lipid profile of egg and blood plasma in time reached a plateau in total ω-3 PUFA/egg in 5.9 to 6.6d, with High birds reaching the target of 300 mg/egg in 5d. Egg enrichment can be modulated by changes to the hen diet.

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| | |
|---|---|
| ACTH | Adrenocorticotropic hormone |
| AHA | American Heart Association |
| CHD | Coronary heart diseases |
| CLA | Conjugated linoleic acid |
| CVD | Cardiovascular diseases |
| DHA | Docosahexaenoic acid |
| DPA | Docosapentaenoic acid |
| EFA | Essential fatty acids |
| EPA | Eicosapentaenoic acid |
| FSH | Follicle-stimulating hormone |
| GC | Gas Chromatography |
| GnRH | Gonadotropin-releasing hormone |
| LA | Linoleic acid |
| LC-PUFA | Polyunsaturated fatty acids |
| LDL | Low density lipoprotein |
| LH | Luteinizing hormone |
| LNA | Linolenic acids |
| MA | Malonaldehyde |
| MDA | Malondialdehyde |
| MUFA | Monounsaturated fatty acids |
| ω-3 PUFA | Omega-3 polyunsaturated fatty acid |
| ω-6 PUFA | Omega-6 polyunsaturated fatty acids |
| PUFA | Polyunsaturated fatty acid |
| RDA | Recommended Daily Allowances |
| SFA | Saturated fatty acids |
| SEM | Standard error of mean |
| TBARS | Thiobarbituric reactive substances |
| TSH | Thyroid stimulating hormone |
| VLDL | Very low-density lipoprotein |
| Vit A | Vitamin A |
| Vit C | Vitamin C |
| Vit E | Vitamin E |
| $Zn^{2+}$ | Zinc |

# CHAPTER 1

# Literature Review

## 1.1 GENERAL INTRODUCTION

The egg is an excellent source of protein and essential fats and is considered by egg researchers as nature's original functional food (Hasler, 2000). Health Canada (Section 2.2) defines a functional food as a product containing additional health-promoting and/or disease-preventing components beyond the basic nutritional function. Since the foray of enriched eggs into the functional food class in the 1990's, these eggs have continued to gain market share and to provide new enrichment options.

Enriched eggs currently in the market have been enriched with one or more of following: omega-3 polyunsaturated fatty acids (ω-3 PUFA), conjugated linoleic acid, vitamin E, lutein, iodine, folic acid and selenium, or similar ingredients (Surai et al., 2000; Bourre and Galea, 2006). These enrichments provide the consumer with the additional potential health benefits. Others eggs with high value include traits such as improved shell quality or modified welfare friendly housing systems (van-Horne et al., 1996, Samman et al., 2009). Overall, these eggs constitute about 15% of shelled egg market in Canada (Egg Producers of Canada, 2008), a value that continues to grow.

The enrichment of eggs occurs through the fortification of the hen diets with enrichment ingredients (Hargis and Van-Elswyk, 1993). These ingredients can be in a lower quality form than would generally be used if included in human diet, reducing the cost of enrichment. These ingredients can also provide benefits to the hen as well, and have been shown to contribute to the hens like an increased number of infection-fighting cells (Wang et al., 2000), or reproductive performance (Al-Daraji et al., 2010).

Most work in the value added arena is directed toward the incorporation of nutrients into eggs by dietary manipulation without concerning the normal biological mechanism of birds. The variation in utilization of a nutrient by the bird contributes to variability in final enriched product. Therefore, research is

required to understand the nutrient utilization and its interaction at gut level in birds, as well as the impact on product uniformity.

## 1.2 AVIAN DIGESTIVE SYSTEM

The avian alimentary canal is a tubular digestive tract with several accessory structures that are unique to birds. The digestive system of the bird has the capability of nutrient uptake from whole seeds, which are part of the typical non-commercial ration (Forbes and Covasa, 1995). The bird temporarily stores recently ingested feed in the crop, which is an out-pouching of the esophagus. The food is masticated in the gizzard, which is a functional analogue to mammalian molars (Hill, 1971). The commercial layer ration is in mash form, which increases digestibility through increasing surface area exposed to digestive enzymes.

Digestive secretions originating from the pancreas, liver, and gall bladder are added to the digesta as it passes through the gut. The liver secretes the bile into the digestive tract. Bile facilitates the emulsification of fats. The exocrine secretion of the pancreas is important for reducing the acidity of the chyme, which is essential for the activity of the enzymes it delivers: primarily amylase, lipase, and proteases. The liver breaks down fats and eliminates toxins from the body (Denbow, 2000). The small intestine is the primary site of chemical digestion and absorption of carbohydrates, amino acids, fatty acids (excluding short-chain fatty acids) and minerals like calcium, phosphate, potassium, and vitamins (Lavin et al., 2008).

### 1.2.1 Small Intestine Anatomy and Development

The intestinal tract of the bird is short relative to what is found in most mammals. The reduced intestinal length results in a lighter body weight, which is a specialized adaptation for flight (Barton and Houston, 1996). The extensive peristaltic and retro-peristaltic contractions in the small intestine of the birds allows for backward and forward mixing of the feed, this compensates for short gut length and thus allow for increased digestion and absorption capacity (Duke, 1982; Caviedes-Vidal et al., 2007). The small intestine is made up of the U-shaped loop of the duodenum followed by the jejunum, and ileum. The large

intestine in laying hens (colon) is much shorter and it consists of a pair of ceca. The intestinal tract is well demarcated morphologically (Duke, 1982).

#### *1.2.1.1 Embryonic Development of Small Intestine*

In the developing chicken embryo, the gut is derived from the endoderm layer, and it is surrounded by the splanchnic mesoderm. It can be distinguished into foregut, midgut, and hindgut by 4 d into the 21 d embryonic development process. During the course of development, the endoderm gives rise to the epithelial lining of the gut and the ducts of the mucous glands, whereas the mesoderm transforms into to the muscular wall and connective tissue (Valérie et al., 2009). The foregut becomes the pharynx, esophagus, stomach, cranial duodenum, and liver and pancreas. The midgut becomes the remaining small intestines, cecum and large intestine and the hindgut give rises to the cloaca and bursa (Gilbert, 1997).

After fully developed, the gut wall can be characterized into five major layers. These regions are the epithelial cell layer, lamina propria, underlying muscularis region, widespread components of the immune system, and the mucus layer (Koutsos and Arias, 2006). In the small intestine, the epithelium is woven into long folds, the villi, which serve to increase the surface area for enzyme secretion and nutrient absorption. The epithelium of the villi is a single layer of columnar cells, which are specialized for mucous secretion (goblet cells), nutrient absorption (enterocytes along with apical proturbance; microvilli), or hormone secretion (entero-endocrine cells) (Dibner and Richards, 2004).

#### *1.2.1.2 Post Hatch Development of Small Intestine*

Small intestinal growth is intense immediately following the hatch, when the increase in small intestine weight is even more rapid than the rate of increase in whole body mass (Yamauchi, 2002). The enterocytes, for example, rapidly develop a well defined polarity and brush border within just few hours of hatch (Geyra et al. 2001). Although this preferential growth of intestine is independent of feed, early access to feed substantially increases its growth rate (Noy and Sklan, 1999). Increases in intestinal weight and length are not identical in each segment, with the duodenum showing earlier rapid growth than either the jejunum

or ileum (Uni et al, 1999). Early vigorous absorptive capacity, due to increased villi numbers, would be centered in the duodenum immediately following the hatch and then extend to the jejunum with increasing age (Yamauchi, 2002). This pattern of the development works well for the avian species; their potential embryonic growth is limited by the space available in the egg. Rapid nutrient absorption after hatch is possible because of early rapid growth of the gut. Absence of feed both at this time and later in life decreases crypt proliferation, limit the number of enterocytes available for villus growth and thus decrease the villus absorptive surface area (Nitsan et al., 1991; Noy et al., 2001).

An increased villus length and width provide more mucosal surface area for the nutrient absorption and thus improve nutrient digestibility (Onderci et al., 2006). A deeper crypt indicates more rapid tissue turnover and is linked to a higher energy and protein demand for gut maintenance (Yason et al., 1987).

### 1.2.2 Physiology of the Digestive System

The digestive system breaks down large macromolecules (proteins, fats and starch), which cannot be absorbed intact. Once in their component forms of amino acids, fatty acids and glucose, they can be absorbed across the wall of the alimentary tube (Denbow, 2000). The key components of the digestive process are secretion of enzymes and mucus into gut, absorption of nutrients and transfer to blood across gut lumen and gut movement (contractions of smooth muscle in the wall of the tube that crush, mix and propel its contents) (Denbow, 2000).

#### *1.2.2.1 Protein and Carbohydrate Metabolism*

Proteins are polymers of amino acids connected by peptide bonds. Amino acids serve as structural and functional units for growth and development. Protease and peptidase enzymes reduce dietary proteins to amino acid by breaking the peptide bonds. The arginine, glutamic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine are important amino acids needed for egg production in mature birds (Johnson & Fisher, 1956). Additionally, the glycine is required for early intestinal growth (Corzo et al., 2004). Dietary protein can also stimulate the secretion of cholecystokinin (Furuse, 1999), a gastrointestinal hormone which stimulates the secretion of pancreatic

juices and further increase the digestion of protein, fat and carbohydrate (Kamisoyama et al., 2009).

Dietary carbohydrates are simple sugars (monosaccharide) or polymers joined by glycosidic bonds. The grains used in chicken rations mostly contain huge polysaccharide polymers. After digestion, simple sugars are transported from the intestinal mucosa via the portal vein to the liver. Replacement of grain-based carbohydrate sources in the poultry ration with fat does not affect lipogenesis in birds. However, replacing it with crude protein induces increased lipogenesis (Rosebrough et al., 1999).

#### *1.2.2.2 Lipid Digestion and Metabolism*

Typical dietary lipids in the pullet and hen ration are triglycerides, phospholipids and sterols. In the gut, the dietary fat particles undergo intestinal emulsification, hydrolytic digestion, micellar solubilization, cell membrane permeation, intracellular esterification and incorporation into lipoproteins before movement into the interstitial fluid (Krogdahl, 1985). A summary of various modifications prior to absorption are presented in Figure 1.1. Larger fat particles are first emulsified by bile into smaller particles, increasing the surface area. The emulsified fat particles then face pancreatic lipase enzyme, which readily hydrolyzes the triglycerides into a monoglyceride and two free fatty acids (Morley and Kuksis, 1972). The hydrolyzed fatty acids then combine with bile salts to form a colloidal aggregate complex called a micelle (Hofmann and Borgstrom, 1964). Micelles are absorbed through the entire free surface of enterocytes by passive diffusion (Holman, 1979). However, the type of fatty acid included in the formation of micelle can affect fat absorption by enterocyte. For example, absorption rate decreases with increasing chain lengths of the fatty acid in the monoglyceride (Garret and Young, 1975). In the enterocytes, micelles dissociate and reform into triglycerides, phospholipids and cholesterols (Robin et al., 1971). Triglycerides are packaged into very low density lipoprotein called portomicrons (Bensadoun and Rothfield, 1972) and reach the liver through the hepatic portal system (Fraser et al., 1986). In contrast, mammals have larger

lipoprotein “chylomicrons” which reach the liver through the lymphatic system and eventually thoracic duct (Zilversmit, 1965).

Approximately 95% of the lipid synthesis in poultry occurs in the liver (O'Hea and Leveille, 1969). The abdominal fat depot is not a significant source of lipid synthesis (Leveille et al., 1975). In the liver the major lipid metabolism processes are lipolysis (breakdown of lipid into fatty acid), beta-oxidation (complete breakdown of fatty acid into two carbon units), and *de novo* lipogenesis (synthesis of fatty acid by the bird).

The net lipid content in birds that accumulate in the avian adipose tissue is derived from either plasma triglyceride-rich lipoproteins from fat sources present in the feed (80 to 85%) or *de novo* lipogenesis (10 to 15%) (Griffin et al., 1992). The common fatty acids present or added in poultry ration are listed in Table 1.1. However, the main fatty acids resulting from hepatic *de novo* synthesis are C 16:0, C16:1 ω-7, C18:0, and C18:1 ω-9 (Crespo & Esteve-Garcia, 2002). A summary of *de novo* synthesis and the metabolism of ω-3 PUFA and ω-6 PUFA are presented in Figure 1.2.

#### *1.2.2.3 Efficiency Estimation in laying hens*

Efficiency is defined as a state of performing or functioning in the best possible manner with the least waste of time and effort (www.dictionary.com). However, in animals, term feed efficiency is more often used and is calculated as the ratio of production weight (body gain or egg mass) to feed intake (Nordskog, 1972). In laying hens, the feed accounts for about 50 to 90% of egg production costs (Iddamalgoda et al., 1988). The feed efficiency can be increased either by reducing feed requirements for maintenance and activity, or by reducing behaviors causing feed wastage (Fairfull and Chambers, 1984).

Koch et al., (1963) first used the residual feed intake (RFI) to differentiate among animals for feed utilization efficiency. Residual feed intake is defined as the difference between the observed and expected maintenance energy (ME) intake after accounting for changes in growth, maintenance or egg production (Bordas et al., 1992; Schulman et al., 1994). In addition, RFI is also used as a measure of the overall energetic efficiency in laying hens (Flock, 1998). The main

cause of variation in feed efficiency is attributed to the variation due to the ME. The variation in ME is due to differences in physical activity, feathering density, basal metabolic rate, body temperature and body composition (Luiting, 1990). The partitioning ME requirements in laying hens has been suggested to be based on maintenance, growth, and production using following model:

$$MEI = aW^b (T) + cDW + dEM,$$

where MEI is ME daily intake, $W^b$ is metabolic body weight, DW is body weight change, EM is egg mass output, T is environmental temperature, a, c, and d are the maintenance, growth and production requirement coefficients, respectively (Sakamura, 2004).

Recently, Romero et al. (2009) proposed a nonlinear model to estimate residual maintenance requirement (RMEm) which provided an unbiased estimate of individual energetic efficiency of broiler breeders by adjusting the maintenance requirement for the effect of dietary thermogenesis. RMEm is the residual of the linear relationship between ME and feed intake. In addition, Romero et al. (2009) also reported that hens with greater RMEm efficiency partitioned more energy toward reproduction than those with lower RMEm. This model was developed for egg laying poultry and, although first publish in broiler breeders, applies equally well for table egg layers because it uses the same input for both bird types.

## 1.3 AVIAN REPRODUCTIVE SYSTEM

Table egg laying hens have now undergone genetic selection for reproductive efficiency for 60 years (Jones et al., 2001). Compared to other poultry types, White Leghorns in particular, are able to lay well because they have a highly organized system of follicular recruitment in the ovary (Johnson, 2000). The ovary is made up of the stroma, which is the base supporting structure that contains undifferentiated follicles, and small white follicles and the hierarchical yolky follicles ready for ovulation (Robinson et al., 2003).

The reproductive system of the female chicken consists of a left ovary and oviduct. The right ovary regresses during the embryonic stage (Johnson, 2000). The ovary and oviduct remain about 0.5 g until the sexual maturation process

commences and they undergo drastic increase in size (Renema et al., 1999). Timing of sexual maturity is affected by age, body weight (Brody et al., 1984) and photoperiod (Chen et al., 2007). Egg formation in laying hens is a multi-step process that is the result of a coordinated effort from many systems to supply both the raw materials and the hormonal control.

### 1.3.1 Hormonal Mechanism

External stimuli such as photoperiod and nutritional status triggering the hormonal mechanisms lead to egg formation. These stimuli activate the hypothalamic secretion of gonadotropin-releasing hormone (GnRH) (Sharp, 1993). GnRH induces the pituitary gland to form and release the reproductive hormones; follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Amin and Gilbert, 1970). FSH signals initiation of follicular growth in the ovary. The growing follicles secrete the ovarian hormones: androgen, progesterone and estrogen. A three-cell model for follicular steroidgenesis was proposed by Porter et al, (1989). According to this model, the progesterone is produced in the granulosa cells of large follicles; androgen is produced in the interstitial cells of theca interna of intermediate follicles and an estrogen is synthesized in theca externa.

Small ovarian follicles are the prime source of estrogen in laying hens (Robinson and Etches, 1986). Estrogens have a significant role in oviduct proliferation, vitellogenesis and mating and nesting behavior (Johnson, 2000). Androgens regulate the sexual behavior and are involved in albumen formation. Progesterone is secreted by the granulosa cells of large follicles and, when not being converted to esterogen in immature large follicles, is released into the blood and triggers the ovulation process. Progesterone stimulates a surge in luteinizing hormone (LH) from the pituitary (Furr et al., 1973; Etches and Duke, 1984). LH stimulates the production of androgen and estrogen in theacal cells and also in the rupture of follicle to release the ovum (Filicori et al., 2002). Other hormones involving the egg production includes adrenocorticotropic hormone (ACTH) and thyroid stimulating hormone (TSH). The ACTH acts on the cells of the adrenal gland stimulating them to produce corticosterones, while TSH stimulates the

thyroid gland to secrete thyroxine. Corticosterones and thyroxine are involved in energy metabolism during initiation and maintenance of egg production (Davis et al., 2000; Siopes, 2007; Groothuis and Schwabl, 2008).

### 1.3.2 Egg Formation

Only the left ovary and oviduct are functional in poultry. The embryonic left ovary has a higher number of estrogen receptors which counteract the effect of Müllerian inhibiting substance responsible for regression of the right ovary at the embryonic stage (Hutson et al., 1981). Following sexual maturity, the ovary of an active laying hen has a hierarchy of yolk follicles that are very uniform in composition (Renema et al., 1999). Once the follicles grow to an approximately 9 mm in diameter, they enter the preovulatory hierarchy. Growth from 3 to 5 mm takes 3 days, from 5 to 8 mm takes 2 days, and from 8 mm to ovulation (40 mm) takes 6 days (Gilbert et al., 1983). The largest follicle is designated as F1 (about 40 mm) and is first to ovulate (Gilbert et al., 1983). At ovulation, the yolk enters the oviduct, where the albumen and shell are added over approximately 24-25 hr period (Table. 1.2) (Roberts, 2000).

### 1.3.3 Egg Quality

The albumen height is important criteria for analysis of internal quality of egg (Silversides et al., 1993). Extended storage time and higher storage temperature decrease the albumen height, and thus degrade the internal quality of the egg (Scott and Silversides, 2000; Raji et al., 2009). However, feeding enriched diets (ω-3 PUFA) did not influence the albumen height or internal quality of the egg (Novak and Scheideler, 2001; Bean and Leeson, 2003; Hayat et al., 2009). The egg shell quality can be measured as egg specific gravity, shell breaking strength, shell weight or shell thickness. The age of strain and hen, environmental and nutritional factors may influence the shell strength (Roberts, 2000).

### 1.3.4 Vitellogenesis

Vitellogenesis is the process of yolk formation through deposition of yolk specific proteins and fats. The process is triggered by estrogen, which rises 10 to 15 fold in the blood serum during sexual maturation (Amin and Gilbert, 1970). Estrogen stimulates the liver to synthesize lipids and proteins that will be

packaged as lipoproteins and transported through the blood to the ovary and for incorporation into the growing oocyte **(**Walzem et al., 1999). The major yolk precursors in poultry are two lipoproteins: vitellogenin (VTG) and yolk-targeted very-low-density lipoprotein (VLDLy); both are synthesized by the liver (Aydin, 2005). Vitellogenin contributes 25% of the yolk solids, 48% of the protein and 7% of the lipids (Speake et al., 1998). In contrast, VLDLy provides much more lipid to the ovarian follicles. The VLDLy is a specialized particle that provides about 60% of the yolk solids, 22% of the protein and 93% of the lipids (Speake et al., 1998). Triglycerides make up approximately two-thirds of the total lipid in yolk VLDLy. Once transported to the developing ovarian follicles, the triglyceride-rich VLDLy will be used as the energy source for the developing embryo (Wallace, 1985).

The VLDLy particles secreted by laying hens for yolk deposition have some very specific structural and biochemical differences from regular VLDL that allow them to be transported safely from liver to the ovary rather than be used to fuel cellular metabolism or be used in a fat depot (Bacon et al., 1978). First, the regular VLDL has at least six apolipoproteins (including apoA-I, apoB and apoC) (Chan et al., 1976; Kudzma et al., 1979; Lin et al., 1986). In contrast VLDLy has specific apoliprotein (VLDL-II) on its surface in addition to the standard apoliprotein B found on regular VLDL particles (Nimpf et al., 1988). The apoliprotein VLDL-II found on each VLDLy particle prevents hydrolysis by lipoprotein lipase enzymes (Aydin, 2005). Second, it has a much smaller and more uniform size than that of regular VLDL. The small size of about 25 to 30 nm allows yolk VLDL to cross the basal lamina surrounding the ovarian follicles (Evans et al., 1979), as well as pass through the interstitial space between individual granulosa cells (Griffin and Perry, 1985).

The uptake of both hepatically synthesized lipoproteins (VLDL and VTG) is mediated through 95-kDa receptors which have ability to bind with apoB of VLDL and the lipovitellin part of vitellogenin (Nimpf and Schneider, 1991; Elkin and Schneider, 1994). However, this receptor-mediated transport can be

influenced by inclusion of specific dietary fatty acids or lipid synthesis enzymes activities.

Dietary manipulations of the poultry ration can influence the amount of saturated and unsaturated fatty acid that ultimately ends up in egg yolk (Milinsk et. al., 2003). In addition, Hermier et al., (1996) suggested that the VLDLy synthesis and transport and steroyl-CoA desaturase enzyme activity limits the lipid exportation to liver. The positive correlation of blood plasma VLDL concentration with steroyl-CoA desaturase enzyme activity increases the amount of monounsaturated fatty acids in plasma as well as in yolk (Legrand & Hermier, 1992). In contrast, linoleic acid (LA) tends to decrease the amount of VLDL and LDL in rabbits (Lee et al., 1994) due to inhibitory action for stearoyl-CoA desaturase enzyme (Ntambi et al., 1999). Moreover, the reduction in amount of yolk in birds fed ω-3 PUFA enriched diets may be associated with the difficulties in transport of ω-3 PUFA laden VLDLy to the ovary (Walzem, 1996; Van Elswyk et al., 1997). The final yolk fatty acid composition is the culmination of differences in dietary fatty acid composition, rate of total fatty acid synthesis in the liver and activities of enzymes like desaturase, elongase and transferase in the liver (Walzem, 1996).

## 1.4 BIOCHEMISTRY OF FATTY ACIDS

### 1.4.1 Basic Structure & Nomenclature

Fatty acids consist of a long hydrocarbon chain structure with a carboxyl group (COOH) at one end and methyl group ($CH_3$) at other end (Figure. 1.3). Fatty acids are the main component of fats and oils and are added to the poultry ration either directly or as a part of the grain or oilseed feed ingredients. There are three nomenclature systems for fatty acids. First, the IUPAC or standard chemistry nomenclature involves counting from the carboxyl carbon (which is numbered 1) and giving the fatty acid a Greek-based name. Second, the carboxyl-reference system counts the same as the IUPAC method, but uses a number to denote length of carbon chain rather than a Greek word (For example: C 18:0 instead of octadecanoic acid). The omega-reference system is most recently

developed naming system. It begins counting from the omega carbon at the methyl end of the fatty acid chain (which is numbered 1) rather than from carboxyl end. For example, The IUPAC name for linoleic acid is 9, 12-octadecanoic acid but omega (**ω**) nomenclature it is designated as C18:6 ω-6. The omega-reference system works best for describing the fatty acids in the ω-3 and ω-6 families; an important area of research in the dietary enrichment of poultry diets and products.

### 1.4.2 Metabolism of Omega-3 Polyunsaturated Fatty Acids

The classification of PUFA can be done on the basis of position of the first double bond from the omega end of their chemical structure. The ω-3 PUFA and ω-6 PUFA are the most important groups of omega family because they are essential fatty acids that cannot be synthesized by birds or mammals. Linolenic acid (18:3 ω-3; LNA), and linoleic acid (18:2 ω-6; LA) are precursors for other long chain polyunsaturated fatty acids (LC PUFA) (Schmitz and Ecker, 2008). The metabolites of LNA are eicosapentaenoic acid (20:5 ω-3, EPA), docosapentaenoic acid (22:5 ω-3, DPA) and docosahexaenoic acid (22:6 ω-3 DHA). Their chemical structures are shown in Figure 1.4. For the ω-6 PUFA, LA is a precursor for arachidonic acid (20:4 ω-3, AA). Dietary ω-6 PUFA is plentiful in the poultry diet due to inclusion of sources like corn and many of the dietary oils. The ω-3 PUFA are limited in commercial poultry rations, making dietary enrichment an ideal way to provide adequate levels in the poultry diets, as well as for the consumers eating the poultry products. However, poultry rations formulated with canola oil do contain some ω-3 PUFA due to presense of 10% LNA in canola.

The metabolism of ω-3 PUFA and ω-6 PUFA proceeds using two mechanisms: (1) beta-oxidation; which takes place in the mitochondria and peroxisomes, or (2) desaturation and elongation (Figure 1.2). Most β-oxidation occurs in the mitochondria where mitochondrial enzymes regulate lipid metabolism, i.e. oxidation and synthesis of long-chain fatty acids (Bartlett and Eaton, 2004). However, β-oxidation of very long chain fatty acids occurs in the peroxisome as fatty acids greater than 22 carbons in length cannot enter the

mitochondria. Most of elongation of fatty acids occurs on the cytosolic surface of smooth endoplasmic reticulum of the cells, whereas the desaturase system is located in the membrane of smooth endoplasmic reticulum (Salway, 1999).

The bioconversion of the 18 carbon LNA and LA to longer chain ω-3 or ω-6 PUFA respectively, is limited due to a shortage of the enzymes to complete the initial step. The initial addition of a double bond to these fatty acids requires delta (Δ)-6 desaturase enzyme. The Δ-6 desaturase enzyme is limited in supply, thus making it a rate limiting step for bioconversion into long chain metabolites (Yamazaki et al., 1992). The Δ indicates that the double bond is created at a fixed position from the carboxyl group of a fatty acid. For example, Δ9 desaturase, Δ6 desaturase and Δ5 desaturase enzymes creates a double bond at the $9^{th}$, $6^{th}$ and $5^{th}$ position from the carboxyl end respectively to create double bond (Nakamura and Nara, 2004). The elongase enzyme is involved in the addition of acetate units to the carboxyl end of the fatty acid. The surrogate measures of desaturase and elongase activities can be estimated from ratio of product to the precursor of the fatty acids involved (Warensjo et al. 2008). The values of these indices are an effective way to approximate expression of desaturase and elongase enzyme activities. These methods have been frequently used in a variety of human and animal studies to estimate enzyme activities (desaturases and elongases) involved in ω-3 PUFA and ω-6 PUFA metabolism (Kinsella, 1991; Pan et al., 1995; Okada et al., 2005; Zhang et al., 2007; Betti et al., 2009; Ntawubizi et al., 2010).

Plants, unlike most animals, have a Δ12 desaturase and a Δ15 desaturase enzyme that facilitate the synthesis of ω-6 and/or ω-3 series of fatty acids (Harwood, 1988; Sayanova et al., 2006). In animals, even though the LNA and LA compete for the same enzymes for their bioconversion into longer chain PUFA, LNA is more efficiently converted than LA (Kinsella, 1991). LNA suppresses ω-6 PUFA bioconversion ten times stronger than LA can suppress ω-3 PUFA bioconversion (Mohrhauer and Holman, 1963). Despite this preferential conversion, the biosynthesis of LNA can still be reduced when LA is more plentiful in diets (Emken et al., 1994; Cherian, 2007). Further, Gerster (1998) suggested that this suppression can reach as high as upto 50% in humans.

## 1.5 OMEGA -3 AND LUTEIN

### 1.5.1 Sources of enrichment

Two of the most common types of enrichment in value added eggs are ω-3 PUFA and lutein (Leeson et al., 2007). Ingredients that can be included in the hen ration as sources of ω-3 PUFA are flaxseed, fish oil, menhaden oil or fish meal, vegetable oils such as canola and soybean, and marine algae (Van Elswyk, 1997). Lutein is supplied through addition of a commercial marigold extract to the hen diet (Hadden et al., 1999).

Flaxseeds contain 40% fat, 58% of which is ω-3 PUFA (Cunnane et al., 1993; Gonzalez-Esquerra and Leeson, 2000). However, in addition to being a rich source of ω-3 PUFA, the raw flaxseed also contains mucilage, phytic acid, antipyrodoxine, trypsin inhibitors and hydrocyanic acid (Madhusudhan et al., 1986; Bhatty, 1993). Many of these components have a negative effect on the feed intake and growth of birds due to their contribution to high viscosity of the gut chyme and irritation of the gut, which results in a reduced AME in diet (Leeson et al., 2000; Novak and Scheideler, 2001). Of these antinutritional factors, the mucilage associated with the hull of flaxseed has greatest impact on the birds. The flaxseed mucilage is primarily a mixture of polysaccharides which can be divided into an acidic and a neutral fraction (Erskine and Jones, 1957). Acid-catalyzed hydrolysis yields rhamnose, fucose, galactose, and galacturonic acid, whereas the neutral fraction consists of arabinose and xylose (Hunt and Jones, 1962). The neutral polysaccharide fractions allow a high water-hydration capacity and thus contribute to higher intrinsic viscosity than the acidic counterpart (Bhatty and Cherdkiatgumchai, 1990; Fedeniuk and Biliaderis; 1994).

The increased viscosity at the gut level decreases the rate of diffusion of substrates and digestive enzymes and hinders their effective interaction at the mucosal surface (Edwards et al., 1988). The gel-like condition of the chyme caused by increased viscosity reduces the mixing of sugars, amino acids, and other nutrients which results in reduced digestion of fats, proteins and carbohydrates (Jaroni et al., 1999). Apart from causing direct impairment of nutrient absorption, the high gut viscosity also stimulates increased proliferation

of anaerobic microflora (Choct et al., 1996). This change in microflora ecology from normally aerobic microbes to increased anaerobic microbes can lead to production of toxins and deconjugation of bile salts which leads to decreased fat digestibility in the small intestine (Carre et al., 1995).

Another possible issue with the feeding of flax is a lower shell thickness resulting in reduced shell quality. In studies where shell thickness has been reduced with the feeding of flax, it has been proposed that calcium absorption may be impaired due to increased digesta viscosity associated with the flax mucilage (Scheideler, and Froning, 1996; Jia et al., 2008). Not all short- or long-term flax feeding studies have resulted in a negative impact on shell thickness, however (Caston et al., 1994; Novak and Scheideler, 2001; Bean and Leeson, 2003).

Flax is usually included in the poultry diet in the ground form. However, modified processing of flax has been demonstrated to reduce the negative attributes of flax. The use of extruded rather than ground flaxseed for ω-3 PUFA enrichment minimizes the impact of anti-nutritional factors associated with flaxseed (Thacker et al., 2005). Co-extrusion of flaxseed with peas at a temperature of 135°C and pressure of 2,750 kPa with a single screw extruder has been reported to increase the energy digestibility of both flax and peas in both pigs (Htoo et al., 2008) and in broiler chickens (Thacker et al., 2005). The extrusion process significantly reduces the antinutritional factors associated with ground flaxseed, including mucilage (Wu et al., 2010), phytic acid, tannins, trypsin inhibitors (El-Hady and Habiba, 2003), and hydrocyanic acid (Wu et al., 2008). These changes allow the extruded flax to be added to the hen ration at a lower level but generates greater amount of ω-3 PUFA enrichment in the products. For example, a hen diet including 15% of the extruded flaxseed and peas (1:1wt/wt), which delivers approximately 7.5% flax, results in same level of total ω-3 PUFA enrichment with the inclusion of about 12% of ground flax (Jia et al., 2008).

Lutein is a carotenoid found in egg yolk (Figure.1.5). Its two oxygenated rings make it more lipophilic and therefore potentially more readily absorbed in

the gastrointestinal tract of hen compared to carotenes (no oxygen) (Olson et al., 2008). In the poultry ration, lutein can be added as a premix prepared from an extraction of marigold (*tagetes erecta*) (Hadden et al., 1999). In its natural state, lutein is esterified, and mainly to lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), and stearic fatty acid (C18:0) (Breithaupt et al., 2003). However, the laying hens efficiently deposit lutein in the free form in the egg yolk irrespective of its saponified or esterified state in the feed (Breithaupt, 2007). This fact allows the freedom to analyze the egg yolk samples without saponification during laboratory analysis compared to other plant sources where analysis should include saponification in order to calculate the free lutein concentration.

### 1.5.2 Benefits for the Bird

Fat-based enrichment ingredients like flaxseed, added to poultry diet usually alter the fatty acid composition of the egg yolk (Skellon and Windsor 1962). Flax enrichment directly supplies LNA, but also triggers an incremental increase of LC ω-3 PUFA (EPA, DPA and DHA) in the birds. In addition to supplying these essential fatty acids, increased LNA intake can also affect aspects of the immune system. The higher LNA amount increases immunoglobin production (IgG) in the hatchling, allowing the hens to provide a greater passive immunity (Wang et al., 2004). Additionally, a higher LNA (low LA-to-LNA ratio) in diet has been reported to increase antibody production in broilers (Friedman and Sklan, 1995). Increased lutein intake impacts the immune response of the laying hen as well, likely through boosting secondary antibody activities (Bedecarrats and Leeson, 2006). Lutein also has an antioxidant effect in the hen that can lead to reduced liver hemorrhage in birds (Leeson et al, 2007), thereby reducing the incidence of fatty liver hemorrhagic syndrome.

### 1.5.3 Benefits for Humans

According to the International Society for the Study of Fatty Acids and Lipids (ISSFAL, 2004) the amount of LNA in the human diet should be 1% of total energy intake from dietary fat (2,000 cal/day). More specifically, 2.22 g/day should be in the form of LNA and 0.65 g/day as EPA and DHA. The ω-3 PUFAs contribute significantly to increased protection from various cardiovascular,

neurological and rheumatological disorders (Yashodhara et al., 2009). A summary of the dietary effects of ω-3 PUFA in humans is presented in Table 1.3. Many of these health effects are associated with the long chain ω-3 PUFA like DHA, DPA and EPA. Less is known about the health effect of LNA, a medium chain ω-3 PUFA which is precursor of the long chain ω-3 PUFAs. While LNA has not been attributed with as many health promoting aspects as EPA, DPA or DHA with regard to cardiovascular diseases, there is a lack of research regarding the role of LNA in this area. The majority of ω-3 PUFA research for human benefits had been conducted in fish, where LNA is not the primary ω-3 PUFA present.

The typical diets of modern Western society are highly unbalanced in terms of ratio of ω-6 PUFA to the ω-3 PUFA (15-20:1 instead of the recommended 1-4:1) (Simopoulos, 2006). The higher content of ω-6 PUFA in the diet can lead to physiological anomalies in body such as increased blood viscosity, vasospasm, vasoconstriction, and decrease in blood clotting time, with these conditions being even more pronounced in people suffering from chronic disease (Benatti et al., 2004). Thus, the lower ratio of ω-6 to ω-3 PUFA in diets can contribute to reduction of chronic diseases, including coronary heart disease, diabetes, arthritis, cancer, osteoporosis and age related macular degeneration (Simopoulos, 2008). This does not imply that ω-6 PUFA are harmful for humans, but the ratio of ω-6 PUFA to the ω-3 PUFA should not be out of balance, as it is in Western diets.

Lutein helps to protect the retina from photo damage by filtering out blue wavelength light (Junghans et al., 2001). Research shows that chronic exposure to blue wavelength light; which is most energetic within the visible spectrum, damages retina cells and is associated with the age-related macular degeneration (Junghans et al., 2001; Shaban and Richter, 2002). Lutein can also act as an antioxidant for retinal cells and protects them from oxidative stress (Hogg and Chakravarthy, 2004; Landrum and Bone, 2001).

Nutrient bioavailability is determined by the amount of ingested nutrient that is absorbed by the intestinal tract and used in metabolic process and storage (Jackson, 1997; Wu et al., 2009). Lutein is present in a lipid matrix in egg yolk,

which contributes to it being the highest bioavailable source to humans compared to other lutein supplements or vegetable sources (Handleman et al., 1999; Chung et al., 2004).

### 1.5.4 Omega-3 Egg: Challenges for Industry

Egg-market survey results suggest that in countries like Canada, Australia, Japan and England, people are prepared to pay a premium for ω-3 PUFA enriched eggs (Pickering, 2003). The egg industry can capitalize on consumer preferences by formulating diets for chicken to create specialty eggs that have the potential to contribute to human health. Increasing the existing enrichment methods can also increase the economic profitability of this sector.

Health Canada (1990) restricts labeling a product as a source of ω-3 PUFA, to those that contain at least 0.3 g of ω-3 PUFA per 50g egg. To enrich the egg for ω-3 PUFA, producers need to add an ω-3 PUFA enriched dietary source in the poultry feed and therby the cost of production of eggs goes up. The addition of 15% flaxseed in layer ration results in about 12% increase in total cost of production (Betancourt and Diaz, 2009).

A potential problem is that due to biological variability in ω-3 PUFA deposition in the eggs, uniformity of the end product is not ensured. Due to differences in feed intake, absorptive conditions and metabolic rate, the amount of ω-3 PUFA ultimately deposited in the egg varies among birds and may also vary among eggs from individual birds. Much of the research and most product testing are done on pooled egg samples, therefore reflecting mean enrichment values. Variability in end product may be ameliorated by increasing the absorption efficiency of value added ingredients through modification of poultry feed or gastrointestinal health. The nutritional treatments to increase flock performance parameters generally do not affect all birds evenly. The nutritional additives help the poorly performing birds or flocks to a greater extent, bringing them closer to the level of already high performing birds. This is likely partly due to differences in the absorbtive capacity of the gut based on surface condition and gut health. As a result, one outcome of treatments to improve enrichment ingredient transfer would be more uniform enrichment of eggs.

## 1.6 EFFICIENCY OF ENRICHMENT AND TRANSFER TO EGG

If minimum enrichment levels can be ensured in value-added eggs with lower levels of enrichment ingredients in the base feed, it will thereby lower the cost of production. Before revising the feeding strategies for enrichment of poultry products through dietary modulation, it will be of utmost importance to consider the bird's absorptive physiology. For example, even the different breeds of chicken (broiler and layer) have physiological differences which can significantly affect the nutrient utilization. The layers have higher fat mobilization efficiency, longer feed intake time, and higher ME compared to broilers (Table 1.4). Strategies to increase absorption could be found in methods to slow nutrient transport time. Adding whole wheat to broiler diets, for example, has been found to cause longer residence in intestinal tract resulting in greater integration of digestive juices and feed, thereby increasing the energy and protein availability (Amerah et al., 2008; Del Alamo et al., 2009). Similar results to inclusion of whole cereals was noted with the addition of wood shavings to layer diets, which resulted in increased gizzard weight and increased gastro-duodenal reflux (Hetland et al., 2003). A greater understanding of the interactions of value added ingradients with internal mechanism of bird would help in understanding of how to maximize enrichment in the eggs.

### 1.6.1 Effect of Dietary Modulation on Microflora

Changing dietary compostion or ingredients can lead to increased nutrient utilization efficiency due to shifts in gut microbial communities and gut morphology (Choct, 2009). These changes can be capitalized on maximize uniformity of egg enrichment as well as level of enrichement. The intestine of the chicken hosts beneficial microbes which facilitate the nutrient recovery from dietary substrate through microbial fermentation and help gut development **(**Tellez et al., 2006). For example, butyric acid is a product of microbial fermentation, acts as an antimicrobial for pathogenic bacteria (Ricke, 2003) and as an energy source for epthilalial cells in the lower intestine (Van Immerseel et al., 2004). In addition to dietary ingredients, litter condition and water and feed quality can affect microbial composition of chicken GI tract either by potentially

providing a continuous source of harmful bacteria or by negatively affecting the gut environment and the immune system of the birds (Apajalahti et al., 2004). The various microbial types attach to the gastrointestinal epithelium with differing efficiencies (Edelman et al., 2003). Gastrointestinal microflora is very diverse even within the same species. The differences may be influenced by physio-chemical conditions in the gastrointestinal tract and are prone to rapid change due to sudden stresses, diseases or even diet changes (Yu et al., 2004; Rehman et al, 2007a).

Dietary manipulation targeted to favor the symbiotic gut microflora and to reduce the competitiveness of beneficial microbes with harmful microbes for nutrient utilization may boost development of the gut. Enzymes, probiotics and prebiotics are common feed additive in poultry diets (Tellez et al., 2006). The some of feed additives (probiotics and prebiotics) limit the concentration of pathogenic bacteria and thereby reduce the local inflammation to intestinal mucosa and reduce intestinal thickening (Van-Immerseel et al., 2002). This results into increased digestibility of feed ingredients (Apajalahti et al., 2004). Therefore, the feeding strategies targeted to increase digestibility in presence of favorable microbes should be balanced with the negative effects resulting from the excessive number of microbes to the gut surface.

Dietary mannanoligosaccharide (MOS) is an example of an alternative to antibiotic growth promoters. It has poor digestibility and thus bacteria bound to them are directly excreted out in feces (Spring et al., 2000; Hill et al., 2009). The MOS, therefore, maintains functional integrity of intestine and thus increases the villus height (jejunum), increases digestive enzymes (Iji et al., 2001). The nutrient and energy-saving effects of MOS on the gut indirectly stimulate the proliferation of microflora and assists in gut development (Yang et al., 2008; Yarim et al 2009).

In addition to feed ingredients (enzymes, probiotics and commercial additives), the feed form also affects the microflora in the gastrointestinal tract of chicken (Engberg et al., 2002). The increased particle size of grain decreases pH at gizzard and increases the pH of small intestine (Engberg et al., 2004). For

example, feeding whole wheat to broiler chickens reduces the pH of gizzard, which in turn prevents the impact of non-acid tolerant bacteria (*Salmonella typhimurium* and *Clostridium perfringens*), in the intestinal tract of the birds (Bjerrum et al., 2005). The altered intestinal microflora through dietary modulation can influence the ability of the birds to digest and absorb dietary nutrients.

### 1.6.2 Effect of Dietary Modulation on Gut Function

The intestinal cells require nutrients to support their proliferation and differentiation as well as the secretion of enzymes and proteins. The GI tract of avian species exhibits considerable flexibility in the face of altered food composition and levels of intake (Karasov, 1990). The absorptive capacity of the intestinal tract of the chicken is influenced by digesta retention time, rate of absorption, total gut surface area, and gut volume (Karasov, 1990). The villi can increase or decrease their length and width and alter surface area. Long villi, for example, are an indicator of excellent gut health and high absorptive efficiency (Caspary, 1992).

In the chick, extensive increases in villi number and activation of digestive enzymes and absorption pathways occur in first 7 days following hatch (Sklan, 2001). The intestinal lumen of the duodenum and jejunum has the ability to shrink during fasting and enlarge again on refeeding due to changes in villus length. Yamauchi (2002) explained that the alteration in villus morphology due to fasting and refeeding suggests that villus morphology is responsive to the type of feed ingredients present for the intestinal absorption. In addition, the villi structure varies in response to the type of nutrient in the diet. For example, an inulin-containing diet results in longer jejunal villi and deeper crypts than control birds without affecting the ratio of villus length to crypt depth (Rehman, 2007b).

Dietary fat content can impacts gut motility and specific cellular functions. The retention time of digesta decreases with increased dietary fat content (Mateos et al., 1982). In addition to the presence of fat, the fatty acid composition of the diet can affect enterocyte dynamics. For example, ω-3 PUFA increases goblet cell number and mucin production, and enhances repair of damaged intestinal tissue

(Koutsos and Arias, 2006). Oxidized poultry fat results in increased intestinal epithelial cell turnover and increased proliferation of hepatocytes; which leads to increased bile synthesis for fat absorption (Dibner et al., 1996). Lipid absorption has been found to be most negatively affected by increased digesta viscosity in chickens due to a reduction in the absorption of free fatty acids (Maisonnier et al., 2001). Dietary modification can alter digestive and absorptive process of intestine and thereby can influence the utilization, metabolism and transfer of nutrients from the diet to the egg. For example, high doses of vitamin E in the diet not only decrease its own transfer efficiency from feed to egg (Galobart et al., 2001) but also interfere with intestinal absorption of long chain PUFA (Meluzzi et al., 2000).

The interaction of dietary components with the intestinal microflora, digestive enzymes and intestinal structures determine the efficiency of absorption and transfer of basic building blocks to the bird. The complex interaction between the components of intestinal ecology can hinder the process of egg enrichment. Likewise, explaining specific strategies to improve passage of enrichment ingredients from the diet to the egg could increase the effectiveness of absorption and depositing processes relating to these ingredients.

## 1.7 RESEARCH APPROACH

There is increasing interest in ω-3 PUFA enriched eggs because of their potential health benefits for the consumer. Egg enrichment is a research area with a lot of potential because of the high bioavailability of enrichment ingredients to the humans through the egg components. Modifying egg lipid profiles is an avenue of research with good potential in generating new enriched egg products.

Research focused on the enrichment process of the egg for the production of value-added eggs through dietary manipulation of hen ration is a growing area. If the inclusion of value added ingredients in poultry ration increases the cost of production, it can be a disincentive for its use by the egg industry. Additionally, detrimental effects on gut condition or other negative effects on bird health and well being would further reduce motivation for its use. Even if a premium can be

charged for the enriched eggs, it may not be of much value if the enrichment process leads to welfare concerns in birds. The bird to bird variability in nutrient absorption can compound these issues.

The extra cost for value added ingredients can be minimized by achieving target of uniformity in absorption and uniformity of enrichment in end product. The ensemble of value-added ingredients to poultry ration should be targeted to improve efficiency of absorption of key ingredients (ω-3 PUFA, lutein etc) and their transfer to egg with no negative effect on growth and production.

### 1.7.1 Objectives

The overall objective of my research was to increase the effectiveness of the value-added egg enrichment process. Improving the process can reduce the variability among enriched egg by increasing the bird's absorption efficiency. These goals were addressed through a series of experiments:

**Experiment 1: <u>Purpose</u>:** To explore alternate laying hen feeding methods to enrich lutein and ω-3 PUFA deposition in table eggs.

**<u>Description</u>:** The study was designed to assess the variation in egg enrichment with ω-3 PUFA and lutein when provided alone in the diet, in combination, or when fed in alternate day patterns. The idea was to reduce the absorptive competition among these enrichments at gut level. A group of 56-wk-old, individually caged Lohmann White Leghorn layers were subjected to dietary treatments with flax and lutein for 60 d. Egg traits were determined and yolks collected at 0 d, 14 d, 28 d and 56 d. The fatty acid composition of the egg yolk and yolk carotenoid profile was analyzed. Laboratory methods were standardized for estimation of lipid and carotenoid concentration in egg yolk. Eggs from 56 d were stored at 4 C for 30 d for assessment of oxidative stability.

**Experiment 2: <u>Purpose</u>:** To explore the effect of metabolic efficiency and intestinal morphometry on variability in enrichment of eggs with ω-3 PUFA in laying hens.

**<u>Description</u>:** Hens were scored as "Efficient" or "Non-Efficient" through calculation of residual maintenance energy requirement (RMEm), which was the difference between observed and predicted maintenance requirements relative to

ME intake. Core temperature telemetry devices were surgically implanted into 20, 56 week old Lohmann White Leghorn laying hens to calculate the energetic efficiency. Birds were then provided a ω-3 PUFA enriched diet for 14 days. Egg traits were determined and yolks collected at 0 d and 14 d, and egg lipid profile was determined. At 14 d, birds were sacrificed to collect gut length and histomorphometric indices.

**Experiment 3: <u>Purpose</u>:** To characterize the omega-3 PUFA enrichment process in laying hens using an extruded flax enrichment source.

**<u>Description</u>:** This study was designed to explore the time required to reach a plateau of ω-3 PUFA concentration in blood plasma and egg yolk in laying hens fed an extruded flax product. Additionally, the effect of enriched diets on the calculated enzymatic action required for the biosynthesis of long chain ω-3 PUFA was investigated. Lohmann White Leghorn layers were divided into three groups (25/group) at 65 wks of age, and placed on a control diet, a moderate or a high omega-3 diet for 18 days. Baseline values were established for, the BW, fatty acid composition in feed, blood plasma and egg yolk prior to dietary treatment. Data was analyzed with Proc Mixed of SAS and broken stick analysis to determine ω-3 PUFA plateau using the NLIN procedure of SAS.

729 **Table 1.1: The classification of various fatty acids present in or added to poultry ration.**

| Common Fatty acid | | | | | | | |
|---|---|---|---|---|---|---|---|
| Saturated | | Unsaturated | | | | | |
| | | Monounsaturated | | Polyunsaturated | | | |
| | | | | Omega-6 | | Omega-3 | |
| Caproic acid | 6:0 | Myristoleic acid | 14:1 ω-7 | Linoleic acid | 18:2 ω-6 | Alpha-linolenic acid | 18:3 ω-3 |
| Caprylic acid | 8:0 | Palmitoleic acid | 16:1 ω-9 | Arachidonic acid | 20:4 ω-6 | Eicosatrienoic acid | 20:3 ω-3 |
| Capric acid | 10:0 | Oleic acid | 18:1 ω-9 | Dihomo-γ linolenic acid | 20:3 ω-6 | Eicosapentaenoic acid | 20:5 ω-3 |
| Lauric acid | 12:0 | Gadoleic acid | 20:1 ω-9 | Arachidonic acid | 20:4 ω-6 | Docosapentaenoic acid | 22:5 ω-3 |
| Myristic acid | 14:0 | Erucic acid | 22:1 ω-9 | Docosadienoic acid | 22:2 ω-6 | Docosahexaenoic acid | 22:6 ω-3 |
| Palmitic acid | 16:0 | | | Adrenic acid | 22:4 ω-6 | | |
| Stearic acid | 18:0 | | | | | | |
| Arachidic acid | 20:0 | | | | | | |

730 The first number donates the number of carbon in chain of fatty acid and second number donates the number of double bond in fatty acid. For
731 example, In Oleic acid, 18:1 ω-9 represents the fatty acid structure has 18 Carbon and one double bond at 9$^{th}$ Carbon from the terminal CH3 end
732 (ω) of the carbon chain of fatty acid. (Adapted from Leskanich and Noble, 1997)

733 **Table 1.2: The regions of the oviduct involved in the egg formation**

| No. | Regions of Oviduct | Duration | Function |
|---|---|---|---|
| 1 | Infundibulum | 15 min | Receives ovum from ovary |
| 2 | Magnum | 3 hrs | Inner and outer shell membranes |
| 3 | Isthmus | 1 hrs | Albumen is secreted |
| 4 | Shell Gland | 20 hrs | Plumping and calciferous shell added |
| 5 | Cloaca | 1 min | Oviposition |

734 Adapted from Roberts, 2000.

735 **Table 1.3 The effect of omega-3 polyunsaturated fatty acid in various chronic**
736 **diseases of humans.**

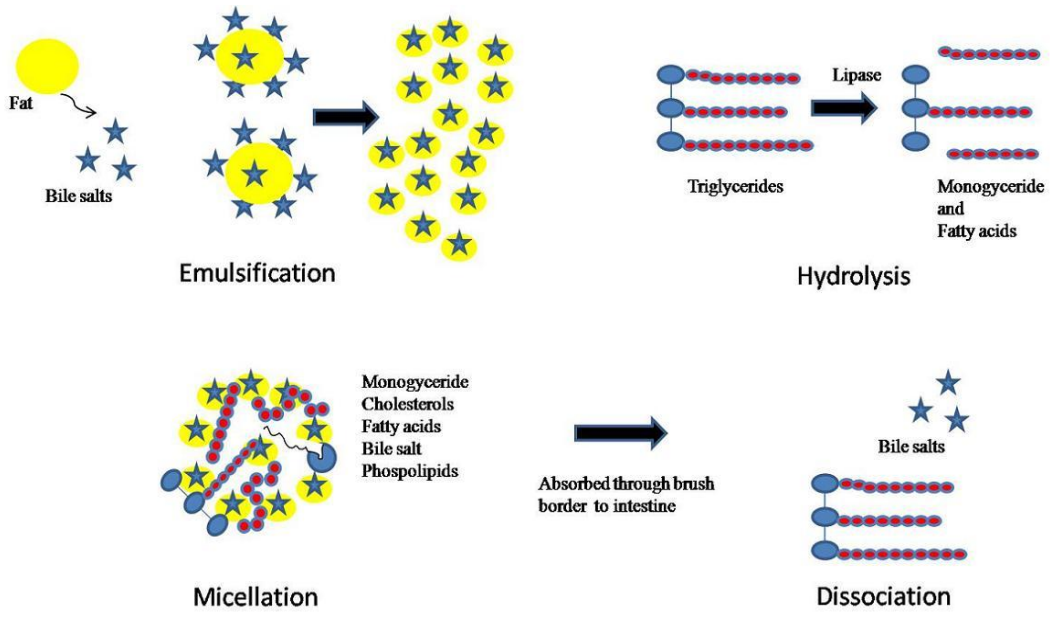
| Disease/condition | Role of omega-3 | Reference |
|---|---|---|
| Cardiovascular disorders | Antiarrhythemic | Marik and Varon, 2009 |
| | Reduction in platlet density | Tremoli, 1995 |
| | Anti-hypertensive (reduce blood pressure) | Cherian, 2007; |
| Dyslipidaemia | Reduce plasma triglyceride | Celebi and Macit, 2008 |
| | Reduce LDL | Griffin et al., 2006 |
| Atherosclerosis | Reduction of plasminogen activator inhibitor | Kinsella, 1990 |
| | Increased endothelial function (flow-mediated dilation) | Morgan et al., 2006 |
| Neurological disorders | Fetal brain development and visual acuity | Lewis et al., 2000; Koletzko, 2007 |
| Immunological disorders | Effective in treatment of autoimmune diseases | Simopoulos, 2002 |
| | Increased indices of immune response (lymphocyte proliferation, natural-killer-cell activity, cytokine production, and delayed-type hypersensitivity) | Kelley et al., 1989; Barone et al., 1989; Meydani et al., 1993; |
| Rheumatological conditions | Acts as anti-inflammatory agent | Galarraga et al., 2008 |

737

738 **Table 1.4: Physiological differences between broiler and layer chickens.**

| No. | Parameters | Broiler | Layer | Reference |
|---|---|---|---|---|
| 1 | Lipid metabolism (Embryonic stage)) | 27% greater yolk utilization | Poor energy conversion efficiency | Sato et al., 2006 |
| 2 | Protein metabolism (Embryonic stage) | Lower | 7 % greater protein synthesis per day | Muramatsu, et al., 1990. |
| 3 | Feeding Pattern | Less time for intake and greater resting period | More time for feed intake and lesser resting period | Masic et al., 1974 |
| 4 | Metabolizable Energy (/g feed) | Lower | 2.5 % Higher | Sibbald and Slinger, 1963 |
| 5 | Protein Accumulation Efficiency (muscle) | Higher (61.6-66.3%) | Lower (17.1-32.4%) | Maeda et al., 1990 |
| 6 | Fat deposition | 4-fold greater | Greater fat mobilization efficiency | Griffin, et al., 1991. |
| 7 | Mean Digesta Retention Time | Longer in crop and gizzard | Longer in duodenum and ilium | Shires et al., 1987 |
| 8 | Immunological Difference | Strong short-term humoral response | Strong long-term humoral response and also a strong cellular response | Koenen et al., 2002 |

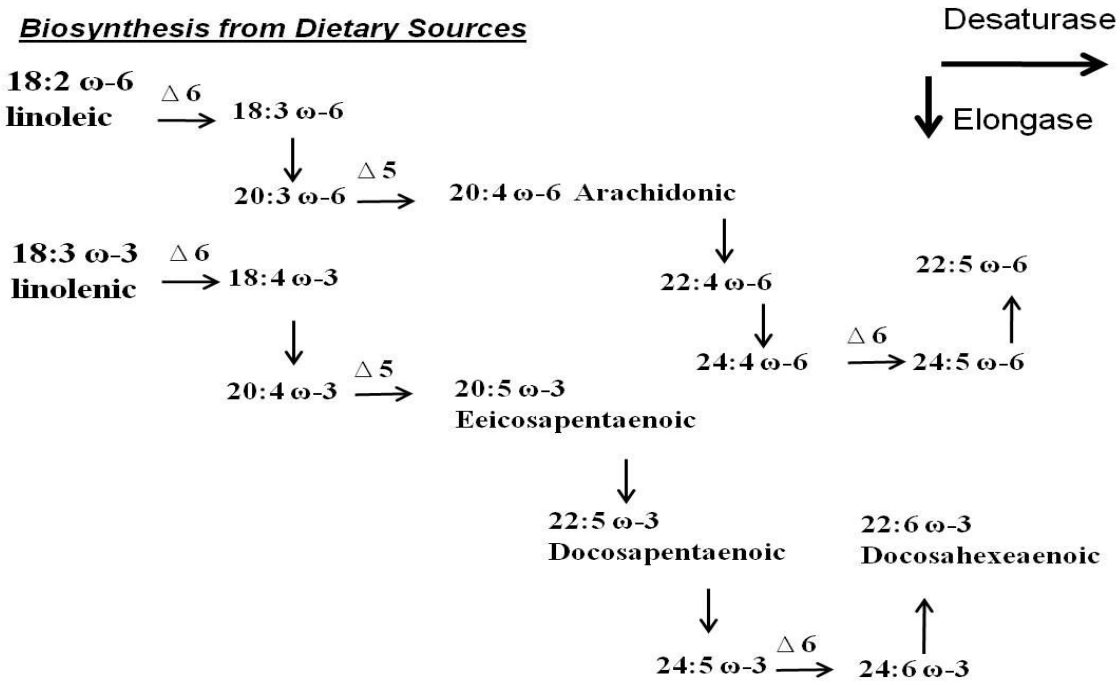
739




740

741 **Figure.1.1: Digestion and absorption of dietary fat in birds.**
742 **(Adapted:http://www.vivo.colostate.edu/hbooks/pathphys/digestion/smallgut/absorb**
743 **_lipids.html).**

**Part: 1**

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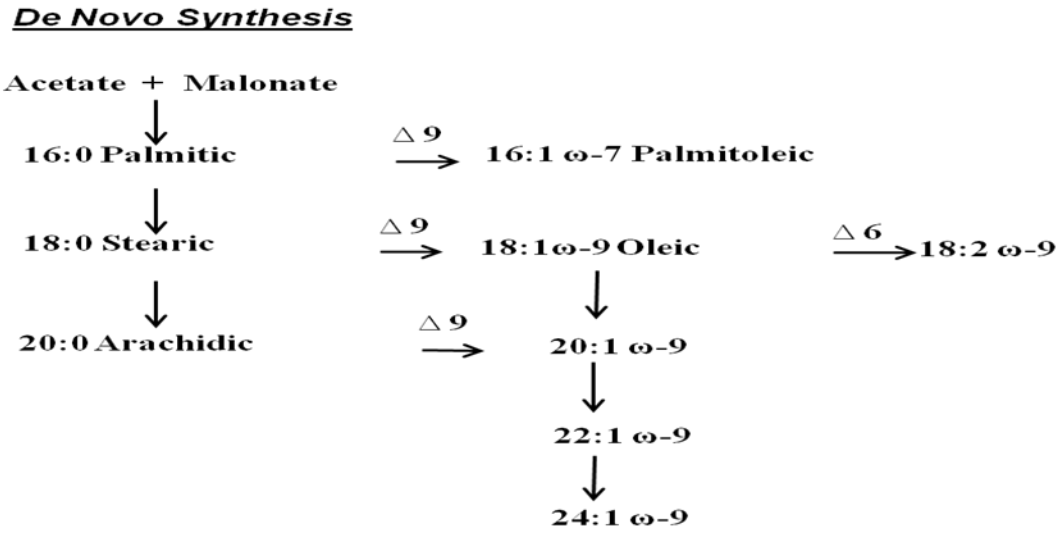



**Figure 1.2: The metabolism of different fatty acids in birds:**
**Part-1: Through desaturation and elongation. Adapted from Walzem, R. L., 1996).**
**Part-2: Through *de novo* Synthesis in liver. (Adapted from Walzem, R. L., 1996).**
**→ represents desaturation step by Δ5, Δ6 or Δ9 desaturase enzyme involved in inserting double bond during at respective carbon number from alpha end of fatty acid.**
**↓ represents the elongation which involves the insertion of acetate to chain of fatty acid.**
**↑ represents partial beta-oxidation in peroxisomes involving removal of acetate from chain of fatty acid.**

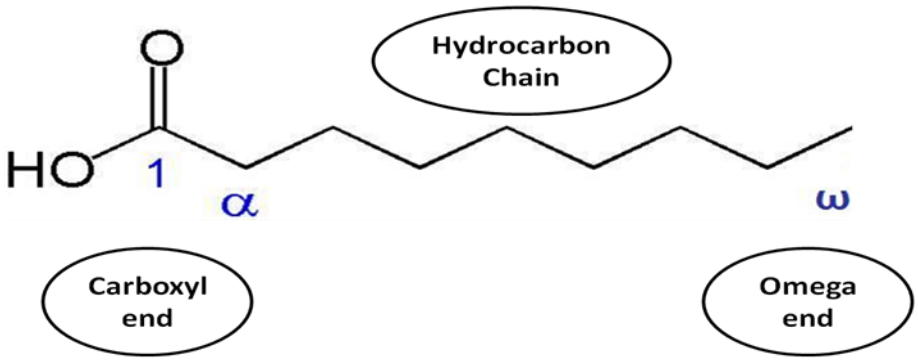
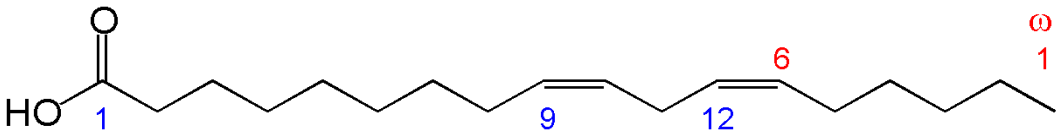
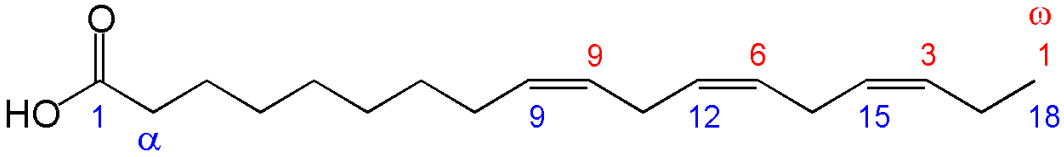


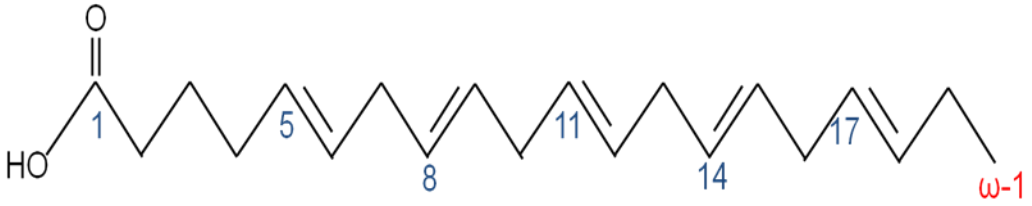

**Figure 1.3: The general structure of fatty acids (Adapted from: http://themedicalbiochemistrypage.org/lipids.html).**



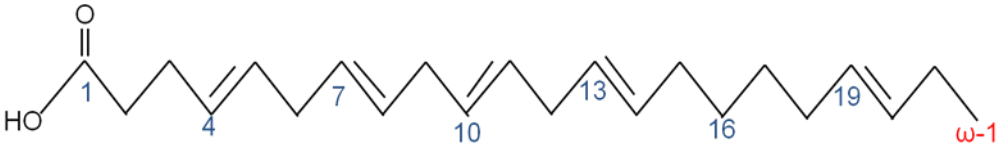

cis- 9, 12 Octadecadienoic acid (**Linoleic Acid)**




cis- 9, 12, 15 Octadecatrienoic acid (**Linolenic Acid)**



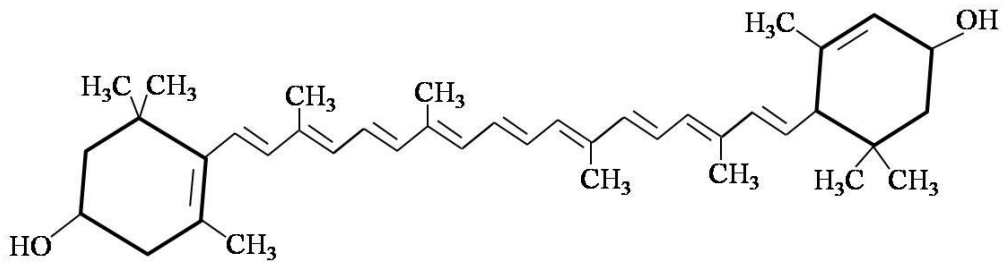

cis- 5, 8, 11, 14, 17- Eicosapentaenoic acid (**EPA)**




cis- 4, 7, 10, 13, 16, 19- Docosahexeaenoic acid (**DHA)**

**Figure 1.4: The structure of omega-6 polyunsaturated fatty acids and omega-3 polyunsaturated fatty acids.**

**(Adapted from: http://themedicalbiochemistrypage.org/lipids.html).**




774

775 **Figure 1.5: Structure of lutein (β,ε-carotene-3,3'-diol), molecular formula $C_{40}H_{56}O_2$**

776 **(Sajilata et al., 2008).**

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# CHAPTER 2

# Exploring Alternate Laying Hen Feeding Methods to Improve Lutein and Omega-3 PUFA Deposition in Table Eggs

## 2.1 INTRODUCTION

Development of nutritionally enhanced eggs containing increased levels of components such as Omega-3 polyunsaturated fatty acids (ω-3 PUFA), lutein or vitamin E, have provided healthy alternatives for the consumer and has increased enriched egg options in the supermarkets. It is well documented that the fatty acid composition of the egg can be modified by the diet (Ziang et al., 1992; Hargis and Van Elswyk, 1993; Sim, 1998). Additional ω-3 PUFA, for example, is often incorporated into the egg through diets containing 10 to 20% flaxseed (Caston et al., 1994; Aymond and Van Elswyk, 1995; Scheideler and Froning, 1996). The consumption of ω-3 PUFA such as alpha-linolenic acid (LNA), eicosapentaenoic acid (EPA), docosapentanoic acid (DPA) and docosahexaenoic acid (DHA) have been associated with reductions in chronic heart diseases, stroke, diabetes, cancer, and arthritis in humans (Kinsella et al., 1990; Marik et al., 2009). Whereas, the increased concentration in unsaturated ω-3 PUFA content in eggs may increase the probability of lipid oxidation, leading to rancidity (Jiang et al., 1992), this can be greatly reduced through the addition of an antioxidant like lutein or vitamin E to the hen ration (Cherian and Sim, 1997; Leeson et al., 2007; Surai et al., 2008). When flaxseed is used as the ω-3 PUFA source, the yolk becomes lighter in color from yellowish towards whitish, which is not acceptable to many consumers (Scheideler et al., 1997). However, the egg yolk colour can be intensified by adding lutein to poultry feed as lutein significantly increased yolk colour to darker yellow (Leeson and Caston, 2004).

Lutein is a pigment concentrated in the retina and lens of the human eye (Bernstein et al., 2001). Humans are unable to synthesize lutein and depend entirely on dietary supplemention from vegetables such as spinach or from egg yolk (Calvo, 2005). Lutein provides protection to the eye, particularly in elderly people, from conditions like cataracts and diseases like age-related macular

degeneration (Bone & Landrum, 2001). Lutein can also contribute to prevention of coronary heart disease by slowing down the process of atherosclerosis as well as contribute to the prevention of cancer, and provide protection to the skin from damage caused by ultraviolet light (Lee et al., 2004; Tsao et al., 2007). Leeson and Caston (2004) showed that eggs could be enriched with lutein by up to five-fold through dietary inclusion of 500 ppm/kg of lutein in a standard layer ration.

In laying hens, lutein acts as potent antioxidant and its incorporation in chickens has been found to prevent liver hemorrhages and benefit birds health by increasing the efficacy of vaccinations (Bedecarrats and Leeson, 2006; Leeson et al., 2007). The egg has been found to be a highly bioavailable source of lutein compared to vegetable sources such as spinach because the lutein in egg yolk is associated with the matrix of the easily digested yolk lipids (Handelman et al., 1999; Chung et al., 2004). The inclusion of dietary lipids promotes efficient absorption of fat-soluble lutein through micelle formation and transportation to the liver as oil droplets (Fredriksson et al., 2006; Huo et al., 2007). Here, lutein is attached to fatty acid binding proteins and is incorporated into VLDL particles during the formation in the liver egg yolk that are subsequently deposited to egg yolk (Surai et al., 2000).

Leeson and Caston (2004) reported that inclusion of ground flaxseed in the hen ration had a negative effect on deposition of lutein in the egg yolk. At a high inclusion rate (greater than 10%), flaxseed can have an anti-nutritional effect through irritation of the mucosal layer of the gut (Leeson et al., 2000; Alzueta et al., 2003). An alternating day feeding program, where a producer switches between a flax or lutein rich diet in an alternative pattern, may allow increased efficiency of absorption of ω-3 PUFA. The present experiment was designed to explore this concept of egg enrichment by using alternating enrichment diets (lutein and flax diet). This could be helpful to increase the effectiveness of the feeding models including multiple enrichments into egg. An alternating day laying hen feeding program with diets containing flax or lutein would also reduce the absorptive interaction at gut level and might increase the simultaneous enrichment of both value-added ingredients. The lutein may also be helpful for

reducing or blocking the production of reactive oxygen species, an outcome of excess unsaturated fatty acid in the egg or hen (Gutteridge and Halliwell, 1990).

## 2.2 MATERIALS AND METHODS

### 2.2.1 Birds and Management

All experiments were conducted in accordance with the principles and guidelines approved by the University of Alberta's Animal Care and Use Committee for Livestock and followed principles established by the Canadian Council on Animal Care (1993). A total of one hundred forty-four (Lohmann White; 56 wk old) laying hens were randomly selected and assigned to individual cages with a light schedule of 14L: 8D per day. The birds were individually wing banded and randomly divided into six dietary treatments (24 hens in each group). The birds were provided ad libitum access to water during the entire experimental period of 60 days.

### 2.2.2 Experimental Design

The experiment had a completely randomized design with six dietary treatments. Each hen was fed one of six diets: control layer diet (C), enriched diets containing 500 ppm of lutein (L), 10% flaxseed (F), a combined diet with 500 ppm lutein and 10% flax (LF), or an alternating diet switching between the L and F diets every 24 h (Alt-1) or every 48 h (Alt-2) (Table 2.1). Daily individual egg production was recorded and body weight and feed intake was recorded weekly. Egg traits including egg weight, yolk weight, albumen height and weight, shell weight and thickness were recorded once every 14 d from the beginning of dietary treatment at day one. The yolks were collected at 0 d, 14 d, 28 d and 56 d were frozen at – 20 C, prior to analysis of egg yolk fatty acids composition. Yolks from eggs stored for 30 d at 4 C (from birds on dietary treatments for 56 d), frozen, and also assessed for fatty acid composition and fatty acid stability compared to unstored samples.

### 2.2.3 Analysis of Fatty Acid Composition

Egg yolks were separated and yolk samples from blocks of 3 hens were combined and homogenized (8 groups / treatment). Duplicate samples of yolk

were analyzed by GC to assess the fatty acid composition. The raw egg yolk samples (1 g) were placed in 25 x 150 mm teflon lined screw capped, test tubes and the modified Folch method used to extract total fat. The egg yolk samples were mixed with 24 mL of Folch solution (Chloroform: Methanol; 2:1 v/v) and kept overnight. Then, 4 mL of 0.88% NaCl (w/v) was added, mixed gently and centrifuged at 1500 x g for 3 min. After phase separation, the top layer was carefully siphoned off. A total of 10 mL of the bottom layer was removed and dried in a block heater under a stream of nitrogen in 20 mL glass vials. The dried fat was resolubilized in 1 mL of chloroform and 50 µL of the reconstituted mixture (extracted fat and chloroform) was then derivatized using 2 mL of methylating reagent (1 N Methanolic HCl, Sigma, Oakville, ON, Canada) in a water bath at 60 C for 60 min. Then, 100 µL of distilled water, a known amount (500 µL) of internal standard (1mg/mL chloroform) (heptadecanoic acid, 17:0, Sigma, Oakville, ON, Canada) and 4 mL of hexane was added, mixed thoroughly, and centrifuged at 1500 x g for 3 min. The top hexane layer was separated and transferred to another test tube containing about 10 to 20 mg of anhydrous sodium sulphate to absorb any moisture from hexane. Finally, after centrifugation (3 min, 1500 x g), and 1 mL of hexane solution (after adjusted to limit 0.2 to 0.3 mg lipids in 1 mL of hexane) was transferred to a GC vial.

Fatty acid composition of the 1 µL of solution injected into the GC (Model 3400, Varian, Palo Alto, CA, USA), equipped with a flame ionization detector and a SGE BP20 capillary column (30 m x 0.25 mm ID x 0.25 µm film thickness; Scientific Instrument Services Inc., Ringoes, NJ, USA) was determined. The initial column temperature was set at 50 C for 0.2 min, increased to 120 C at a rate of 20 C/min, and held for 5 min. Then the column temperature was elevated to 230 C at rate of 10 C/min and held at the final temperature for total running time (30 min). Helium was used as the carrier gas at a flow rate of 3.0 mL/min. A Cool-on-Column injection method was used, with an initial and final injector temperature ($CO_2$) of 60 C (0.2 min) and 230 C (28 min) respectively, increasing at a rate of 150 C/min. The detector was set at 240 C and the column head pressure of the carrier gas (helium) was 25 PSI. The Cool-on-Column injection

method used in our study is best suited because it eliminates the sample discrimination, does not alter the sample due to its inert nature, and provides high analytical precision and accuracy that is needed for detection of LC-PUFA (Yuwono and Indrayanto, 2009). The standard (15A" NU-CHEK PREP, INC. Elysian, MN, USA) was injected after every 10 samples to monitor the chromatographic conditions during the course of duration of sample analysis. The fatty acid peak integration was performed using the Galaxie Chromatography Data System (Varian). Fatty acids were quantified using heptadecanoic acid (17:0) as an internal standard (Varian Walnut Creek, California 94598-1675 USA) and were identified by comparison of authentic standards (GLC-463 NU-CHEK PREP, INC. Elysian, MN, USA). The fatty acid content of egg yolk was calculated as concentration (mg/g) = peak area of a given fatty acid × concentration of internal standard (mg/mL)/peak area of internal standard/sample weight (g).

Total ω-3 PUFA levels were calculated as 18:3 ω-3 (linolenic acid; LNA) + 20:5 ω-3 (ecosapentenoic acid; (EPA) + 22:5 ω-3 (docosapentanoic acid; (DPA) + 22:6 ω-3 (docosahexanoic acid (DHA). Total ω-6 PUFA levels were calculated as 18:2 ω-6 + 18:3 ω-6 + 20:2 ω-6 + 20:3 ω-6 + 20:4 ω-6 + 22:4 ω-6. Saturated fatty acid (SFA) levels were calculated as 14:0 + 16:0 + 18:0 + 20:0. Monounsaturated fatty acid (MUFA) levels were calculated as 16:1 ω-7 + 18:1 ω-7 + 18:1 ω-9 + 20:1 ω-9. Total polyunsaturated fatty acid (PUFA) levels were calculated as sum of total ω-3 acids + total ω-6 fatty acids. Long chain ω-3 PUFA (LC ω-3 PUFA) levels were calculated as sum of EPA + DPA + DHA.

**2.2.4 TBARS Assay**

The method of Slater and Sawyer (1971) for thiobarbituric acid-reactive substance (TBARS) measurement was adapted to determine lipid peroxidation and oxidative status of egg yolk. Egg yolk samples (2 g) were weighed into a test tube (16 x 125 mm) with a Teflon-lined screw-cap. 4 mL of 1.15% KCl in water was immediately added into test tube and the mixture was homogenized for 30 s using a polytron homogenizer (Brinkmann Instruments, Rexdale, ON, Canada), and then 1 mL of 80 mM Tris/maleate buffer solution and 4 mL of TCA-TBA-

HCl (15% w/v trichloroacetic acid; 0.375% w/v thiobarbituric acid; 0.25 N hydrochloric acid) were added. The tube was capped and shaken rapidly, then filtered through #1 Whatman Filter Paper. The tubes with filtrate were incubated overnight at room temperature. The spectrophotometric absorbance at 532 nm was recorded (UV/VIS Spectrophotometer). An extinction coefficient of 1.56 x 105/M/cm was used to estimate the malondialdehyde (MDA) values (Slater and Sawyer, 1971). The TBA values were expressed as mg malondialdehyde/kg (MDA/kg) yolk.

**2.2.5 Carotenoid Analysis**

Yolk samples (300 mg) were weighed into 25 x 150 mm screw cap tubes, 0.7 mL of 5% NaCl was added followed by the 2 mL of ethanol (85%). This mixture was homogenized for 30 s using a Polytron homogenizer (Brinkmann Instruments, Rexdale, ON, Canada), and then 1 mL water (HPLC grade) was used to wash down the homogenizer. Then, 2 mL of dichloromethane were added to the homogenate, mixed thoroughly, and centrifuged at 1500 x g for 3 min to separate the phases. The upper phase was discarded and 1 mL of clear dichloromethane from the bottom phase was transferred to the HPLC vial. The standard curve for lutein, canthaxanthin, β-carotene, retinol, and lycopene (Sigma Aldrich, St Louis MO, USA) were plotted to quantify the absolute amount of each respective pigment.

A Shimadzu Prominence full HPLC system (CBM-20A system controller, Sil-20A autosampler, LC-20AT Pump with SPD-M20A type photodiode array detector) (Shimadzu Instruments, Tokyo, Japan) was used for estimation of carotenoid concentration in egg yolk. For chromatographic separation, a 4.6 x 150 mm column of Supelcosil LC-18 DB with a particle size of 3 µm (Supelco, Bellafonte PA, USA) was used. The mobile phase of acetonitrile/dichloromethane/methanol (6:2:1, v/v/v) was eluted from 5 min to 17 min at 0.1 mL/min. The photodiode array detector recorded the chromatograms simultaneously at wavelengths in the range of 235 to 700 nm to determine peak spectra for carotenoid identification. EZStart 7.4 SP1 (Shimadzu Instruments, Tokyo, Japan) chromatography data software was used for the data acquisition

and peak integration. The retention times of lutein, canthaxanthin, lycopene, β-carotene and retinol were 4.74 min, 6.60 min, 12.55 min, 14.60 min and 3.65 min respectively**.** The peaks of individual carotenoids were identified by comparison with the retention times of known standard peaks of respective carotenoids. The calibration curves were obtained for each carotenoid using the various concentrations of respective standards. The maximum absorbance for lutein, canthaxanthin, lycopene and β-carotene was measured at 446 nm and for retinol it was measured at 325 nm.

**2.2.6 Stastical Analysis**

The egg traits, fatty acid composition and carotenoid composition of egg yolk were analyzed as a two-way ANOVA using the MIXED procedure of SAS with six levels of dietary treatment (C, F, L, LF, Alt-1 and Alt-2) and four durations (0 d, 14 d, 28 d and 56 d) as fixed effects. The experimental unit was individual bird. Least squares means were adjusted using Tukey's honest test, and were reported as significant at $P < 0.05$ level (SAS System, 2002). The model used:

$$Y_{ijk} = \mu + D_i + T_j + D_iT_j + \varepsilon_{ijk}$$

where $Y_{ijk}$ = dependent variable for $k^{th}$ bird, $\mu$ = overall mean, $D_i$ = $i^{th}$ dietary treatment effect, $T_j$ = $j^{th}$ duration of feeding effect, $D_iT_j$ = the interaction effect between the $i^{th}$ dietary treatment and $j^{th}$ duration of feeding, $\varepsilon_{ijk}$ = the residual error.

The storage effect on fatty acid composition and carotenoid composition were analyzed as a two way ANOVA using the MIXED procedure of SAS with six levels of dietary treatment and two condition of egg yolk (unstored and stored) as fixed effects. Least squares means were adjusted using Tukey's honest test, and were reported as significant at $P < 0.05$ level (SAS System, 2002). The model used:

$$Y_{ijk} = \mu + D_i + T_j + D_iT_j + \varepsilon_{ijk}$$

where $Y_{ijk}$ = dependent variable for $k^{th}$ bird, $\mu$ = overall mean, $D_i$ = $i^{th}$ dietary treatment effect, $T_j$ = $j^{th}$ storage condition of egg yolk effect, $D_iT_j$ = the interaction

effect between the $i^{th}$ dietary treatment and $j^{th}$ condition of egg yolk, $\varepsilon_{ijk}$ = the residual error.

The relationship between the different carotenoid compositions in egg yolk was established with the help of Pearson's correlation coefficient with Corr procedure of SAS (SAS System, 2002).

## 2.3 RESULTS AND DISCUSSION

### 2.3.1 Egg Traits

Egg production was statistically similar among all the dietary treatments during the experiment (P= 0.22). The hens on C, F, L, LF, Alt-1 and Alt-2 diets diet had an 86.7%, 92.7%, 90.9%, 94.0%, 89.3% and 89.7% rate of lay respectively. The results for egg production parameters agrees with findings that inclusion of lutein (from 125 to 1000 ppm) (Leeson and Caston, 2004) or 10% flaxseed (Bean and Leeson, 2003) in the poultry ration do not affect rate of lay.

Results for egg quality traits including the egg weight, albumen height, yolk weight, shell weight, shell thickness were measured at 0 d, 14 d, 28 d, and 56 d of experiment are summarized in Table 2.2. With the exception of shell thickness, egg weight and egg quality traits were not affected by feeding treatment, and there were no interaction of treatments with time duration. At 14 d, the eggs from hens on C diet had thicker shells compared to those from F and Alt-1 diet. The reduced shell thickness in F and Alt-1 could be associated with impairment in calcium absorption due to increased digesta viscosity associated with the flax mucilage (Scheideler and Froning, 1996; Jia et al., 2008). However, the shell thickness in LF and Alt-2 eggs were intermediate, and not statistically different from eggs from the C diet or other test diets. This result, in combination with the lack of difference at 0, 28 and 56 d comparisons, suggest that there is not likely a specific negative effect of feeding flax on shell deposition once birds acclimated to the test diets.

The egg weight and shell weight each remained statistically similar with time in all dietary treatments. In contrast, egg specific gravity gradually decreased with the duration of feeding the experimental diets (P<0.5), which may be related

to the fact that egg specific gravity decreases as laying hens get older (Roberts, 2004). Overall, eggs from hens on the Alt-1 diet had lower egg specific gravity than eggs from C, F, L, and LF diets, while Alt-2 egg remained intermediate. Egg specific gravity is usually positively correlated to the shell thickness (Foster and Weatherup, 1979). In the present study we observed the same trend with the lowest value for shell thickness and specific gravity in the eggs from the Alt-1 diet. This suggests that the daily switching of enriched diet might have created an issue with acclimatization of gut microflora and structures due to rapid dietary changes (Dibner et al., 1996) and thus ultimately affecting the calcium absorption and shell quality of egg. Further, results for egg shell thickness for the hens on the Alt-2 diet did not statistically differ from the other treatments, suggesting that keeping hens on enriched diets for two consecutive days was less disruptive to absorptive ability of birds. However, further research is needed to provide proof of this theory.

A higher albumen height and Haugh unit value are indicators of higher internal quality of eggs (Haugh, 1937; Baker and Vadehra, 1970). During the course of this study, there was no affect of either the different dietary treatment or the interaction of diet with duration of feeding experimental diet on these traits. However, the albumen height and Haugh unit values changed with duration of feeding and was higher at 56 d of experiment than at 0, 14, and 28 d (Table 2.2). Normally the albumen height will decrease as the hen ages (Silversides and Scott, 2001).Therefore, the results substantiate the finding that feeding flaxseed and lutein as in enriched ration does not negatively affect the internal quality of eggs through the experimental period.

The yolk weight was not affected by the interaction of dietary treatment with duration of feeding the experimental diets. Overall, yolk from birds on C birds weighed more than all other treatments. This appears to have been a preexisting condition. A decline in yolk weight could be attributed to differences in the hen's capacity to move VLDL for yolk synthesis with differences in fatty acid profile (Walzem, 1996). The enriched ingredients (lutein and ω-3 PUFA) fed included in the hen ration are transported to egg yolk as part of the yolk-VLDL.

Furthermore, a reduction in yolk weight in flaxseed-fed laying hens may be associated with a reduction in circulating estrogen caused either by high amount of ω-3 PUFA (Whitehead et al., 1993) in flaxseed or by the phytoestrogenic effects of flaxseed (Van Elswyk et al., 1997). Since yolk weight on an absolute or a percentage basis did not change significantly in time, it is difficult to identify a specific cause for this difference. Yolk weight often increases in birds with a reduced rate of lay. At 87%, the rate of lay of these birds was numerically the lowest in the study.

**2.3.2 Fatty Acid Composition**

The fatty acid profile of egg yolk can be influenced by the composition of dietary ingredient of layer ration. The use of flax in feed is the most common way to enrich the egg with ω-3 PUFA. The total SFA, PUFA, total ω-3 PUFA, ω-6 PUFA and LC ω-3 PUFA in eggs from hens of L diet was similar to that of C eggs at all durations of feeding tested (Table 2.3a, Table 2.3b and Table 2.3c). This is reasonable, considering the L diet had no additional dietary fatty acid source. The overall SFA, PUFA, total ω-3 PUFA and ω-6 PUFA of the eggs from all flax-fed treatments was consistent after 14 days on the test diets (Table 2.3b and Table 2.3c). The inclusion of flax in the diets significantly increased the total ω-3 PUFA concentration in all the flax containing diets at all durations tested after the start of the treatments compared to C and L diets.

There were no differences in the total yolk LC ω-3 PUFA concentration among the dietary treatments (Table 2.3a). Among the individual LC ω-3 PUFA, the EPA was higher in egg yolks from C and F hens on compared to LF, Alt-1 and Alt-2 hens at 0 d (Table 2.3b and Table 2.3c). However, the amount of EPA in the yolk from all dietary treatment was similar at 14 d, 28 d and 56 d. Flax is the highest vegetable source of LNA (57% of total fatty acids) (Cunnane et al., 1993) and hence the inclusion of flax in the diet significantly increased the LNA concentration in egg yolk. The increased supplementation of LNA in the diets ration leads to increased concentration of LC ω-3 PUFA in the egg yolk through successive metabolic desaturation and elongation reactions (Cherian and Sim, 1991). The similar total LC ω-3 PUFA concentration among dietary treatments in

the present study at all durations is indicative of the low bioconversion of LNA to LC ω-3 PUFAs in the birds fed a flax-based ω-3 PUFA source.

The total ω-3 PUFA in egg yolk was significantly higher in LF and F diet than the L and C diets at 14 d, 28 d and 56 d (Table 2.3a, Table 2.3b and Table 2.3c). However, these values are based largely on LNA, which is supplied by the dietary flax. The alternate day feeding method (Alt-1 and Alt-2) resulted in about 43% less enrichment of ω-3 PUFA in the egg yolks compared to the daily feeding of enriched diet (F and LF) by the end of experiment (56 d) when compared with initial values at 0 d. It is important to note that the hens on alternate diets consumed about half the amount of flax compared to the daily fed flax diets. To compensate for this shortfall, the alternating day diets would need to include 20% flax to supply the same overall enrichment to the daily-fed flax diets containing 10% flax. However, the additional complications of feeding 20% flax, such as reduced egg production, could have appeared (Leeson et al., 2000). The higher amount of flax may have reduced fatty acid digestibility due to the increased viscosity at gut level and the potential anti-nutritional effects of mucilage, linatine and linamarin present in flaxseed (Bhatty, 1993; Rebole et al., 2002). Additionally, Leeson et al., 1998 indicated that inclusion of flaxseed at greater than 10% levels resulted into decreased overall egg acceptability due to "fishy" taint in the enriched egg. It is not clear if the dilution of a 20% flax ration through feeding on alternating days wita a standard ration would eliminate the negative effects feeding a high flax ration to the hen.

The total ω-6 PUFA was significantly reduced in the egg yolk from all dietary treatment enriched in flaxseed (F, LF, Alt-1 and Alt-2) after 56 d compared to the values at 0 d, with total ω-6 PUFA in egg yolk from hens on C and L being statistically similar at 0 d, 28 d and 56 d. The decrease in the ω-6 PUFA along with the increase in ω-3 PUFA amount in flax enriched dietary treatments resulted in a significant reduction in the ω-6:ω-3 ratio in egg yolk. In humans, a diet with a lower ω-6:ω-3 ratio (3:1) is associated with potential health benefits (Simopoulos, 1991).

The MUFA amount in the eggs yolk was similar among all dietary treatments at 0 d, 14 d and 28 d (Table 2.3a, Table 2.3b and Table 2.3c). However, by 56 d, the amount of MUFA in egg yolks from hens on F and LF diet was significantly lower than eggs from the C and L diets, with the Alt-1 and Alt-2 diets at an intermidate level. The total SFA in egg yolk from C, F, L, LF and Alt-1 was quite consistant throughout the 56 d experimental period, with the exception of yolks from hens on diet Alt-2, which had a lower amount of SFA at 56 d compared to values at 0 d. However, the SFA in egg yolk from hens on Alt-2 diet was statistically similar at 14 d, 28 d and 56 d. The reduced level of MUFA found in eggs from the in flax enriched diet may relate to maintainance of a consistant ratio of unsaturated (MUFA and PUFA) to SFA in the yolk, which allows maintenance of cell membrane fluidity (Asghar et al., 1990).

Storage of eggs for 30 d had variable effects on fatty acid composition. The amount of MUFA in unstored eggs from the C diet was higher than F diet eggs, but this difference disappeared in 30 d stored eggs, suggesting that MUFA was being lost during storage (Table 2.4a and 2.4b). Total ω-3 PUFA content in egg yolk from Alt-1 and Alt-2 was similar to those from L diet in unstored egg yolk. However, following 30 d storage, the egg yolks from the hens on Alt-1 and Alt-2 had more ω-3 PUFA than those from the L diet. Although this result could suggest loss of some ω-3 PUFA from L eggs during storage, total ω-3 PUFA in egg yolks from unstored egg and stored egg was similar irrespective of dietary treatment indicating that overall, storage was not detrimental to total ω-3 PUFA content.

Both the overall amount of PUFA and total ω-6 PUFA were reduced in egg yolk from stored egg compared to egg yolk from unstored egg in hens on the C diet, whereas egg yolk from all other diets were not affected by the interaction of storage condition and the different dietary treatment. In unstored eggs, the SFA amount in egg yolk from hens on the C diet was higher than from L and LF diets but at 30 d storage, this difference was not significant. Moreover, the SFA content in unstored egg yolk from C diet was higher compared to at 30 d stored egg yolk from C diet.

The storage of egg at 4 C for even 20 d has been reported to reduce SFA and LC ω-3 PUFA amount in yolk (Hayat et al., 2010). In contrast, both Marshall et al. (1994) and Cherian et al. (1996) have reported that storage of egg does not change fatty acid profiles in egg stored at 4 C for 28 d or 40 d, respectively. In the present study, there was variation in the fatty acid profiles between unstored and 30 d stored eggs. The reduction of PUFA during storage is related to increased susceptibility of unsaturated fatty acid to oxidative damage due to free peroxidation radicals (Krinsky and Deneke, 1982). Further, Castrillon et al. (1996) reported that the amount of PUFA decreases in fish even during frozen (-30 C) storage. The SFA are less susceptible to oxidative damages compared to PUFA (Zhang et al., 2007). However, Cherian et al. (2007) reported a significant reduction in SFA and total lipid following storage for 60 d. Similar results for reduction of SFA and total fatty acid were observed in the current study in egg yolks from eggs stored for 30 d from hens on C diet compared to those from unstored eggs from the C diet.

There was no significant interaction effect of storage condition and dietary treatments on LC ω-3 PUFA content. However, egg yolk following 30 d of storage had significantly less LC ω-3 PUFA (EPA, DPA and DHA) compared to unstored eggs. The storage of egg affected the LC ω-3 PUFA, with EPA particularly most affected. The loss of EPA during storage was about 74.4 to 74.5% in LF, Alt-1 and Alt-2 diets compared to C (94.7%), L (81.6%) and F (72.4%) egg yolks. In contrast the medium chain ω-3 PUFA, LNA was unaffected by storage for 30 d in egg yolk from all dietary treatment, except the eggs from Alt-2 diet had higher amount of LNA in stored compared to unstored. The higher susceptibility of LC ω-3 PUFA to oxidative damage during storage has been reported earlier (Grune et al., 2001; Cherian et al., 2007). Although lutein is a natural antioxidant (Krinsky, 1989), this was not evident in the lutein-containing rations based on this test since oxidative damage to LC ω-3 PUFA was similar among all groups in the 30 d stored eggs. The oxidative status of egg following storage was further analyzed through TBARS analysis in the stored and unstored eggs.

### 2.3.3 Egg Fatty Acid Stability

The TBARS value is an indicator of lipid peroxidation and oxidative status of stored food products (Botsoglou et al., 1994). During lipid oxidation unsaturated fatty acids oxidize, producing hydroperoxides capable of reacting with thiobarbituric acid to produce a colored complex which can be measured spectrophotometrically (Turner et al., 1954; Yu and Sinnhuber, 1957). In the present study, the TBARS number for egg yolk from F, L, LF, and Alt-2 were statistical similar within the dietary treatments for the stored and unstored eggs (Table 2.5). However, the TBARS value increased significantly for eggs from hens on C and Alt-1 diet in 30 d stored eggs compared to unstored eggs, indicating a possibility of lipid peroxidation following 30 d storage. A higher TBARS value indicates the higher lipid oxidation of fatty acid in egg yolk and indicates the egg yolk fatty acids are more reactive making them less stable and more susceptible to oxidative damage (Pikul and Kummerow, 1991).

In addition, The TBARS number in eggs from hens on lutein L and C diets were similar in unstored eggs but after 30 d storage the TBARS number in egg from L diets were significantly lower than C diets, demonstrating a role of lutein as an antioxidant in protection from oxidative damages during storage. Earlier, Cherian et al. (2007) has suggested that egg storage promotes lipid oxidation and the accumulation of TBARS. Therefore, dietary inclusion of an antioxidant source (lutein) along with ω-3 PUFA source for the egg enrichment is required to increase the oxidative stability during storage (Chen and Hsu, 2003; 2004). However, most enrichment diets already include vitamin E as an anti-oxidant (Meluzzi et al., 2000; Chen and Hsu, 2004). Addition of lutein provides additional protection from oxidation (Surai et al., 2001).

Fresh (unstored) eggs are quite resistant to oxidative damages due to the presence of natural antioxidant fractions in the egg yolk like phosvitins and lecithin (Pike and Peng, 1985). However, the presence of the double bonds in ω-3 PUFA makes them susceptible to oxidative deterioration (Frankel, 1984). The LC ω-3 PUFA in egg yolks have multiple double bonds thus further increasing their susceptibility to lipid oxidation (Cherian et al., 2007). This could explain why

significant reductions were observed in the LC ω-3 PUFA during 30 d storage compared to medium chain ones (LNA). However, there was no dietary differentiation for the reduction in LC ω-3 PUFA in stored eggs. Since the lower TBARS number in the stored egg yolk from L diets compared to C and F diets suggests a protective role of lutein in oxidative damages, further work in this direction is required to substantiate the antioxidant role of lutein in protection from oxidative damages during storage.

### 2.3.4 Carotenoid Composition and Storage Stability

Concentrations of LC ω-3 PUFA (susceptible to oxidation) are the highest in the macular region of the eye (Aveldano de Caldironi and Bazan). Of the carotenoids present in this region, lutein concentration is at the hightest level (Rapp et al., 2000; Bernstein et al., 2001). Laying hens efficiently deposit lutein as the free form in egg yolk irrespective of its form in feed; either saponified or esterified (Breithaupt, 2007). However, intestinal absorption of lutein can be influenced by the amount (Roodenberg et al., 2000) and type of the dietary fat consumed (Lakshminarayana et al., 2009). For example, olive oil, which is rich in oleic acid (C18:1ω-9) favors the incorporation of lutein into micelles at the intestinal level and thus increases the absorption (Nidhi and Bhaskaran, 2010). In the present study, lutein, canthaxanthin, β-carotene and retinol were the main carotenoids detected in egg yolk (Table 2.6), wheras lycopene was too low to even be detected in yolks from many of the diets (data not shown). Lutein concentration was positively correlated to canthaxanthin ($r= 0.65$, $P=0.0001$) and β-carotene ($r= 0.40$, $P=0.0001$). Retinol, however, was not correlated to lutein concentration in egg yolk. Therefore, enrichment of lutein in egg yolk can increase the overall carotenoid profile of the egg. This will result in deeply hued yolks (higher pigments), which are preferred by consumers in most countries (Karunajeewa et al., 1984; Beardsworth and Hernandez, 2004).

Egg yolk lutein concentration from hens fed the lutein enriched diets (L, LF, Alt-1, Alt-2) were higher than from C and F diets after 14 d on experimental diets (Table 2.6). In addition, yolk lutein amounts from hens on L diet and LF diets were similar at all times tested, with the exception of 14 d, when yolk lutein

concentration from hens fed the L diet was significantly higher than that of the LF diet. The lower lutein in egg yolk from LF compared to L at 14 d can be explained as a reduction in ability to form a micelle with lutein, which is required for absorption at gut level, in presence of higher level of PUFA (due to flaxseed) (Hou et al., 2007). One of the reasons for designing the experiment by feeding the alternate diets was to avoid the simultaneous presence of flaxseed and lutein from ration at gut level and to avoid possible negative effect on transfer of enrichment from diet to egg yolk. Leeson & Caston (2004) reported that it was possible to enrich egg yolk up to 5- to 8-fold with 500 ppm lutein in the diet fed for 28 d. In the current study, the yolk lutein peaked at 28 d of feeding and was 7- to 8-fold higher in egg yolks from the enriched L and LF diets compared to egg yolk from hens on the C diet through dietary inclusion of 500 ppm. However, at 14 d and 56 d on dietary treatment, the L and LF had about 3 to 4 fold higher level of lutein than C and F diets. The Alt-1 and Alt-2 eggs contained approximately half the enrichment of the L treatments at each testing point.

The lower level of lutein enrichment in our study at 14 d and 56 d compared to that of Leeson and Caston (2004) could be the result of use of a wheat-based diet compared to their use of corn-based diet. Corn contains 35 µg/g carotenoids compared to 1.9 to 9.6 µg/g in common Canadian wheat varieties (Abdel-Aal et al., 2007). The use of a corn-based diet may further increase the level of lutein increment in egg yolk as corn contains significant amounts of lutein, zeaxanthin, and other carotenoids. Additionally, dietary lutein has been shown to have a synergistic effect when fed with other carotenoids that leads to an increased overall level of carotenoid deposition (Scott & Eldrigde 2005; Stahl et al. 1998).

The amount of yolk canthaxanthin was similar among all dietary treatments until 14 d on experimental diets (Table 2.6). The egg yolks from hens on daily lutein enriched diets (L and LF) were higher at 28 d compared to C and F. The egg yolk from alternating diets (Alt-1 and Alt-2) was statistically similar to the daily lutein fed diets. The canthaxanthin concentration reached the peak at 56 d, when in egg yolks from hens fed L diet had highest amount compared to other

treatment and the LF, Alt-1 and Alt-2 diets has higher amount compared to the diets without lutein (C and F). Although rate of increase was slowing at that time, it may not yet have reached its maximum level within the range tested in this experiment.

The amount of β-carotene in the egg yolk was more variable (Table 2.6). It was significantly elevated in hens fed L and Alt-2 diets after 14 d compared to C, F, and LF diets. At 28 d, hens fed L and Alt-1 diets had higher β-carotene in egg yolk compared to C, F, and LF diets. These findings indicate that the along with lutein enrichment, the amount of canthaxanthin and β-carotene in the egg yolk was also increased. However, adding dietary lutein did not increased all the carotenoids measured. In contrast to canthaxanthin and β-carotene, the amount of retinol was reduced in LF, Alt-1 and Alt-2 compared to C and F diets with those from L diets at intermediate level at 14 d. At 28 d there was no dietary difference in retinol content. However at 56 d the retinol amount in egg yolk was higher in hens on the LF treatment compared to the F, Alt-1 and Alt-2 treatments. The differentiation in the carotenoid accumulation in the egg yolk may be related to the disparity in isomerization as well as structural differences among carotenoids, which can lead to differences in the absorption, transport and delivery of carotenoids due to the *trans* form of Lutein being more bioavailable than the *cis* form (Parker, 1996). The variability in quantitative differences of different carotenoids in egg yolk following lutein enrichment may be due to tissue specificity of various carotenoids (Surai et al., 2000).

At 56 d on the dietary treatments, the storage stability of carotenoids was assessed in unstored eggs and in eggs stored for 30 d from this point. There was a significant interaction of the storage condition with dietary treatment for the lutein, canthaxanthin and β-carotene (Table 2.7). The lutein concentration in stored egg yolk from hens fed C and F was lower compared to the unstored egg yolk. However, in all other treatments which include lutein in diet (L, LF, Alt-1 and Alt-2) had similar concentration of lutein in stored egg and in unstored eggs yolk, indicating a higher stability of lutein in egg yolk during storage. The β-carotene in unstored eggs from hens on each diet was similar, however after 30 d

of storage, the amount of β-carotene was reduced in egg yolks from C and F diets compared to the lutein-enriched diets (L, LF Alt-1 and Alt-2). This indicates that β-carotene along with lutein had good stability during the 30 d storage in lutein-enriched diets. Although low to begin with, the canthaxanthin concentration was similar in unstored and stored eggs from the C and F diets. However, when canthaxanthin was present at a higher level in the lutein enriched treatments (L, LF, Alt-1 and Alt-2), content was significantly reduced following storage, which indicates a low stability of the yolk canthaxanthin originating from the lutein-enrichment product during storage. The dietary lutein product used in this experiment has enrichment claims specifically for lutein rather than for the other carotenoids. In this case, if canthaxanthin enrichment were desired, specific, stable enrichment of canthaxanthin would be possible through the use of other dietary enrichment products (Grashorn and Steinberg. 2002). Like canthxanthin, retinol also lacked stability during storage, with eggs from all dietary treatments losing retinol content during the 30 d of storage. Overall, egg yolk from LF eggs had more retinol than F, Alt-1 and Alt-2 eggs.

The changing relationship among treatment results with time (Table 2.6 and 2.7) suggests there may be a time based acclimation effect with these diets, or perhaps evidence of time based impairment in gut conditions that may alter the gut absorptive capacity for the carotenoids. At start of the experiment, eggs contained 4 to 5 fold more lutein than β-carotene; the next most plentiful carotenoid (Table 2.6). Once on the lutein-rich diets, egg lutein increased over 300% while β-carotene, canthaxanthin and retinol levels did not change to near the same degree. As a result, measured reductions in these minor carotenoids during storage do not carry the same biological significance as they would have for lutein, which did not change. Lutein is a more stable carotenoid compared to β-carotene and canthaxanthin present in egg yolk (Delgado-Vargas et al, 2000).

The carotenoids vary in their action against the lipid peroxidation (McNulty et al., 2007). Canthaxanthin reduces the hydroperoxide formation more efficiently than β-carotene and lutein due to the presence of oxo groups (=O) (Terao, 1989) and thus might have been depleted at a faster rate in order to

prevent the oxidative damages during the 30 d storage. In addition, the carotenoids are unstable molecules whose stability in feed is drastically affected by storage condition, light, humidity, and temperature (Lai et al., 1996). Exposure to light and higher storage temperature (35 C) may degrade the lutein (Delgado-Vargas et al, 1998). In the current study, feed was stored in covered containers kept at ambient temperature (21 C). The degradation of carotenoids in the feed is possible and would have reduced the amount available for deposition in the egg. For example, the β-carotene content of eggs from hens fed C diet was higher at the start of the experiment than at 14 d (Table 2.6).

In the present study, the results for levels of ω-3 PUFA and lutein enrichment were most promising (higher) for the dietary treatment including the combination of ω-3 PUFA and lutein (LF). There was no negative interaction of feeding 10% of flaxseed with 500 ppm lutein for the multi enrichment of eggs, indicating that this level of flax does not negatively affect gut condition to the point of affecting the absorptive capability of other enrichment ingredients like lutein. In a similar study, Rajesha et al., (2009) suggested that feeding layers with ω-3 PUFA sources along with a spirulina-based diet (high in lutein and β-carotene) increased the total ω-3 PUFA content of eggs. In the current study, yolk lutein enrichment increased the overall carotenoids profile in the unstored egg. Inclusion of a dietary lutein source also reduced the TBARS value, suggesting the antioxidant role of lutein against oxidative damages in ω-3 PUFA enriched eggs. However, the strength of this finding was unclear based on the fatty acid profile of unstored compared to stored eggs.

Feeding combinations of enrichment ingredients or using alternating feeding programs are both functional strategies to create an enriched egg. The potential to increase the stability of enriched ω-3 PUFA with the antioxidant properties of lutein can benefit value-added egg quality by allowing a longer, more stable storage time. Since there was no additional advantage of using the diets fed alternating days, it is recommended that the use of a combined ration like that of the LF treatment be used because it is much more practical to implement. Further studies in this area could focus on incorporation of higher

quality ingredients or testing of diets with ingredients that normally can be fed together without interferences with absorption.

**Table 2.1: Experimental diet[1] composition and nutrient content.**

| Ingredient (%) | Control (C) | Flax (F) | Lutein (L) | Lutein-Flax (LF) |
|---|---|---|---|---|
| Wheat | 68.69 | 64.66 | 68.69 | 64.66 |
| Soybean meal | 14.38 | 10.28 | 14.38 | 10.28 |
| Flax seed | -- | 10.00 | -- | 10.00 |
| Lutein[2] (ppm) | -- | -- | 500 | 500 |
| Canola Oil | 5.08 | 3.25 | 5.08 | 3.25 |
| Limestone | 8.35 | 8.33 | 8.35 | 8.33 |
| Dicalcium phosphate | 1.01 | 1.00 | 1.01 | 1.00 |
| Salt | 0.28 | 0.29 | 0.28 | 0.29 |
| DL-Methionine | 0.09 | 0.09 | 0.09 | 0.09 |
| Layer Premix[3] | 2.10 | 2.10 | 2.10 | 2.10 |
| Enzyme[4] | 0.50 | 0.50 | 0.50 | 0.50 |
| Calculated Nutrient Analysis: | | | | |
| Crude Protein (%) | 16.50 | 16.50 | 16.50 | 16.50 |
| Metabolizable Energy (kcal/kg) | 2,700 | 2,700 | 2,700 | 2,700 |

[1]There were 2 additional dietary treatments that include F and L diets and were fed in an alternating pattern as follows: Alt-1 = Lutein diet and flax diets alternately every other day; Alt-2 = Lutein diet and flax diets alternately every two days.

[2]Lutein was in form of ORO GLO® brand 15 Dry Pigmenter: A dry stabilized source of saponified yellow carotenoids from marigolds for use in poultry feed. (Kemin Agri-Foods North America, Inc., Iowa, USA)

[3]The Layer premix contained per kilogram of diet: vitamin A (retinyl acetate), 12,000 IU; cholecalciferol, 3,000 IU; vitamin E (DL-α-tocopheryl acetate), 40 IU; vitamin K, 2.0 mg; pantothenic acid, 14 mg; riboflavin, 6.5 mg; folacin, 1.0 mg; niacin, 40 mg; thiamine, 3.3 mg; pyridoxine, 6.0 mg; vitamin B12, 0.02 mg; biotin, 0.2 mg; iodine, 0.5 mg; Mn, 75 mg; Cu, 15 mg; Zn, 80 mg, Se, 0.1 mg; and Fe, 100 mg; choline chloride, 100 mg.

[4]Enzyme: Avizyme 1302, Xylanase enzyme, Danisco Animal Nutrition, Marlborough, Wiltshire, UK.

**Table 2.2: Egg trait parameters from hens (56wks) fed dietary tretaments[1] for 56 d.**

| Treatment | Duration | Egg Weight (g) | Yolk Weight (g) | Shell Weight (g) | Shell Thickness (mm) | Specific Gravity | Albumen Height (mm) | Haugh Unit |
|---|---|---|---|---|---|---|---|---|
| C | | 60.90 | 18.18$^{a}$ | 5.229 | 0.337$^{ab}$ | 76.93$^{ab}$ | 7.189 | 84.04 |
| F | | 60.79 | 17.28$^{b}$ | 5.303 | 0.337$^{ab}$ | 77.83$^{a}$ | 7.286 | 84.76 |
| L | | 60.68 | 17.35$^{b}$ | 5.222 | 0.334$^{ab}$ | 76.64$^{ab}$ | 7.462 | 85.71 |
| LF | | 59.96 | 17.04$^{b}$ | 5.175 | 0.339$^{a}$ | 77.46$^{a}$ | 7.128 | 83.93 |
| Alt-1 | | 61.40 | 17.41$^{b}$ | 5.123 | 0.327$^{b}$ | 73.56$^{c}$ | 7.290 | 84.33 |
| Alt-2 | | 60.18 | 17.11$^{b}$ | 5.125 | 0.328$^{ab}$ | 75.21$^{bc}$ | 7.351 | 85.22 |
| SEM | | 0.448 | 0.165 | 0.056 | 0.003 | 0.526 | 0.092 | 0.591 |
| | 0 d | 59.91 | 17.51 | 5.211 | 0.332$^{bc}$ | 77.67$^{a}$ | 7.086$^{b}$ | 83.69$^{b}$ |
| | 14 d | 61.02 | 17.22 | 5.198 | 0.344$^{a}$ | 75.86$^{bc}$ | 7.176$^{b}$ | 83.96$^{b}$ |
| | 28 d | 60.79 | 17.50 | 5.165 | 0.324$^{c}$ | 76.66$^{ab}$ | 7.210$^{b}$ | 84.23$^{b}$ |
| | 56 d | 60.89 | 17.35 | 5.211 | 0.335$^{ab}$ | 74.89$^{c}$ | 7.665$^{a}$ | 86.78$^{a}$ |
| SEM | | 0.368 | 0.134 | 0.046 | 0.003 | 0.429 | 0.075 | 0.483 |
| C | 0 d | 60.88 | 18.15 | 5.258 | 0.332$^{bcd}$ | 79.65 | 7.100 | 83.50 |
| F | | 59.30 | 17.44 | 5.470 | 0.343$^{abc}$ | 78.17 | 7.158 | 84.46 |
| L | | 58.72 | 17.77 | 5.230 | 0.331$^{bcd}$ | 78.17 | 7.422 | 86.13 |
| LF | | 59.84 | 16.95 | 5.074 | 0.331$^{bcd}$ | 78.50 | 7.008 | 83.19 |
| Alt-1 | | 60.77 | 17.40 | 5.188 | 0.332$^{bcd}$ | 74.70 | 6.861 | 81.94 |
| Alt-2 | | 59.95 | 17.32 | 5.043 | 0.324$^{bcd}$ | 76.83 | 6.967 | 82.91 |
| C | 14 d | 60.89 | 17.85 | 5.413 | 0.367$^{a}$ | 75.65 | 7.161 | 84.04 |
| F | | 61.11 | 16.75 | 5.171 | 0.334$^{bcd}$ | 77.83 | 7.225 | 84.34 |
| L | | 61.38 | 16.79 | 5.139 | 0.343$^{abc}$ | 76.78 | 7.300 | 84.42 |
| LF | | 59.84 | 17.43 | 5.196 | 0.354$^{ab}$ | 77.50 | 6.975 | 83.13 |
| Alt-1 | | 62.01 | 17.56 | 5.130 | 0.331$^{bcd}$ | 72.50 | 7.167 | 83.50 |
| Alt-2 | | 60.91 | 16.93 | 5.142 | 0.337$^{abcd}$ | 74.92 | 7.229 | 84.36 |
| C | 28 d | 60.68 | 18.34 | 5.100 | 0.322$^{cd}$ | 77.83 | 6.942 | 82.69 |
| F | | 61.12 | 17.39 | 5.392 | 0.340$^{abcd}$ | 78.08 | 7.246 | 84.39 |
| L | | 61.27 | 17.42 | 5.274 | 0.331$^{bcd}$ | 77.27 | 7.248 | 84.27 |
| LF | | 60.15 | 16.96 | 5.225 | 0.331$^{bcd}$ | 78.67 | 6.954 | 82.85 |
| Alt-1 | | 61.92 | 17.59 | 4.979 | 0.308$^{d}$ | 73.13 | 7.496 | 85.51 |
| Alt-2 | | 59.57 | 17.31 | 5.021 | 0.311$^{cd}$ | 75.00 | 7.375 | 85.67 |
| C | 56 d | 61.17 | 18.36 | 5.146 | 0.328$^{bcd}$ | 74.58 | 7.554 | 85.94 |
| F | | 61.64 | 17.52 | 5.178 | 0.333$^{bcd}$ | 77.22 | 7.513 | 85.84 |
| L | | 61.33 | 17.43 | 5.245 | 0.332$^{bcd}$ | 74.35 | 7.878 | 88.00 |
| LF | | 60.03 | 16.82 | 5.205 | 0.342$^{abc}$ | 75.18 | 7.574 | 86.55 |
| Alt-1 | | 60.89 | 17.08 | 5.196 | 0.335$^{abcd}$ | 73.92 | 7.638 | 86.39 |
| Alt-2 | | 60.28 | 16.88 | 5.295 | 0.341$^{abc}$ | 74.09 | 7.832 | 87.95 |
| SEM | | 0.905 | 0.331 | 0.110 | 0.006 | 1.050 | 0.183 | 1.181 |
| Source of Variation | | ----------Probability---------- | | | | | | |
| Treatment | | 0.2454 | 0.0001 | 0.1758 | 0.0153 | 0.0001 | 0.1525 | 0.1426 |
| Duration | | 0.1279 | 0.3624 | 0.8792 | 0.0001 | 0.0010 | 0.0001 | 0.0001 |
| Duration*Treatment | | 0.9353 | 0.7218 | 0.2913 | 0.0010 | 0.6752 | 0.8275 | 0.7856 |

[1]C= control diet; F= Flax enriched diet; L=lutein enriched diet; LF= 1:1 Mix of Flax and Lutein enriched diets; Alt-1 = Lutein and Flax enriched diets alternately every other day; Alt-2=Lutein and Flax enriched diets alternately every second day

[2]Haugh Unit = 100 log (h-.01*5.6745(30w^.37-100) +1.9) where h=Albumen height and w=Egg weight.

[a]-[d] Means within different dietary treatments, within duration of feeding dietary treatments and within their interactions across the column with no common superscript are significant different (P<0.05).

**Table 2.3a: Fatty acid profile (mg fatty acid/g of egg yolk) of egg yolk from hens (56 wks) fed dietary treatment[1] for 56 days.**

| | Treatment | | | | | | | Duration | | | | | Probability | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fatty acid | C | F | L | LF | Alt-1 | Alt-2 | SEM | 0d | 14d | 28d | 56d | SEM | T | D | T*D |
| 14:0 | 0.760$^{a}$ | 0.641$^{b}$ | 0.745$^{a}$ | 0.640$^{b}$ | 0.745$^{a}$ | 0.754$^{a}$ | 0.02 | 0.749$^{a}$ | 0.716$^{ab}$ | 0.730$^{ab}$ | 0.659$^{b}$ | 0.02 | 0.0001 | 0.0036 | 0.0271 |
| 16:0 | 67.47$^{ab}$ | 57.44$^{c}$ | 63.01$^{abc}$ | 60.65$^{bc}$ | 66.61$^{ab}$ | 67.99$^{a}$ | 1.79 | 67.86$^{a}$ | 61.86$^{b}$ | 67.84$^{a}$ | 57.90$^{b}$ | 1.45 | 0.0001 | 0.0001 | 0.0018 |
| 16:1 ω-7 | 5.540 | 5.198 | 5.242 | 5.267 | 5.283 | 5.779 | 0.33 | 5.441$^{a}$ | 5.443$^{a}$ | 6.247$^{a}$ | 4.405$^{b}$ | 0.27 | 0.8003 | 0.0001 | 0.5037 |
| 18:0 | 24.00 | 21.92 | 22.91 | 22.07 | 24.15 | 22.66 | 0.66 | 23.14$^{ab}$ | 22.41$^{ab}$ | 24.50$^{a}$ | 21.76$^{b}$ | 0.53 | 0.0758 | 0.0082 | 0.4120 |
| 18:1 ω-7 | 5.680$^{a}$ | 4.226$^{b}$ | 5.225$^{ab}$ | 4.533$^{ab}$ | 4.854$^{ab}$ | 4.084$^{b}$ | 0.29 | 4.457$^{bc}$ | 5.018$^{ab}$ | 5.597$^{a}$ | 3.997$^{c}$ | 0.23 | 0.0007 | 0.0001 | 0.0001 |
| 18:1 ω-9 | 127.5$^{a}$ | 106.1$^{c}$ | 115.4$^{bc}$ | 105.9$^{c}$ | 120.4$^{ab}$ | 119.9$^{ab}$ | 2.81 | 116.7$^{ab}$ | 115.0$^{b}$ | 125.6$^{a}$ | 106.3$^{c}$ | 2.26 | 0.0001 | 0.0001 | 0.0001 |
| 18:2 ω-6 | 31.98$^{ab}$ | 29.95$^{b}$ | 32.11$^{ab}$ | 32.16$^{ab}$ | 34.61$^{a}$ | 33.40$^{a}$ | 0.81 | 37.17$^{a}$ | 30.61$^{c}$ | 33.44$^{b}$ | 28.27$^{d}$ | 0.65 | 0.0035 | 0.0001 | 0.0207 |
| 18:3 ω-3 | 2.500$^{d}$ | 6.402$^{a}$ | 3.947$^{c}$ | 6.205$^{a}$ | 5.904$^{ab}$ | 4.892$^{bc}$ | 0.25 | 2.791$^{c}$ | 5.736$^{a}$ | 6.462$^{a}$ | 4.911$^{b}$ | 0.20 | 0.0001 | 0.0001 | 0.0001 |
| 18:3 ω-6 | 0.350 | 0.305 | 0.464 | 0.464 | 0.407 | 0.527 | 0.07 | 0.623$^{a}$ | 0.272$^{b}$ | 0.398$^{b}$ | 0.382$^{b}$ | 0.05 | 0.1878 | 0.0001 | 0.0068 |
| 20:1 ω-9 | 0.740$^{ab}$ | 0.570$^{c}$ | 0.580$^{c}$ | 0.620$^{bc}$ | 0.700$^{abc}$ | 0.790$^{a}$ | 0.03 | 0.660$^{ab}$ | 0.650$^{ab}$ | 0.750$^{a}$ | 0.610$^{b}$ | 0.03 | 0.0001 | 0.0107 | 0.0001 |
| 20:3 ω-3 | 0.359 | 0.398 | 0.555 | 0.568 | 0.345 | 1.132 | 0.31 | 1.040 | 0.698 | 0.199 | 0.301 | 0.25 | 0.4599 | 0.0613 | 0.3865 |
| 20:3 ω-6 | 0.507 | 0.508 | 0.441 | 0.541 | 0.746 | 1.947 | 0.48 | 1.664$^{a}$ | 0.472$^{ab}$ | 0.629$^{ab}$ | 0.363$^{b}$ | 0.39 | 0.1965 | 0.0349 | 0.0152 |
| 20:4 ω-6 | 5.740$^{a}$ | 4.337$^{c}$ | 5.199$^{ab}$ | 4.654$^{bc}$ | 4.728$^{bc}$ | 4.619$^{bc}$ | 0.18 | 5.440$^{a}$ | 4.323$^{b}$ | 5.541$^{a}$ | 4.215$^{b}$ | 0.14 | 0.0001 | 0.0001 | 0.0001 |
| 20:5 ω-3 | 1.044$^{ab}$ | 1.123$^{a}$ | 0.993$^{abc}$ | 0.813$^{c}$ | 0.839$^{bc}$ | 0.932$^{abc}$ | 0.06 | 1.389$^{a}$ | 1.315$^{a}$ | 0.229$^{c}$ | 0.896$^{b}$ | 0.05 | 0.0007 | 0.0001 | 0.0001 |
| 22:5 ω-3 | 0.415 | 0.421 | 0.415 | 0.449 | 0.461 | 0.535 | 0.04 | 0.452$^{ab}$ | 0.406$^{b}$ | 0.410$^{ab}$ | 0.529$^{a}$ | 0.03 | 0.2486 | 0.0178 | 0.0083 |
| 22:6 ω-3 | 4.445 | 4.854 | 4.534 | 4.826 | 4.576 | 4.774 | 0.12 | 4.138$^{b}$ | 4.377$^{b}$ | 5.192$^{a}$ | 4.832$^{a}$ | 0.10 | 0.0640 | 0.0001 | 0.0960 |
| SFA[2] | 92.23$^{a}$ | 80$^{b}$ | 86.66$^{ab}$ | 83.37$^{ab}$ | 91.51$^{a}$ | 91.4$^{a}$ | 2.22 | 91.75$^{a}$ | 84.98$^{b}$ | 93.07$^{a}$ | 80.32$^{b}$ | 1.79 | 0.0002 | 0.0001 | 0.0056 |
| MUFA[3] | 139.4$^{a}$ | 116.1$^{c}$ | 126.5$^{bc}$ | 116.4$^{c}$ | 131.2$^{ab}$ | 130.5$^{ab}$ | 3.11 | 127.2$^{b}$ | 126.1$^{b}$ | 138.2$^{a}$ | 115.3$^{c}$ | 2.51 | 0.0001 | 0.0001 | 0.0001 |
| PUFA[4] | 47.34$^{bc}$ | 47.30$^{c}$ | 48.66$^{abc}$ | 50.68$^{abc}$ | 52.70$^{ab}$ | 52.76$^{ab}$ | 1.35 | 54.70$^{a}$ | 48.21$^{bc}$ | 52.50$^{ab}$ | 44.70$^{c}$ | 1.09 | 0.0022 | 0.0001 | 0.0010 |
| LC ω-3 PUFA[5] | 6.263 | 6.521 | 6.497 | 6.656 | 6.296 | 7.372 | 0.34 | 7.019 | 6.796 | 6.030 | 6.558 | 0.27 | 0.2154 | 0.1262 | 0.0927 |
| Total ω-3 PUFA[6] | 8.763$^{c}$ | 12.92$^{a}$ | 10.44$^{bc}$ | 12.86$^{a}$ | 11.20$^{ab}$ | 11.26$^{ab}$ | 0.48 | 9.81$^{b}$ | 12.53$^{a}$ | 12.49$^{a}$ | 11.47$^{a}$ | 0.38 | 0.0001 | 0.0001 | 0.0001 |
| Total ω-6 PUFA[7] | 38.58$^{ab}$ | 35.10$^{b}$ | 38.22$^{ab}$ | 37.82$^{ab}$ | 40.50$^{a}$ | 40.50$^{a}$ | 1.07 | 44.89$^{a}$ | 35.68$^{c}$ | 40.01$^{b}$ | 33.23$^{c}$ | 0.86 | 0.0050 | 0.0001 | 0.0040 |
| Ratio ω-6:ω-3[8] | 4.420$^{a}$ | 3.126$^{c}$ | 3.870$^{b}$ | 3.214$^{c}$ | 3.376$^{c}$ | 3.465$^{c}$ | 0.10 | 4.728$^{a}$ | 3.031$^{c}$ | 3.486$^{b}$ | 3.069$^{c}$ | 0.08 | 0.0001 | 0.0001 | 0.0001 |
| Total | 279.0$^{a}$ | 243.4$^{c}$ | 261.8$^{abc}$ | 250.4$^{bc}$ | 276.2$^{a}$ | 274.7$^{ab}$ | 6.15 | 273.7$^{ab}$ | 259.3$^{b}$ | 283.8$^{a}$ | 240.3$^{c}$ | 4.96 | 0.0001 | 0.0001 | 0.0007 |

*SEM = standard error of mean, D= Duration, T= Treatment, LC = Long Chain, SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

[1]C= control diet; F= Flax enriched diet; L=lutein enriched diet; LF= 1:1 Mix of Flax and Lutein enriched diets; Alt-1 = Lutein and Flax enriched diets alternately every other day; Alt-2=Lutein and Flax enriched diets alternately every second day.

[2]SFA levels were calculated as 14:0 + 15:0 + 16:0 + 18:0 + 20:0 + 22:0.

[3]MUFA levels were calculated as 16:1ω-7 + 18:1ω-7 + 18:1ω-9 + 20:1ω-9.

[4]PUFA levels were calculated as 18:2ω-6 + 18:3ω-3 + 18:3ω-6 + 20:3ω-3 + 20:3ω-6 + 20:4ω-6 + 20:5ω-3 + 22:5 ω-3 + 22:6ω-3.

[5]LC- ω-3 PUFA was calculated as 20:5ω-3 + 22:5ω-3 + 22:6ω-3.

[6]Total ω-3 PUFA was calculated as 18:3ω-3 + 20:5ω-3 + 22:5ω-3 + 22:6ω-3.

[7]Total ω-6 PUFA was calculated as 18:2ω-6 + 20:4ω-6 + 20:3ω-6.

[8]Ratio ω-6:ω-3 was calculated as total ω-6 PUFA/total ω-3 PUFA.

[a-d] Means within different dietary treatments and within duration of feeding dietary treatments across the rows with no common superscript are significant different (P<0.05) .

2019 **Table -2.3 b : Interaction effect on fatty acid profile (mg fatty acid/g of egg yolk) from hens (56 wks) fed dietary treatment[1] for 56 days**

| Fatty | 0 days | | | | | | 14 days | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Acids | C | F | L | LF | Alt-1 | Alt-2 | C | F | L | LF | Alt-1 | Alt-2 |
| 14:0 | 0.727$^{abc}$ | 0.646$^{abc}$ | 0.775$^{ab}$ | 0.681$^{abc}$ | 0.821$^{ab}$ | 0.842$^{a}$ | 0.716$^{abc}$ | 0.668$^{abc}$ | 0.657$^{abc}$ | 0.704$^{abc}$ | 0.753$^{abc}$ | 0.800$^{ab}$ |
| 16:0 | 67.43$^{abcd}$ | 54.59$^{cde}$ | 67.12$^{abcd}$ | 65.37$^{abcde}$ | 72.18$^{ab}$ | 80.47$^{a}$ | 60.80$^{bcde}$ | 59.29$^{bcde}$ | 55.25$^{cde}$ | 61.32$^{bcde}$ | 67.26$^{abcd}$ | 67.23$^{abcd}$ |
| 16:1 ω-7 | 5.683 | 5.615 | 6.022 | 5.721 | 4.490 | 5.117 | 5.061 | 4.718 | 4.856 | 5.472 | 5.676 | 6.877 |
| 18:0 | 24.09 | 22.08 | 22.96 | 22.35 | 25.03 | 22.36 | 22.22 | 21.21 | 21.36 | 22.24 | 24.05 | 23.37 |
| 18:1 ω-7 | 5.018$^{ab}$ | 4.610$^{abc}$ | 5.407$^{a}$ | 5.256$^{a}$ | 4.274$^{abc}$ | 2.179$^{cd}$ | 5.015$^{ab}$ | 5.362$^{a}$ | 4.762$^{abc}$ | 5.393$^{a}$ | 5.245$^{ab}$ | 4.331$^{abc}$ |
| 18:1 ω-9 | 116.7$^{abcd}$ | 104.3$^{cdef}$ | 115.0$^{abcd}$ | 112.0$^{abcde}$ | 123.8$^{abcd}$ | 128.0$^{abc}$ | 118.8$^{abcd}$ | 112.3$^{abcde}$ | 099.8$^{def}$ | 107.8$^{bcdef}$ | 126.1$^{abcd}$ | 125.0$^{abcd}$ |
| 18:2 ω-6 | 37.48$^{abc}$ | 32.23$^{bcdefg}$ | 36.95$^{abcd}$ | 36.80$^{abcde}$ | 39.85$^{a}$ | 39.69$^{ab}$ | 27.36$^{gh}$ | 29.36$^{efgh}$ | 28.07$^{fgh}$ | 33.43$^{abcdefg}$ | 32.62$^{abcdefg}$ | 32.83$^{abcdefg}$ |
| 18:3 ω-3 | 2.760$^{fgh}$ | 2.373$^{h}$ | 2.760$^{gh}$ | 2.706$^{gh}$ | 2.863$^{fgh}$ | 3.287$^{efgh}$ | 2.330$^{h}$ | 7.287$^{bc}$ | 4.206$^{defgh}$ | 9.048$^{ab}$ | 5.800$^{cde}$ | 5.743$^{cd}$ |
| 18:3 ω-6 | 0.258$^{b}$ | 0.282$^{b}$ | 0.625$^{ab}$ | 0.727$^{ab}$ | 0.621$^{ab}$ | 1.227$^{a}$ | 0.273$^{b}$ | 0.234$^{b}$ | 0.269$^{b}$ | 0.334$^{b}$ | 0.256$^{b}$ | 0.266$^{b}$ |
| 20:1 ω-9 | 0.643$^{bcd}$ | 0.491$^{cd}$ | 0.502$^{cd}$ | 0.670$^{abcd}$ | 0.666$^{abcd}$ | 0.982$^{a}$ | 0.640$^{bcd}$ | 0.635$^{bcd}$ | 0.391$^{d}$ | 0.594$^{bcd}$ | 0.801$^{abc}$ | 0.863$^{ab}$ |
| 20:3 ω-3 | 0.572 | 0.470 | 0.703 | 0.654 | 0.485 | 3.353 | 0.717 | 0.497 | 0.944 | 1.019 | 0.351 | 0.658 |
| 20:3 ω-6 | 0.460$^{b}$ | 0.430$^{b}$ | 0.475$^{b}$ | 0.629$^{b}$ | 1.371$^{b}$ | 6.621$^{a}$ | 0.421$^{b}$ | 0.459$^{b}$ | 0.420$^{b}$ | 0.528$^{b}$ | 0.537$^{b}$ | 0.464$^{b}$ |
| 20:4 ω-6 | 5.863$^{ab}$ | 5.276$^{abcd}$ | 5.775$^{ab}$ | 5.603$^{abc}$ | 5.365$^{abcd}$ | 4.755$^{abcde}$ | 5.041$^{abcde}$ | 4.239$^{bcdefg}$ | 3.614$^{efg}$ | 3.883$^{defg}$ | 4.574$^{abcdef}$ | 4.585$^{abcdef}$ |
| 20:5 ω-3 | 2.019$^{a}$ | 2.058$^{a}$ | 1.764$^{ab}$ | 0.754$^{efgh}$ | 0.742$^{fghi}$ | 0.998$^{defg}$ | 1.209$^{cdef}$ | 1.169$^{cdef}$ | 1.283$^{bcd}$ | 1.327$^{bcd}$ | 1.310$^{bcde}$ | 1.590$^{abc}$ |
| 22:5 ω-3 | 0.397$^{b}$ | 0.341$^{b}$ | 0.410$^{b}$ | 0.375$^{b}$ | 0.405$^{b}$ | 0.785$^{a}$ | 0.342$^{b}$ | 0.362$^{b}$ | 0.392$^{b}$ | 0.418$^{ab}$ | 0.479$^{ab}$ | 0.443$^{ab}$ |
| 22:6 ω-3 | 4.247 | 3.626 | 3.986 | 4.045 | 4.518 | 4.406 | 3.854 | 4.002 | 4.228 | 4.788 | 4.735 | 4.657 |
| SFA[2] | 92.24$^{abcd}$ | 77.31$^{cde}$ | 90.86$^{abcd}$ | 88.40$^{abcde}$ | 98.02$^{ab}$ | 103.70$^{a}$ | 83.73$^{abcde}$ | 81.16$^{bcde}$ | 77.26$^{cde}$ | 84.26$^{abcde}$ | 92.06$^{abcd}$ | 91.39$^{abcd}$ |
| MUFA[3] | 128.1$^{abc}$ | 115.0$^{cde}$ | 126.9$^{abc}$ | 123.7$^{abcd}$ | 133.2$^{abc}$ | 136.3$^{abc}$ | 129.5$^{abc}$ | 123.0$^{abcd}$ | 109.8$^{cde}$ | 119.2$^{bcde}$ | 137.8$^{abc}$ | 137.1$^{abc}$ |
| PUFA[4] | 54.05$^{abcd}$ | 47.08$^{bcde}$ | 53.45$^{abcd}$ | 52.30$^{bcde}$ | 56.21$^{abc}$ | 65.13$^{a}$ | 41.55$^{de}$ | 44.53$^{cde}$ | 46.50$^{bcde}$ | 54.78$^{abc}$ | 50.66$^{bcde}$ | 51.24$^{bcde}$ |
| LC ω-3 PUFA[5] | 7.235 | 6.494 | 6.864 | 5.828 | 6.150 | 9.542 | 6.122 | 6.030 | 6.848 | 7.553 | 6.875 | 7.348 |
| Total ω-3 PUFA[6] | 9.99$^{defgh}$ | 8.87$^{fgh}$ | 9.62$^{efgh}$ | 8.53$^{gh}$ | 9.01$^{efgh}$ | 9.83$^{bcdefg}$ | 8.45$^{gh}$ | 14.13$^{abcd}$ | 10.24$^{defgh}$ | 16.60$^{ab}$ | 12.67$^{bcdefgh}$ | 13.09$^{abcdef}$ |
| Total ω-6 PUFA[7] | 44.06$^{abc}$ | 38.21$^{bcde}$ | 43.83$^{abc}$ | 43.76$^{abc}$ | 47.20$^{ab}$ | 52.30$^{a}$ | 33.09$^{def}$ | 34.29$^{cdef}$ | 32.37$^{def}$ | 38.18$^{bcdef}$ | 37.99$^{bcdef}$ | 38.15$^{bcde}$ |
| Ratio ω-6:ω-3[8] | 4.406$^{abcd}$ | 4.307$^{bcde}$ | 4.559$^{abc}$ | 5.128$^{ab}$ | 5.246$^{a}$ | 4.722$^{abc}$ | 3.932$^{cdef}$ | 3.647$^{defg}$ | 2.348$^{hi}$ | 2.333$^{hi}$ | 2.988$^{fghi}$ | 2.935$^{ghi}$ |
| Total | 274.4$^{ab}$ | 239.4$^{bcd}$ | 271.2$^{ab}$ | 264.4$^{abc}$ | 287.5$^{ab}$ | 305.1$^{a}$ | 254.8$^{abcd}$ | 248.7$^{abcd}$ | 233.6$^{bcd}$ | 258.3$^{abcd}$ | 280.6$^{ab}$ | 279.8$^{ab}$ |

2020 *SEM = standard error of mean, LC = Long Chain, SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.
2021 [1]C= control diet; F= Flax enriched diet; L=lutein enriched diet; LF= 1:1 Mix of Flax and Lutein enriched diets; Alt-1 = Lutein and Flax enriched diets alternately every other day;
2022 Alt-2 = Lutein and Flax enriched diets alternately every second day.
2023 [2]SFA levels were calculated as 14:0 + 15:0 + 16:0 + 18:0 + 20:0 + 22:0.
2024 [3]MUFA levels were calculated as 16:1ω-7 + 18:1ω-7 + 18:1ω-9 + 20:1ω-9.
2025 [4]PUFA levels were calculated as 18:2ω-6 + 18:3ω-3 + 18:3ω-6 + 20:3ω-3 + 20:3ω-6 + 20:4ω-6 + 20:5ω-3 + 22:5 ω-3 + 22:6ω-3.
2026 [5]LC- ω-3 PUFA was calculated as 20:5ω-3 + 22:5ω-3 + 22:6ω-3.
2027 [6]Total ω-3 PUFA was calculated as 18:3ω-3 + 20:5ω-3 + 22:5ω-3 + 22:6ω-3.
2028 [7]Total ω-6 PUFA was calculated as 18:2ω-6 + 20:4ω-6 + 20:3ω-6.
2029 [8]Ratio ω-6:ω-3 was calculated as total ω-6 PUFA/total ω-3 PUFA.
2030 [a-i] Means within the interaction of different dietary treatments and duration of feeding dietary treatments across the rows with no common superscript are significant different (P<0.05).

**Table -2.3 c: Continue: Interaction effect on fatty acids profile (mg fatty acid/g of egg yolk) from hens (56 wks) fed dietary treatment[1] for 56 days.**

| Fatty | 28 days | | | | | | 56 days | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Acids | C | F | L | LF | Alt-1 | Alt-2 | C | F | L | LF | Alt-1 | Alt-2 | SEM |
| 14:0 | 0.779$^{abc}$ | 0.730$^{abc}$ | 0.814$^{ab}$ | 0.571$^{abc}$ | 0.759$^{abc}$ | 0.724$^{abc}$ | 0.798$^{ab}$ | 0.522$^{c}$ | 0.735$^{abc}$ | 0.603$^{bc}$ | 0.647$^{abc}$ | 0.648$^{abc}$ | 0.480 |
| 16:0 | 71.26$^{abcd}$ | 66.85$^{abcde}$ | 68.41$^{abcde}$ | 63.14$^{abcde}$ | 71.38$^{abcd}$ | 66.01$^{abcde}$ | 70.41$^{abc}$ | 49.04$^{e}$ | 61.25$^{bcde}$ | 52.79$^{de}$ | 55.64$^{bcde}$ | 58.27$^{bcde}$ | 3.540 |
| 16:1 ω-7 | 6.268 | 6.204 | 6.292 | 5.611 | 6.841 | 6.268 | 5.135 | 4.257 | 3.800 | 4.261 | 4.123 | 4.852 | 0.659 |
| 18:0 | 25.03 | 25.37 | 23.80 | 23.34 | 26.37 | 23.06 | 24.68 | 19.02 | 23.50 | 20.37 | 21.14 | 21.84 | 1.296 |
| 18:1 ω-7 | 6.395$^{a}$ | 5.352$^{abc}$ | 6.218$^{a}$ | 4.999$^{abc}$ | 5.424$^{abc}$ | 5.194$^{abc}$ | 6.299$^{a}$ | 1.579$^{d}$ | 4.515$^{abc}$ | 2.484$^{bcd}$ | 4.475$^{abc}$ | 4.631$^{abc}$ | 0.568 |
| 18:1 ω-9 | 137.3$^{ab}$ | 122.5$^{abcd}$ | 128.8$^{abcd}$ | 115.0$^{abcdef}$ | 127.4$^{abcd}$ | 122.8$^{abcd}$ | 137.1$^{a}$ | 85.5$^{f}$ | 118.2$^{abcd}$ | 88.9$^{ef}$ | 104.3$^{cdef}$ | 103.6$^{cdef}$ | 5.544 |
| 18:2 ω-6 | 33.38$^{abcdefgh}$ | 33.75$^{abcdefg}$ | 32.05$^{abcdefg}$ | 31.71$^{abcdefgh}$ | 36.62$^{abcdef}$ | 33.11$^{abcdefgh}$ | 29.72$^{defgh}$ | 24.45$^{h}$ | 31.38$^{cdefgh}$ | 26.71$^{gh}$ | 29.37$^{efgh}$ | 27.98$^{fgh}$ | 1.595 |
| 18:3 ω-3 | 2.91$^{defgh}$ | 9.85$^{ab}$ | 3.19$^{defgh}$ | 11.48$^{a}$ | 5.15$^{acdef}$ | 5.58$^{cdef}$ | 2.00$^{h}$ | 7.19$^{bc}$ | 2.55$^{h}$ | 7.29$^{bc}$ | 5.47$^{cde}$ | 4.96$^{defg}$ | 0.494 |
| 18:3 ω-6 | 0.414$^{b}$ | 0.381$^{b}$ | 0.526$^{ab}$ | 0.433$^{b}$ | 0.378$^{b}$ | 0.259$^{b}$ | 0.437$^{b}$ | 0.324$^{b}$ | 0.435$^{b}$ | 0.364$^{b}$ | 0.374$^{b}$ | 0.358$^{b}$ | 0.134 |
| 20:1 ω-9 | 0.815$^{abc}$ | 0.700$^{abcd}$ | 0.821$^{abc}$ | 0.714$^{abcd}$ | 0.722$^{abcd}$ | 0.751$^{abcd}$ | 0.856$^{ab}$ | 0.465$^{cd}$ | 0.614$^{bcd}$ | 0.507$^{cd}$ | 0.628$^{bcd}$ | 0.572$^{bcd}$ | 0.068 |
| 20:3 ω-3 | 0.060 | 0.305 | 0.152 | 0.210 | 0.370 | 0.095 | 0.086 | 0.318 | 0.420 | 0.387 | 0.175 | 0.421 | 0.607 |
| 20:3 ω-6 | 0.602$^{b}$ | 0.833$^{b}$ | 0.545$^{b}$ | 0.663$^{b}$ | 0.659$^{b}$ | 0.469$^{b}$ | 0.544$^{b}$ | 0.308$^{b}$ | 0.326$^{b}$ | 0.346$^{b}$ | 0.418$^{b}$ | 0.234$^{b}$ | 0.944 |
| 20:4 ω-6 | 6.115$^{ab}$ | 5.053$^{abcde}$ | 5.925$^{abcd}$ | 6.113$^{ab}$ | 4.993$^{abcdef}$ | 5.050$^{abcde}$ | 5.941$^{a}$ | 2.780$^{g}$ | 5.482$^{abcd}$ | 3.018$^{fg}$ | 3.982$^{cdefg}$ | 4.085$^{cdefg}$ | 0.346 |
| 20:5 ω-3 | 0.104$^{j}$ | 0.372$^{ghij}$ | 0.112$^{ij}$ | 0.210$^{hij}$ | 0.360$^{ghij}$ | 0.218$^{hij}$ | 0.844$^{defgh}$ | 0.893$^{defg}$ | 0.811$^{defgh}$ | 0.961$^{defg}$ | 0.942$^{defg}$ | 0.922$^{defg}$ | 0.110 |
| 22:5 ω-3 | 0.327$^{b}$ | 0.467$^{ab}$ | 0.309$^{b}$ | 0.359$^{ab}$ | 0.568$^{ab}$ | 0.428$^{ab}$ | 0.594$^{ab}$ | 0.515$^{ab}$ | 0.548$^{ab}$ | 0.645$^{ab}$ | 0.392$^{b}$ | 0.482$^{ab}$ | 0.079 |
| 22:6 ω-3 | 4.967 | 5.322 | 4.778 | 5.345 | 5.644 | 5.097 | 4.713 | 5.145 | 4.466 | 5.125 | 4.606 | 4.934 | 0.234 |
| SFA[2] | 97.07$^{abcd}$ | 92.95$^{abcde}$ | 93.02$^{abcde}$ | 87.05$^{abcde}$ | 98.52$^{abcd}$ | 89.79$^{abcde}$ | 95.89$^{abc}$ | 68.59$^{e}$ | 85.49$^{abcde}$ | 73.75$^{de}$ | 77.43$^{bcde}$ | 80.75$^{bcde}$ | 4.385 |
| MUFA[3] | 150.7$^{ab}$ | 134.7$^{abc}$ | 142.1$^{abc}$ | 126.4$^{abcde}$ | 140.3$^{abc}$ | 135.1$^{abc}$ | 149.4$^{a}$ | 91.8$^{e}$ | 127.2$^{abc}$ | 96.2$^{de}$ | 113.5$^{cde}$ | 113.7$^{cde}$ | 6.139 |
| PUFA[4] | 48.87$^{bcde}$ | 56.33$^{abcde}$ | 47.59$^{bcde}$ | 50.82$^{abcde}$ | 61.07$^{ab}$ | 50.31$^{abcde}$ | 44.88$^{cde}$ | 41.25$^{e}$ | 47.10$^{bcde}$ | 44.84$^{cde}$ | 45.73$^{bcde}$ | 44.37$^{cde}$ | 2.657 |
| LC ω-3 PUFA[5] | 5.458 | 6.466 | 5.351 | 6.124 | 6.943 | 5.839 | 6.237 | 6.924 | 6.192 | 7.118 | 6.116 | 6.760 | 0.673 |
| Total ω-3 PUFA[6] | 08.36$^{efgh}$ | 16.31$^{abc}$ | 08.54$^{efgh}$ | 18.90$^{abc}$ | 11.10$^{bcdefgh}$ | 11.42$^{bcdefgh}$ | 08.24$^{h}$ | 13.39$^{abcde}$ | 09.48$^{efgh}$ | 14.40$^{abcd}$ | 11.59$^{cdefgh}$ | 11.72$^{cdefg}$ | 0.940 |
| Total ω-6 PUFA[7] | 40.51$^{abcde}$ | 40.01$^{bcdef}$ | 39.05$^{bcdef}$ | 38.92$^{bcdef}$ | 42.65$^{abcd}$ | 38.89$^{bcdef}$ | 36.64$^{cdef}$ | 27.86$^{f}$ | 37.62$^{bcdef}$ | 30.43$^{ef}$ | 34.14$^{cdef}$ | 32.66$^{def}$ | 0.193 |
| Ratio ω-6:ω-3[8] | 4.855$^{abc}$ | 2.464$^{hi}$ | 4.593$^{abcd}$ | 3.275$^{efgh}$ | 2.319$^{hi}$ | 3.411$^{defgh}$ | 4.487$^{abcd}$ | 2.086$^{i}$ | 3.978$^{cde}$ | 2.120$^{i}$ | 2.950$^{ghi}$ | 2.793$^{ghi}$ | 0.346 |
| Total | 296.7$^{ab}$ | 284.0$^{abc}$ | 282.7$^{abc}$ | 264.2$^{abcd}$ | 299.9$^{ab}$ | 275.2$^{abc}$ | 290.2$^{ab}$ | 201.6$^{d}$ | 259.7$^{abc}$ | 214.8$^{cd}$ | 236.7$^{bcd}$ | 238.8$^{bcd}$ | 12.14 |

*SE = standard error of mean, LC = Long Chain, SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

[1]C= control diet; F= Flax enriched diet; L=lutein enriched diet; LF= 1:1 Mix of Flax and Lutein enriched diets; Alt-1 = Lutein and Flax enriched diets alternately every other day; Alt-2=Lutein and Flax enriched diets alternately every second day.

[2]SFA levels were calculated as 14:0 + 15:0 + 16:0 + 18:0 + 20:0 + 22:0.

[3]MUFA levels were calculated as 16:1ω-7 + 18:1ω-7 + 18:1ω-9 + 20:1ω-9.

[4]PUFA levels were calculated as 18:2ω-6 + 18:3ω-3 + 18:3ω-6 + 20:3ω-3 + 20:3ω-6 + 20:4ω-6 + 20:5ω-3 + 22:5 ω-3 + 22:6ω-3.

[5]LC- ω-3 PUFA was calculated as 20:5ω-3 + 22:5ω-3 + 22:6ω-3.

[6]Total ω-3 PUFA was calculated as 18:3ω-3 + 20:5ω-3 + 22:5ω-3 + 22:6ω-3.

[7]Total ω-6 PUFA was calculated as 18:2ω-6 + 20:4ω-6 + 20:3ω-6.

[8]Ratio ω-6:ω-3 was calculated as total ω-6 PUFA/total ω-3 PUFA.

[a-j] Means within the interaction of different dietary treatments and duration of feeding dietary treatments across the rows with no common superscript are significant different (P<0.05).

**Table-2.4a: Fatty acid profile (mg fatty acid/g egg yolk) of unstored eggs and 30 day stored eggs (stored at 4 C) from hens (56wks) fed dietary tretaments[1] for 56 d.**

| | Condition (SC) | | | Treatment (T) | | | | | | | Probability | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fatty Acid | Unstored | Stored | SEM | C | F | L | LF | Alt-1 | Alt-2 | SEM | Condition | Treatment | SC*T |
| 14:0 | 0.659 | 0.614 | 0.02 | 0.674$^{ab}$ | 0.542$^{b}$ | 0.699$^{ab}$ | 0.568$^{ab}$ | 0.718$^{a}$ | 0.618$^{ab}$ | 0.04 | 0.1891 | 0.0154 | 0.0389 |
| 16:0 | 57.90 | 54.45 | 1.45 | 61.24$^{a}$ | 50.34$^{b}$ | 58.44$^{ab}$ | 51.58$^{ab}$ | 60.84$^{ab}$ | 54.61$^{ab}$ | 2.55 | 0.1031 | 0.0093 | 0.0063 |
| 16:1 ω-7 | 4.405 | 4.845 | 0.26 | 4.758 | 4.481 | 4.241 | 4.540 | 4.845 | 4.885 | 0.45 | 0.2406 | 0.9118 | 0.6455 |
| 18:0 | 21.76$^{a}$ | 18.95$^{b}$ | 0.57 | 20.62 | 18.79 | 20.97 | 18.99 | 22.28 | 20.47 | 1.00 | 0.0011 | 0.1491 | 0.0120 |
| 18:1 ω-7 | 3.997 | 4.626 | 0.26 | 5.631$^{a}$ | 2.950$^{b}$ | 4.776$^{ab}$ | 3.267$^{b}$ | 5.227$^{a}$ | 4.018$^{ab}$ | 0.45 | 0.0921 | 0.0002 | 0.0103 |
| 18:1 ω-9 | 106.3 | 106.1 | 2.96 | 120.6$^{a}$ | 092.6$^{b}$ | 115.9$^{a}$ | 090.3$^{b}$ | 117.9$^{a}$ | 099.8$^{ab}$ | 5.20 | 0.9665 | 0.0001 | 0.0037 |
| 18:2 ω-6 | 28.27 | 28.32 | 0.73 | 25.53$^{b}$ | 25.76$^{b}$ | 30.09$^{ab}$ | 27.72$^{ab}$ | 32.98$^{a}$ | 27.70$^{ab}$ | 1.29 | 0.9595 | 0.0009 | 0.0024 |
| 18:3 ω-3 | 4.911$^{b}$ | 6.054$^{a}$ | 0.32 | 1.802$^{c}$ | 7.739$^{a}$ | 2.623$^{c}$ | 7.812$^{a}$ | 6.705$^{ab}$ | 6.215$^{b}$ | 0.38 | 0.0001 | 0.0001 | 0.0053 |
| 18:3 ω-6 | 0.382$^{a}$ | 0.285$^{b}$ | 0.03 | 0.298$^{a}$ | 0.303$^{a}$ | 0.413$^{a}$ | 0.260$^{a}$ | 0.468$^{a}$ | 0.259$^{a}$ | 0.05 | 0.0288 | 0.0284 | 0.2228 |
| 20:1 ω-9 | 0.607 | 0.687 | 0.03 | 0.823$^{a}$ | 0.515$^{c}$ | 0.708$^{abc}$ | 0.530$^{c}$ | 0.746$^{ab}$ | 0.558$^{bc}$ | 0.05 | 0.0540 | 0.0001 | 0.0328 |
| 20:3 ω-3 | 0.301$^{a}$ | 0.108$^{b}$ | 0.04 | 0.059 | 0.195 | 0.245 | 0.273 | 0.159 | 0.297 | 0.08 | 0.0036 | 0.2996 | 0.6825 |
| 20:4 ω-6 | 4.215$^{a}$ | 3.336$^{b}$ | 0.10 | 4.822$^{a}$ | 2.743$^{d}$ | 4.734$^{ab}$ | 2.844$^{d}$ | 4.073$^{bc}$ | 3.437$^{cd}$ | 0.18 | 0.0001 | 0.0001 | 0.0001 |
| 20:5 ω-3 | 0.896$^{a}$ | 0.194$^{b}$ | 0.02 | 0.444 | 0.570 | 0.480 | 0.603 | 0.592 | 0.579 | 0.04 | 0.0001 | 0.0552 | 0.8249 |
| 22:5 ω-3 | 0.529$^{a}$ | 0.361$^{b}$ | 0.04 | 0.469 | 0.458 | 0.412 | 0.540 | 0.377 | 0.416 | 0.07 | 0.0040 | 0.6316 | 0.8111 |
| 22:6 ω-3 | 4.832$^{a}$ | 3.783$^{b}$ | 0.11 | 3.816 | 4.124 | 4.388 | 4.595 | 4.485 | 4.437 | 0.19 | 0.0001 | 0.0738 | 0.0895 |
| SFA[2] | 80.32$^{a}$ | 74.01$^{b}$ | 2.00 | 82.54$^{a}$ | 69.68$^{a}$ | 80.11$^{a}$ | 71.14$^{a}$ | 83.84$^{a}$ | 75.70$^{a}$ | 3.51 | 0.0319 | 0.0221 | 0.0065 |
| MUFA[3] | 115.3 | 116.3 | 3.31 | 131.8$^{a}$ | 100.6$^{b}$ | 125.6$^{a}$ | 98.7$^{b}$ | 128.7$^{a}$ | 109.3$^{ab}$ | 5.81 | 0.8388 | 0.0001 | 0.0045 |
| PUFA[4] | 44.70 | 43.22 | 1.12 | 37.70$^{b}$ | 42.26$^{ab}$ | 43.76$^{ab}$ | 45.52$^{ab}$ | 50.35$^{a}$ | 44.16$^{ab}$ | 1.97 | 0.3638 | 0.0018 | 0.0023 |
| LC ω-3 PUFA[5] | 6.558$^{a}$ | 4.446$^{b}$ | 0.15 | 4.788$^{b}$ | 5.346$^{ab}$ | 5.525$^{ab}$ | 6.011$^{a}$ | 5.612$^{ab}$ | 5.729$^{ab}$ | 0.26 | 0.0001 | 0.0411 | 0.1239 |
| Total ω-3 PUFA[6] | 11.47 | 10.43 | 0.36 | 06.59$^{b}$ | 13.08$^{a}$ | 08.15$^{b}$ | 14.32$^{a}$ | 12.32$^{a}$ | 12.44$^{a}$ | 0.56 | 0.1666 | 0.0001 | 0.0114 |
| Total ω-6 PUFA[7] | 33.23 | 32.39 | 0.83 | 31.11$^{bc}$ | 29.18$^{c}$ | 35.61$^{ab}$ | 31.20$^{bc}$ | 38.04$^{a}$ | 31.71$^{bc}$ | 1.47 | 0.4865 | 0.0006 | 0.0009 |
| Ratio ω-6:ω-3[8] | 3.069$^{b}$ | 3.394$^{a}$ | 0.04 | 4.837$^{a}$ | 2.237$^{d}$ | 4.469$^{b}$ | 2.182$^{d}$ | 3.095$^{c}$ | 2.569$^{d}$ | 0.07 | 0.0001 | 0.0001 | 0.0001 |
| Total | 240.3 | 233.5 | 6.30 | 252.0$^{ab}$ | 212.5$^{b}$ | 249.4$^{ab}$ | 215.3$^{b}$ | 262.9$^{a}$ | 229.1$^{ab}$ | 11.1 | 0.4541 | 0.0068 | 0.0039 |

*SEM = standard error of mean, SC= Storage condition, T= Treatment, LC = Long Chain, SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

[1]C= control diet; F= Flax enriched diet; L=lutein enriched diet; LF= 1:1 Mix of Flax and Lutein enriched diets; Alt-1 = Lutein and Flax enriched diets alternately every other day; Alt-2=Lutein and Flax enriched diets alternately every second day.

[2]SFA levels were calculated as 14:0 + 15:0 + 16:0 + 18:0 + 20:0 + 22:0.

[3]MUFA levels were calculated as 16:1ω-7 + 18:1ω-7 + 18:1ω-9 + 20:1ω-9.

[4]PUFA levels were calculated as 18:2ω-6 + 18:3ω-3 + 18:3ω-6 + 20:3ω-3 + 20:4ω-6 + 20:5ω-3 + 22:5 ω-3 + 22:6ω-3.

[5]LC- ω-3 PUFA was calculated as 20:5ω-3 + 22:5ω-3 + 22:6ω-3.

[6]Total ω-3 PUFA was calculated as 18:3ω-3 + 20:5ω-3 + 22:5ω-3 + 22:6ω-3.

[7]Total ω-6 PUFA was calculated as 18:2ω-6 + 20:4ω-6.

[8]Ratio ω-6:ω-3 was calculated as total ω-6 PUFA/total ω-3 PUFA.

[a-e] Means within different dietary treatments and within duration of feeding dietary treatments and within their interactions across the rows with no common superscript are significant different ($P<0.05$).

**Table-2.4b: Means of interaction of storage and dietary treatment on fatty acid profile (mg fatty acid/g egg yolk) of unstored eggs and 30 day stored eggs (stored at 4 C) from hens (56wks) fed dietary tretaments[1] for 56 d.**

| | Unstored egg | | | | | | Stored egg | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fatty acids | C | F | L | LF | Alt-1 | Alt-2 | C | F | L | LF | Alt-1 | Alt-2 | SEM |
| 14:0 | 0.798$^{a}$ | 0.522$^{b}$ | 0.735$^{ab}$ | 0.603$^{ab}$ | 0.647$^{ab}$ | 0.648$^{ab}$ | 0.550$^{ab}$ | 0.562$^{ab}$ | 0.663$^{ab}$ | 0.532$^{ab}$ | 0.789$^{ab}$ | 0.589$^{ab}$ | 0.06 |
| 16:0 | 70.41$^{a}$ | 49.04$^{b}$ | 61.25$^{ab}$ | 52.79$^{b}$ | 55.64$^{b}$ | 58.27$^{ab}$ | 52.07$^{b}$ | 51.64$^{b}$ | 55.63$^{ab}$ | 50.38$^{b}$ | 66.04$^{ab}$ | 50.95$^{b}$ | 3.56 |
| 16:1 ω-7 | 5.135 | 4.257 | 3.800 | 4.261 | 4.123 | 4.852 | 4.381 | 4.704 | 4.682 | 4.818 | 5.567 | 4.917 | 0.63 |
| 18:0 | 24.68$^{a}$ | 19.02$^{bc}$ | 23.50$^{ab}$ | 20.37$^{abc}$ | 21.14$^{abc}$ | 21.84$^{abc}$ | 16.57$^{c}$ | 18.56$^{abc}$ | 18.44$^{abc}$ | 17.61$^{bc}$ | 23.42$^{abc}$ | 19.09$^{abc}$ | 1.39 |
| 18:1 ω-7 | 6.299$^{a}$ | 1.579$^{c}$ | 4.515$^{ab}$ | 2.484$^{bc}$ | 4.475$^{ab}$ | 4.631$^{ab}$ | 4.962$^{ab}$ | 4.321$^{abc}$ | 5.038$^{ab}$ | 4.049$^{abc}$ | 5.980$^{a}$ | 3.405$^{abc}$ | 0.63 |
| 18:1 ω-9 | 137.1$^{a}$ | 085.5$^{c}$ | 118.2$^{ab}$ | 088.9$^{c}$ | 104.3$^{bc}$ | 103.6$^{bc}$ | 104.1$^{abc}$ | 099.7$^{bc}$ | 113.5$^{abc}$ | 091.7$^{bc}$ | 131.6$^{ab}$ | 096.0$^{bc}$ | 7.24 |
| 18:2 ω-6 | 29.72$^{abc}$ | 24.45$^{bc}$ | 31.38$^{ab}$ | 26.71$^{bc}$ | 29.37$^{abc}$ | 27.98$^{abc}$ | 21.33$^{c}$ | 27.07$^{abc}$ | 28.8$^{abc}$ | 28.74$^{abc}$ | 36.58$^{a}$ | 27.41$^{abc}$ | 1.80 |
| 18:3 ω-3 | 2.002$^{e}$ | 7.193$^{ab}$ | 2.552$^{e}$ | 7.287$^{ab}$ | 5.471$^{bc}$ | 4.959$^{cd}$ | 1.602$^{e}$ | 8.284$^{a}$ | 2.694$^{de}$ | 8.336$^{a}$ | 7.939$^{ab}$ | 7.470$^{a}$ | 0.53 |
| 18:3 ω-6 | 0.437 | 0.324 | 0.435 | 0.364 | 0.374 | 0.358 | 0.159 | 0.282 | 0.392 | 0.155 | 0.563 | 0.160 | 0.07 |
| 20:1 ω-9 | 0.856$^{a}$ | 0.465$^{c}$ | 0.614$^{abc}$ | 0.507$^{c}$ | 0.628$^{abc}$ | 0.572$^{bc}$ | 0.791$^{abc}$ | 0.566$^{abc}$ | 0.802$^{abc}$ | 0.552$^{abc}$ | 0.865$^{ab}$ | 0.544$^{abc}$ | 0.07 |
| 20:3 ω-3 | 0.086 | 0.318 | 0.420 | 0.387 | 0.175 | 0.421 | 0.032 | 0.071 | 0.071 | 0.158 | 0.142 | 0.172 | 0.11 |
| 20:4 ω-6 | 5.941$^{a}$ | 2.780$^{d}$ | 5.482$^{a}$ | 3.018$^{cd}$ | 3.982$^{bc}$ | 4.085$^{b}$ | 3.703$^{bcd}$ | 2.707$^{d}$ | 3.985$^{bcd}$ | 2.669$^{d}$ | 4.164$^{bc}$ | 2.788$^{cd}$ | 0.25 |
| 20:5 ω-3 | 0.844 | 0.893 | 0.811 | 0.961 | 0.942 | 0.922 | 0.045 | 0.246 | 0.149 | 0.245 | 0.241 | 0.236 | 0.06 |
| 22:5 ω-3 | 0.594 | 0.515 | 0.548 | 0.645 | 0.392 | 0.482 | 0.344 | 0.401 | 0.276 | 0.435 | 0.361 | 0.350 | 0.10 |
| 22:6 ω-3 | 4.713 | 4.466 | 5.145 | 5.125 | 4.606 | 4.934 | 2.918 | 3.782 | 3.631 | 4.066 | 4.364 | 3.939 | 0.27 |
| SFA[2] | 95.89$^{a}$ | 68.59$^{b}$ | 85.49$^{ab}$ | 73.75$^{b}$ | 77.43$^{ab}$ | 80.75$^{ab}$ | 69.19$^{b}$ | 70.76$^{b}$ | 74.73$^{ab}$ | 68.52$^{b}$ | 90.25$^{ab}$ | 70.64$^{b}$ | 4.90 |
| MUFA[3] | 149.4$^{a}$ | 091.8$^{d}$ | 127.2$^{abc}$ | 096.2$^{cd}$ | 113.5$^{bcd}$ | 113.7$^{bcd}$ | 114.2$^{abcd}$ | 109.3$^{bcd}$ | 124.0$^{abcd}$ | 101.2$^{bcd}$ | 144.0$^{ab}$ | 104.9$^{bcd}$ | 8.10 |
| PUFA[4] | 44.88$^{ab}$ | 41.25$^{bc}$ | 47.10$^{ab}$ | 44.84$^{ab}$ | 45.73$^{ab}$ | 44.37$^{ab}$ | 30.52$^{c}$ | 43.27$^{abc}$ | 40.42$^{abc}$ | 46.20$^{ab}$ | 54.98$^{a}$ | 43.94$^{abc}$ | 2.75 |
| LC ω-3 PUFA[5] | 6.237 | 6.192 | 6.924 | 7.118 | 6.116 | 6.760 | 3.339 | 4.500 | 4.127 | 4.903 | 5.109 | 4.697 | 0.37 |
| Total ω-3 PUFA[6] | 08.24$^{cd}$ | 13.39$^{a}$ | 09.48$^{bc}$ | 14.40$^{a}$ | 11.59$^{ab}$ | 11.72$^{ab}$ | 04.94$^{d}$ | 12.78$^{ab}$ | 06.82$^{cd}$ | 13.24$^{a}$ | 13.05$^{ab}$ | 12.17$^{ab}$ | 0.77 |
| Total ω-6 PUFA[7] | 36.64$^{ab}$ | 27.86$^{c}$ | 37.62$^{ab}$ | 30.43$^{bc}$ | 34.14$^{abc}$ | 32.66$^{abc}$ | 25.58$^{c}$ | 30.49$^{abc}$ | 33.59$^{abc}$ | 31.97$^{abc}$ | 41.93$^{a}$ | 30.77$^{abc}$ | 2.04 |
| Ratio ω-6:ω-3[8] | 4.487$^{b}$ | 2.086$^{f}$ | 3.978$^{c}$ | 2.120$^{f}$ | 2.950$^{d}$ | 2.793$^{de}$ | 5.187$^{a}$ | 2.387$^{ef}$ | 4.959$^{ab}$ | 2.245$^{f}$ | 3.240$^{d}$ | 2.345$^{ef}$ | 0.10 |
| Total | 290.2$^{a}$ | 201.6$^{c}$ | 259.7$^{abc}$ | 214.8$^{bc}$ | 236.7$^{abc}$ | 238.8$^{abc}$ | 213.9$^{bc}$ | 223.3$^{abc}$ | 239.2$^{abc}$ | 215.9$^{abc}$ | 289.2$^{ab}$ | 219.5$^{abc}$ | 15.4 |

*SEM = standard error of mean, LC = Long Chain, SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.
1C= control diet; F= Flax enriched diet; L=lutein enriched diet; LF= Mix of Flax and Lutein enriched diets; Alt-1 = Lutein and Flax enriched diets alternately every other day; Alt-2=Lutein and Flax enriched diets alternately every second day.
[2]SFA levels were calculated as 14:0 + 15:0 + 16:0 + 18:0 + 20:0 + 22:0.
[3]MUFA levels were calculated as 16:1ω-7 + 18:1ω-7 + 18:1ω-9 + 20:1ω-9.
[4]PUFA levels were calculated as 18:2ω-6 + 18:3ω-3 + 18:3ω-6 + 20:3ω-3 + 20:4ω-6 + 20:5ω-3 + 22:5 ω-3 + 22:6ω-3.
[5]LC- ω-3 PUFA was calculated as 20:5ω-3 + 22:5ω-3 + 22:6ω-3.
[6]Total ω-3 PUFA was calculated as 18:3ω-3 + 20:5ω-3 + 22:5ω-3 + 22:6ω-3.
[7]Total ω-6 PUFA was calculated as 18:2ω-6 + 20:4ω-6.
[8]Ratio ω-6:ω-3 was calculated as total ω-6 PUFA/total ω-3 PUFA.

2072 [a-f] Means within the interaction of different dietary treatments and duration of feeding dietary treatments across the rows with no common superscript are significant different (P<0.05).

**Table 2.5: Thiobarbituric acid reactive substances (TBARS) values (mg malondialdehyde/kg yolk (MA/kg) of unstored eggs and 30 day stored eggs (stored at 4 C) from hens (56wks) fed dietary tretaments[1] for 56 d.**

| Treatment | Storage | TBARS Value |
|---|---|---|
| C | -- | 0.6850[a] |
| F | -- | 0.7035[a] |
| L | -- | 0.5296[b] |
| LF | -- | 0.6056[ab] |
| Alt-1 | -- | 0.6175[ab] |
| Alt-2 | -- | 0.5874[b] |
| SEM | | 0.0250 |
| | Unstored | 0.5665[b] |
| | Stored | 0.6772[a] |
| SEM | | 0.0144 |
| C | Unstored | 0.6142[bc] |
| F | Unstored | 0.6552[ab] |
| L | Unstored | 0.4960[c] |
| LF | Unstored | 0.5679[bc] |
| Alt-1 | Unstored | 0.5192[c] |
| Alt-2 | Unstored | 0.5465[bc] |
| C | Stored | 0.7523[a] |
| F | Stored | 0.7557[a] |
| L | Stored | 0.5632[bc] |
| LF | Stored | 0.6490[ab] |
| Alt-1 | Stored | 0.7149[ab] |
| Alt-2 | Stored | 0.6283[bc] |
| SEM | | 0.0359 |
| Source of variation | ----------Probability---------- | |
| Treatment | | 0.0001 |
| Storage | | 0.0007 |
| Treatment *Storage | | 0.0233 |

[1]C= control diet; F= Flax enriched diet; L=lutein enriched diet; LF= Mix of Flax and Lutein enriched diets; Alt-1 = Lutein and Flax enriched diets alternately every other day; Alt-2=Lutein and Flax enriched diets alternately every second day

[a-c] Means within storage condition, within dietary treatments and within their interaction across the column with no common superscript are significant different ($P<0.05$).

**Table 2.6: Carotenoid composition of egg yolk (µg/g Egg Yolk) from hens (56wks) fed dietary tretaments[1] for 56 d.**

| Treatment | Duration | Lutein | Canthaxanthin | β-Carotene | Retinol |
|---|---|---|---|---|---|
| C | 0 d | 09.43$^{f}$ | 0.051$^{de}$ | 2.188$^{abcde}$ | 0.963$^{ab}$ |
| F | | 09.70$^{f}$ | 0.041$^{e}$ | 2.101$^{bcde}$ | 0.827$^{abcde}$ |
| L | | 09.40$^{f}$ | 0.042$^{de}$ | 2.004$^{bcde}$ | 0.915$^{abc}$ |
| LF | | 09.38$^{f}$ | 0.040$^{de}$ | 1.283$^{cdef}$ | 0.791$^{bcdef}$ |
| Alt-1 | | 09.43$^{f}$ | 0.035$^{de}$ | 1.595$^{cdef}$ | 0.843$^{abcde}$ |
| Alt-2 | | 09.48$^{f}$ | 0.038$^{de}$ | 1.857$^{bcdef}$ | 0.864$^{abcd}$ |
| C | 14 d | 10.04$^{f}$ | 0.015$^{de}$ | 0.521$^{f}$ | 0.844$^{abcdef}$ |
| F | | 10.59$^{f}$ | 0.013$^{de}$ | 0.468$^{ef}$ | 0.877$^{abcd}$ |
| L | | 30.17$^{bc}$ | 0.261$^{de}$ | 3.553$^{a}$ | 0.731$^{bcdefgh}$ |
| LF | | 22.49$^{d}$ | 0.175$^{de}$ | 1.265$^{def}$ | 0.469$^{gh}$ |
| Alt-1 | | 18.31$^{e}$ | 0.128$^{de}$ | 1.747$^{bcdef}$ | 0.432$^{h}$ |
| Alt-2 | | 19.66$^{de}$ | 0.153$^{de}$ | 2.769$^{abc}$ | 0.558$^{efgh}$ |
| C | 28 d | 05.27$^{g}$ | 0.012$^{cde}$ | 0.052$^{def}$ | 0.701$^{bcdefgh}$ |
| F | | 06.04$^{g}$ | 0.015$^{de}$ | 0.398$^{cdef}$ | 0.554$^{fgh}$ |
| L | | 39.45$^{a}$ | 1.045$^{c}$ | 3.142$^{ab}$ | 0.724$^{bcdefg}$ |
| LF | | 34.59$^{ab}$ | 0.447$^{d}$ | 1.748$^{bcdef}$ | 0.626$^{defgh}$ |
| Alt-1 | | 20.76$^{de}$ | 0.145$^{bc}$ | 3.677$^{a}$ | 0.609$^{defgh}$ |
| Alt-2 | | 20.73$^{de}$ | 0.494$^{d}$ | 1.286$^{cdef}$ | 0.652$^{cdefgh}$ |
| C | 56 d | 08.12$^{fg}$ | 0.123$^{cde}$ | 1.542$^{bcdef}$ | 0.856$^{abcd}$ |
| F | | 08.22$^{fg}$ | 0.065$^{de}$ | 1.800$^{abcdef}$ | 0.741$^{bcdefg}$ |
| L | | 29.87$^{c}$ | 1.834$^{a}$ | 2.440$^{abcd}$ | 0.840$^{abcde}$ |
| LF | | 31.72$^{bc}$ | 1.294$^{b}$ | 2.403$^{abcd}$ | 1.098$^{a}$ |
| Alt-1 | | 20.81$^{de}$ | 1.081$^{bc}$ | 1.459$^{def}$ | 0.707$^{bcdefgh}$ |
| Alt-2 | | 22.42$^{d}$ | 1.244$^{b}$ | 1.944$^{bcde}$ | 0.774$^{bcdef}$ |
| SEM | | 0.730 | 0.244 | 0.634 | 0.059 |
| Source of Variation | | ----------Probability---------- | | | |
| Treatment | | 0.0001 | 0.0001 | 0.0001 | 0.0001 |
| Duration | | 0.0001 | 0.0001 | 0.4422 | 0.0001 |
| Treatment*Duration | | 0.0001 | 0.0001 | 0.0001 | 0.0001 |

*SEM = standard error of mean.

[1]C= control diet; F= Flax enriched diet; L=lutein enriched diet; LF= Mix of Flax and Lutein enriched diets; Alt-1 = Lutein and Flax enriched diets alternately every other day; Alt-2=Lutein and Flax enriched diets alternately every second day.

[a-c] Means within the interaction of dietary treatments and the duration of feeding experimental diets across the column with no common superscript are significant different ($P<0.05$).

**Table 2.7: Carotenoid composition (µg/g egg yolk) of unstored eggs and 30 day stored eggs (stored at 4 C) from hens (56wks) fed dietary tretaments[1] for 56 d.**

| Condition | Treatment | Lutein | Canthaxanthin | β-Carotene | Retinol |
|---|---|---|---|---|---|
| Unstored | - | 20.19 | $0.940^{a}$ | 1.931 | $0.836^{a}$ |
| Stored | - | 19.83 | $0.223^{b}$ | 1.946 | $0.683^{b}$ |
| SEM | | 0.385 | 0.087 | 0.169 | 0.028 |
| | C | $5.396^{d}$ | $0.071^{b}$ | $0.865^{d}$ | $0.779^{ab}$ |
| | F | $5.474^{d}$ | $0.043^{b}$ | $0.934^{cd}$ | $0.679^{b}$ |
| | L | $31.45^{b}$ | $1.001^{a}$ | $2.944^{a}$ | $0.826^{ab}$ |
| | LF | $34.22^{a}$ | $0.751^{a}$ | $2.896^{ab}$ | $0.920^{a}$ |
| | Alt-1 | $22.15^{c}$ | $0.716^{a}$ | $2.170^{bc}$ | $0.697^{b}$ |
| | Alt-2 | $21.37^{c}$ | $0.908^{a}$ | $1.824^{cd}$ | $0.654^{b}$ |
| SEM | | 0.689 | 0.189 | 0.397 | 0.051 |
| Unstored | C | $8.115^{d}$ | $0.123^{bcde}$ | $1.542^{abcd}$ | 0.856 |
| Unstored | F | $8.220^{d}$ | $0.065^{cde}$ | $1.800^{abcd}$ | 0.741 |
| Unstored | L | $29.86^{b}$ | $1.834^{a}$ | $2.440^{abc}$ | 0.840 |
| Unstored | LF | $31.72^{b}$ | $1.294^{ab}$ | $2.403^{abc}$ | 1.098 |
| Unstored | Alt-1 | $20.80^{c}$ | $1.081^{bcd}$ | $1.459^{cd}$ | 0.707 |
| Unstored | Alt-2 | $22.42^{c}$ | $1.244^{bc}$ | $1.944^{bc}$ | 0.774 |
| Stored | C | $2.677^{e}$ | $0.020^{e}$ | $0.188^{d}$ | 0.702 |
| Stored | F | $2.729^{e}$ | $0.021^{e}$ | $0.068^{d}$ | 0.618 |
| Stored | L | $33.04^{ab}$ | $0.169^{e}$ | $3.447^{a}$ | 0.812 |
| Stored | LF | $35.52^{ab}$ | $0.208^{e}$ | $3.388^{a}$ | 0.742 |
| Stored | Alt-1 | $23.49^{c}$ | $0.350^{e}$ | $1.881^{bc}$ | 0.687 |
| Stored | Alt-2 | $20.31^{c}$ | $0.572^{de}$ | $1.704^{bc}$ | 0.535 |
| SEM | | 0.974 | 0.328 | 0.502 | 0.072 |
| Source of variation | | ----------Probability---------- | | | |
| Condition | | 0.5037 | 0.0001 | 0.9425 | 0.0002 |
| Treatment | | 0.0001 | 0.0001 | 0.0001 | 0.0016 |
| Condition*Treatment | | 0.0001 | 0.0001 | 0.0001 | 0.1385 |

[1]C= control diet; F= Flax enriched diet; L=lutein enriched diet; LF= Mix of Flax and Lutein enriched diets; Alt-1 = Lutein and Flax enriched diets alternately every other day; Alt-2=Lutein and Flax enriched diets alternately every second day

[a-c] Means within storage condition, within dietary treatments and within their interaction across the column with no common superscript are significant different ($P<0.05$).

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# CHAPTER 3

# Effect of Metabolic Efficiency and Intestinal Morphometry on Variability in Enrichment of Eggs with Omega-3 PUFA in Laying Hens

## 3.1 INTRODUCTION

A diet rich in omega-3 polyunsaturated fatty acids (**ω-3 PUFA**) has been demonstrated to prevent coronary heart disease, cancer, arthrosclerosis, diabetes and aids in visual and fetal neural development in humans (Kinsella et al., 1990; Lewis et al., 2000; Yashodhara et al., 2009). Eggs can be enriched with ω-3 PUFA through modification of the hen diet (Celebi & Macit, 2008). This enrichment occurs in part due to the inhibition of *de novo* lipogenesis and reduces total fat deposition in the body when dietary PUFA is fed compared to when dietary monounsaturated fatty acid (**MUFA**) or saturated fatty acid (**SFA**) are fed (Crespo and Esteve-Gracia, 2002). The inclusion of more PUFA instead of SFA during the micellar formation increases the solubility of micelles in bile. This increases fat digestibility and ultimately provides higher metabolic energy (Garret and Young, 1975). Wiseman et al. (1984; 1991) reported higher digestibility for PUFA in young broilers based on AME values, and that this was linearly increased with incremental increases in unsaturated/saturated fatty acid ratios (Whitehead, 1993). In laying hens, the presence of reproductive hormones such as estrogen triggers large increases in lipid synthesis for the support of egg yolk formation (Walzem, 1999).

Absorptive efficiency is modulated by the condition of the absorptive surface of the gut. Morphologically, the length of intestinal villi and crypt depth are indicative of the absorptive capacity of the intestine (Caspary, 1992). The long villus in small intestine lead to increased nutrient absorption, while shallow crypts are indicative of villus mucosal cell turn over (Xu et al., 2003). The metabolic cost of gut maintenance accounts for 20% of body energy expenditure (Choct, 2009). The crypt is regarded as the villus factory and hence it has a high demand of energy for new tissue synthesis (Yason et al., 1987). However, unlike in other

vertebrates, enterocyte proliferation of birds is not only restricted to the crypt region at the base of villus, but is also observed throughout the length of the villus (Uni et al., 1998). Additionally, feed composition has an impact on gut dimensions. For example, a high fiber diet in the chicken can result in increased size and length of the small intestine to compensate for the nutrient inadequacy primarily due to increased viscosity of digesta (Iji, et al., 2001; Montagne et al., 2003). Villus size increases with low energy- high protein diets in hens (Yamauchi and Tarachai, 2000). The shrinking and expansion of villus dimensions due to fasting and refeeding clearly indicates that villus morphology is affected by nutrient absorption process in the small intestine (Shamoto & Yamauchi, 1999). In addition, the macro and micro-structural integrity of the gut influences the nutrient uptake and growth efficiency of the laying hen (Choct, 2009). Therefore, a gut surface that is in poor condition may have reduced effectiveness for absorption of feed with ingredients used to enrich the eggs being of particular concern.

The intestinal physiological parameters of the laying hen should be considered in order to maximize egg enrichment through dietary modifications. This is likely even more important in laying hens, as White Leghorns have smaller villus dimensions than broilers (Yamauchi et al., 1996). Hen energetic efficiency has not been widely used as a selection trait because the genetic selection in layers is focused on egg production, weekly BW from hatch to 6 weeks, egg traits including egg, albumen, yolk, and shell weight and shell thickness (Schreiweis et al., 2006). Residual maintenance requirement (**$RME_m$**) is the residual of the linear relationship between maintenance energy requirement and feed intake (Romero et al., 2009). In the current study, the $RME_m$ methodology used to group birds on efficiency score was meant to provide an unbiased estimate of energetic efficiency by adjusting the maintenance requirement for the effect of diet induced thermogenesis (Romero et al., 2009). Hence, the present experiment was designed to explore potential linkages among metabolic efficiency, intestinal length and morphology, and transfer of ω-3 PUFA

to the egg. In this study we also characterized the extent of individual bird to bird variability in transfer of ω-3 PUFA and how the transfer of enriched ingredients to egg is modulated by hen efficiency along with gut morphological parameters.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Stocks and Management

The experimental protocols were in compliance with the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993) and were approved by a University Animal Care and Use Committee. A group of 24, 56-wk old Lohmann White Leghorn laying hens were housed individually in wire battery cages (30 x 46 cm) with individual light sources (Renema et al., 2001). Prior to the experiment, birds were provided a standard layer ration (16% CP; 2,750 Kcal/kg) (Table 3.1). The enriched diet was formulated to similar specifications (16% CP; 2,750 Kcal/kg), but included 15% LinPRO® (O&T Farms., Regina, SK, Canada) as the source of ω-3 PUFA enrichment (Table 3.1). The birds had *ad libitum* access to water. A lighting program of 16L: 8D with a light intensity of 50 lux at bird level was used for the entire experimental period.

### 3.2.2 Surgical Procedure

#### *3.2.2.1 Implant preparation*

Temperature biotelemetry devices were surgically implanted into 24 laying hens to provide the core body temperature data needed to determine individual bird efficiency. Cylindrical remote temperature sensors (Model: 1010T, H.A.B.I.T. Research LTD., Victoria, BC), which were covered in inert plastic to create a 5 cm long x 1 cm diameter implant with rounded ends. The sensors were calibrated in a temperature-controlled water bath at 5 temperature calibration points: 36.0, 38.5, 41.0, 43.5 and 46.0 C. Live implant temperatures were recorded using a wireless Osprey VHF receiver (H.A.B.I.T. Research LTD., Victoria, BC), and water bath temperature readings were recorded using an Omega temperature monitor calibrated by a NIST traceable mercury thermometer.

The clean temperature sensors were kept submerged in an 85% ethyl alcohol solution for a minimum of 30 min for sterilization prior to surgery.

#### *3.2.2.2 Surgery*

Birds were fasted overnight and water was withheld for three hours prior to surgery. Birds were anaesthetized with 0.75 to 1.5% isoflurane at rate of 1.5 L/min. Feathers were plucked from the surgical area on the right ventral abdominal and the area was cleaned aseptically with Hibitane$^{TM}$ antiseptic (Chlorhexidine 2%, Ayerst Vetrinary Laboratories). A 3 cm incision was made on bird's right ventral abdominal flank, 5 cm caudal to the junction of last rib to the sternum. The implants were inserted and directed caudally in the abdominal space. Following implantation, the muscle layers and skin were sutured with synthetic absorbable material (3/0 Polydioxone Suture). Subcutaneous injection of long term acting analgesic, meloxicam (0.1mg/Kg/5%) (Metacam, Boehringer Ingelheim) and a short term acting analgesic, buprenorphine (0.01 mg/kg) (Buprenex®, Norwich Eaton) were provided to prevent discomfort after surgery. A broad spectrum antibiotic, Ampicilin (50 mg/kg) was administered intramuscularly. Birds were returned to their respective cages after surgery and feed and water was provided *ad libitum.* Birds were checked a minimum of every 30 minutes during initial recovery (1 to 2 hrs) and twice daily once recovery was complete. Approximately 7 d after surgery, 3 birds out of 24 surgically treated birds, were treated with 3 d course of ampicilin, when light fever was noted.

### 3.2.3 Enrichment and Efficiency Score

#### *3.2.3.1 Omega-3 PUFA Enrichment*

Energetic efficiency score (Efficient or Non-efficient) and feeding duration (0 d *vs*. 14 d) were tested in 2 x 2 factorial design. Calculated energetic efficiency score was based on individual metabolic maintenance energy requirement. The individually caged birds were fed an ω-3 PUFA diet containing 15% LinPRO for 14 days. LinPRO is a ground-extruded mix of flaxseed and peas (1:1 wt/wt) high in ω-PUFA (Jia et al. 2008). Flax is a rich source of α- linolenic acid (18:3 ω-3) (LNA) (Cunnane et al., 1993).

Egg traits were measured throughout the period of feeding the ω-3 PUFA enriched diet. Egg traits included yolk weight, shell weight and shell thickness, and were determined at 0 d and 14 d. Yolks were separated at 0 d and 14 d and individually frozen at -20 C pending laboratory analysis. Egg yolk lipid profile was determined by GC analysis. Representative feed samples were collected for determination of dietary fatty acid composition. The feed intake, BW and egg mass during the period of feeding the ω-3 PUFA enriched diet were used to score hens for energetic efficiency.

For comparative purposes, birds were re-classified during data analysis on the basis of feed intake and gut length. The new variable of feed intake (High or Low) and gut length (Short or Long) was then correlated with the efficiency scores of the laying hens and with the total ω-3 PUFA concentration in the egg yolk.

***3.2.3.2 Energetic Efficiency Determination***

The hens were individually monitored for BW change, feed intake and egg production. Core body temperature was recorded remotely from the implanted sensors twice per minute per bird. Cloacal temperature was also measured with a thermometer at the start of the study to validate the accuracy of the internal temperature sensors. The temperature of individual cage microclimates was recorded every ten minutes using HOBO® temperature loggers (Onset Computers, 2003). Using this information, the birds were scored for energetic efficiency through calculation of RMEm determined using a model that accounted for metabolic maintenance energy (**MEm**) **(**Kcal ME/$BW^{0.67}$**)** requirement for BW, BW gain, continuous core body temperature and egg mass production (Romero et al., 2009). The estimates of the MEm for individual hens with respect to average daily ME intake (Kcal/d) during the 14 d duration of feeding enriched diet were calculated. The regression of MEm and ME intake was plotted that included the individual birds (Figure 3.1). The vertical distance between every point (birds) and the regression line corresponded to the RMEm value. Any birds below the regression line were considered more energetically efficient because

they had lower maintenance requirements than predicted (negative RMEm value) (**Efficient**). Birds above the line were considered less energetically efficient **(Non-efficient)**. Of the birds receiving the surgical temperature telemetry implants, the data from only 20 birds could be used for the energetic efficiency calculations as 4 implants ceased transmitting.

### 3.2.4 Fatty Acid Analysis

#### *3.2.4.1 Feed*

Triplicate samples of the control diet and the 17% LinPRO enriched experimental diet were analyzed for dietary fatty acid composition. Following grinding for 1 min, a 50 mg fine powdered feed sample was placed in a 10 mL, teflon lined screw capped, tubes and kept overnight with 100 µl of chloroform at room temperature. After shaking well, 2 mL of methylating reagent (1 N Methanolic HCl, Sigma, Oakville, ON, Canada) was added to the mixture and kept in a water bath at 60 C for 120 min for derivatization of fat. Water was added to make methanol/water (95/5 v/v). A known amount of internal standard (500 µL) (heptadecanoic acid, 17:0, Sigma, Oakville, ON, Canada) and 3 mL of hexane was added, mixed thoroughly, and centrifuged at 1,500 x g for 5 min. 1 mL of the clear hexane layer was transferred to GC vial after adjusting fat content to around 0.3 to 0.5 mg/mL.

#### *3.2.4.2 Egg yolk*

Fatty acid profile of duplicate yolk samples from individual birds was analyzed. The total lipids were extracted using the modified Folch extraction method and fatty acid composition was analyzed with GC as described in chapter 2. The Galaxie Chromatography Data System (Varian) was used for the fatty acid peak integration. Total ω-3 fatty acid levels were calculated as 18:3 ω-3 (**LNA**) + 20:5 ω-3 (**EPA**) + 22:5 ω-3 **(DPA**) + 22:6 ω-3 (**DHA**). Total ω-6 fatty acid levels were calculated as 18:2 ω-6 + 18:3 ω-6 + 20:2 ω-6 + 20:3 ω-6 + 20:4 ω-6 + 22:4 ω-6. Saturated fatty acids (**SFA**) levels were calculated as 14:0 + 16:0 + 18:0 + 20:0. Monounsaturated fatty acids (**MUFA**) levels were calculated as 16:1 ω-7 + 18:1 ω-7 + 18:1 ω-9 + 20:1 ω-9. Polyunsaturated fatty acids (**PUFA**) levels were

calculated as sum of total ω-3 acids + total ω-6 fatty acids. Total fatty acid was calculated as SFA + MUFA + PUFA.

### 3.2.5 Intestinal Morphometrics

After 14 d on the enriched diet, all birds were euthanized and dissected for measurement of liver weight and lengths of the duodenum, jejunum, ileum and colon. A 5 cm sample from the middle of duodenum was excised and fixed in 10% formalin. Duodenum samples were serially dehydrated in progressively more concentrated ethanol. This was followed by a hydrophobic clearing agent (xylene) to remove the alcohol, and finally embedded in molten paraffin wax. Blocks were cut to provide 5 µm transverse sections of duodenum. Four 5-µm sections were placed on a microscopic slide and stained with haematoxylin and eosin. The slides were examined with a Zeiss Axiovert 200M inverted microscope and digital images were captured for morphometric analysis. The villus height from the tip to the base of the lamina properia, villus width at one-third and two-thirds of the length of villus and crypt depth from the base of the villus to the mucosa was measured using the image analysis software MetaMorph (Version 6.2, Universal Imaging, Downington, PA, USA). The ratio of villus to crypt (V/C) was estimated by dividing the villus length by crypt depth (Kettunen et al., 2001). In addition, the duodenal surface area of villus was estimated considering villus as a cylindrical structure (Solis de los Santos et al., 2005).

Equation 1: $Villus\ Surface\ Area = 2\pi \times (Average\ Villus\ Width/2) \times Villus\ length$

### 3.2.6 Statistical Model

Nonlinear regressions were performed using the NLMIXED procedure of SAS (SAS System, 2002), which used maximum likelihood and with the Random statement, it allowed specifying a distribution of random effects, which were clustered by subject. The model used was:

$$MEI = (a+u) * MetBW^{0.67} + ADG + 2.40 * EM$$

where, MEI = ME intake (kcal/d); BW = BW (kg); ADG = ADG (g/d); EM = egg mass (g/d); u = hen related random term.

Residual maintenance requirement was analyzed thorough linear regressions of MEm and MEI for each hen: The regression model was:

$MEm = 91.4 + 0.27*MEI + e$; ($r^2 = 0.45$; $P=0.001$)

where MEm = predicted maintenance ME requirement; MEI = average ME intake, e = residual error.

The egg trait and gut morphometric parameters were analyzed as a one way ANOVA using the MIXED procedure of SAS with two levels of efficiency score (Efficient and Non-efficient) as fixed effects. Least squares means were adjusted using Tukey's honest test, and were reported as significant at $P < 0.05$ level (SAS System, 2002). The model used:

$$Y_{ik} = \mu + E_i + \varepsilon_{ik}$$

where $Y_{ik}$ = dependent variable for $k^{th}$ Bird, $\mu$ = overall mean, $E_i$ = $i^{th}$ efiicency score effect, $\varepsilon_{ik}$ = the residual error.

The fatty acid composition for the efficiency scores was analyzed as a two way ANOVA using the MIXED procedure of SAS with two durations of dietary treatment (0 d and 14 d) and two levels of efficiency score (Efficient and Non-efficient) as fixed effects. Least squares means were adjusted using Tukey's honest test, and were reported as significant at $P < 0.05$ level (SAS System, 2002). The model used:

$$Y_{ijk} = \mu + D_i + E_j + D_iE_j + \varepsilon_{ijk}$$

where $Y_{ijk}$ = dependent variable for $k^{th}$ bird, $\mu$= overall mean, $D_i$ = $i^{th}$ duration of dietary treatment effect, $E_j$ = $j^{th}$ efficiency score, $D_iE_j$ = the interaction effect between the $i^{th}$ duration of dietary treatment and $j^{th}$ efficiency score, $\varepsilon_{ijk=}$ the residual error.

The yolk fatty acid composition of eggs from birds sorted based on gut length or total feed intake was done as a 2-way ANOVA using the MIXED procedure of SAS. Following the 14 d experimental period, two levels of Efficiency score (Efficient and Non-efficient) and two levels of either feed intake (High and Low) or gut length (Short and Long) were assessed as the fixed effects.

Least squares means were adjusted using Tukey's honest test, and were reported as significant at $P < 0.05$ level (SAS System, 2002). The model used:

$$Y_{jkm} = \mu + E_j + N_k + N_kE_j + E_jN_k + \varepsilon_{jkm}$$

where $Y_{jkm}$ = dependent variable for $m^{th}$ bird; $\mu$= overall mean; $E_j = j^{th}$ efficiency score; and $k^{th}$ level of new variable (gut length or feed intake); $N_kE_j$ = the interaction effect between the $k^{th}$ level of new variable (gut length or feed intake) and $j^{th}$ efficiency score; $E_jN_k$ = the interaction effect between the $j^{th}$ efficiency score and $k^{th}$ level of new variable (gut length or feed intake); $\varepsilon_{jk=}$ the residual error.

Pearson's correlation coefficient with the Proc Corr procedure of SAS (SAS System, 2002) was used to determine if relationships existed among efficiency measures, gut histology and enrichment level. Significance was reported at the $P<0.05$ level.

Coefficients of variation (CV) were presented as measure of variability. The variability differences in total ω-3 PUFA in egg yolk were determined using the Means procedure of SAS (SAS System, 2002).

## 3.3 RESULTS AND DISCUSSION

### 3.3.1 Production Parameters

There was no difference in BW between Efficient and Non-efficient treatment birds at 0 d (Data not shown). However, the Efficient hens had a higher BW than the Non-efficient hens after the 14 d feeding of the enriched diet ($P=0.036$). In addition, the rate of lay was 96.0% in efficient birds compared to 88.6% in Non-efficient birds ($P=0.046$). There was no interaction of energetic efficiency score with the duration of feeding enriched diet on the egg traits (Table 3.2). However, the average egg weight was higher in efficient birds than non-efficient birds.

The yolk weight, percentage yolk and shell thickness were not affected by energetic efficiency (Table 3.2). The increased egg production in efficient birds was an expected result as energetically efficient birds had lower maintenance

requirement and therefore they are able to partition more energy for reproduction (Romero et al. 2009). The yolk weight and yolk as a percentage of egg weight declined significantly with after the 14 d feeding of enriched diet. This reduction in yolk weight following the enrichment of ω-3 PUFA into yolk is likely due to difficulties at liver level in lipid synthesis and transport to egg yolk and reductions in the hen's capacity to mobilize very low density lipoprotein (VLDL) with high PUFA to the yolk instead of SFA (Walzem, 1996; Van Elswyk et al., 1997).

### 3.3.2 Fatty acid Analysis

Fatty acid analysis of the feed indicated that ω-3 PUFA made up only 4.9% of the total fatty acids in the original basal diet compared to 26.5% in the ω-3 PUFA enriched diet fed during this study (Table 3.3). The ω-3 PUFA enriched diet was lower in both MUFA (32.0%) and SFA (11.4%) compared to the original hen diet (48.8% MUFA and 18.7% of SFA). The ω-6 PUFA was 19.2% higher in ω-3 PUFA enriched feed compared to the pre-experimental diet.

The fatty acid composition of feed was reflected in the fatty acid composition of eggs, with the exception of ω-6 PUFA and long chain ω-3 PUFA (**LC ω-3 PUFA**) (Table 3.4). The ω-6 PUFA concentration in Efficient hens was reduced in the egg from 901 mg/egg at 0 d to 789 mg/egg at 14 d, a decline of 12.4%. The reduction in ω-6 PUFA concentration in the egg yolk may relate to fact that the higher inclusion of ω-3 PUFA in experimental diet, which contained 5-fold more ω-3 PUFA than the pre-experimental diet, may suppress the synthesis of the long chain metabolites from medium chain precursors (Holman, 1998). The same set of desaturase and elongase enzymes are required by 18:2 ω-6 (LA) and 18:3 ω-3 (LNA) for the synthesis of their respective long chain metabolites (Garg et al. 1990, Shimizu et al. 2001, Tocher et al. 2006). As this competitive metabolism for synthesis of long chain metabolites takes place within the bird, it can change final fatty acid concentrations of the egg yolk. By 14 d on the enriched diet, concentration of ω-3 PUFA had risen by 104% in the egg (151.6 vs. 315.8 mg/egg), while the amount of SFA, MUFA and ω-6 PUFA were reduced ($P<0.0001$) (Table 3.4). The total LC ω-3 PUFA after 14 d of feeding was 137.4

mg/g yolk compared to 112.7 mg/g yolk at the start of the experiment. These LC ω-3 PUFA were not provided by the enriched diet (Table 3.2), indicating biosynthesis of LC ω-3 PUFA from the medium chain LNA was occurring in the bird. The eggs from the pre-experimental diet period did contain some LC ω-3 PUFA, which could be partly due to the use of canola oil in that ration. The fat in flax oil, canola oil, sunflower oil and soyabean oil contains 54.8%, 13.1%, 0.3% and 11.2% LNA respectively (Mazalli et al., 2004). The switch from the pre-experimental diet to the enriched diet in the current study led to an increase in DHA from 1.92% (99.1 mg/egg) at 0 d to 2.84% (115.0 mg/egg) at 14 d (Table 3.4). Mazalli et al. (2004) reported that the average yolk DHA from birds fed canola oil or flax oil based diet was 1.26% and 1.87% respectively, compared to 0.43% and 0.46% in yolk from sunflower oil and soybean oil fed birds. In the current study, use of 10% ground flaxseed with 0.5% canola oil resulted in a higher yolk DHA concentration than reported by Mazzalli et. al. (2004). The use of canola compared to soybean oil led to higher DHA concentration, which is likely a result of the competitive effect of the 4-fold greater ω-6 PUFA found in soybean compared to canola on the long-chain ω-3 PUFA biosynthesis pathway. Mazalli et al. (2004) concluded that the inclusion of 3% flax oil or 9% gound flaxseed compared to 3% fish oil or other vegetable oil sources (at 3%) led to the highest concentrations of DHA in egg yolks. When 4% oil inclusion has been tested, use of fish oil compared to linseed oil has resulted in 3.18% and 1.56% DHA, respectively, in the yolk (Baucells et al., 2000).

There was no statistical interaction of energetic efficiency with the duration of feeding the enriched diet on the fatty acid composition of egg yolks from birds scored for energetic efficiency (Table 3.4). The only significant difference observed among ω-3 PUFA composition was in 18:3ω-3 (LNA) and 22:5ω-3 (DPA) concentration in egg yolks. Eggs from the Non-efficient contained more LNA than the Efficient birds while eggs from Efficient birds contained more DPA. In addition, the LC ω-3 PUFA amount in egg yolk from Efficient hens was numerically higher in compared to Non-efficient ones (P=0.08). These results

suggests that Efficient birds could have increased ability for upconversion of medium chain ω-3 PUFA to long chain ω-3 PUFA compared to Non-efficient ones.

The liver weight was higher in Efficient hens than the Non-efficient ones (36.53 vs 32.99; P=0.038). As discussed earlier, the Efficient hens also had higher rate of lay compared to Non-efficient hens. A higher demand for support of egg production in Efficient birds would require a greater amount of fat to be moved through the liver to egg yolk. However, since liver weight as a percentage of BW was similar between Efficient and Non-efficient hens, it suggests that there may have been a difference in lipid utilization efficiency. Dietary fat in the pre-experimental diet and enrichment diet were 5.0 and 5.3%, respectively (Table 3.1), neither of which would be expected to be limiting to the support of yolk lipid formation. In addition, Efficient hens had lower abdominal fat weight compared to Non-efficient hens (43 g vs 52 g; P=0.05). These results for the impact of energetic efficiency on abdominal fat deposition in laying hens concurs with the findings of Washburn et al. (1975) and Whitehead et al. (1984), who did their work in broilers, and reported that the increase in feed efficiency lead to reduced abdominal fat pad weight.

The variability of total ω-3 PUFA in egg yolk as measured by coefficient of variation (CV) analysis, changed during the course of the experiment. At 0 d there was no difference in CV for total ω-3 PUFA in egg yolks from efficient and non efficient birds (27.7 vs. 28.2). However, by 14 d the CV for total ω-3 PUFA in egg yolk from efficient hens was lower than that of Non-efficient birds (11.1 vs. 21.4). This result supports the potential for using efficient hens for value added egg production could improve the uniformity of enrichment of the end product.

### 3.3.3 Feed Intake

Hen energetic efficiency did not significantly affect feed intake (data not shown). However, the birds with High feed intake had increased liver weight (r=0.52, P=0.02). The feed intake was negatively correlated with abdominal fat

weight (r = -0.52; P=0.02) and was also negatively correlated with body weight (r= - 0.39; P=0.05). In addition, birds with High feed intake also had a greater rate of lay (r= 44; P=0.04). This reduction in body weight despite increased feed intake is likely related to increased nutrient demands to support egg production (NRC, 1994). The reduced abdominal fat and increased liver weight in birds with High feed intake suggests that birds might be in a metabolic state that is more conducive for mobilization of fat for support of egg production (Freeman, 1983). Furthermore, the reduction of body weight in birds having High feed intake could also be related to a lower absorptive efficiency in the gut due to the presence of non-starch polysaccharide associated with flax based diets (Slominski et al., 2006). The lack of effect of energetic efficiency on enrichment of ω-3 PUFA in the current study may ultimately have been due to variability in nutrient absorption.

### 3.3.4 Intestinal morphometric Analysis

The results of the current study indicate that the Efficient hens had longer villi than in Non-efficient birds (Table 3.5). The average villus length for Efficient hens was 529 μm and for Non-efficient hens was 466 μm (P=0.019). There was no significant difference for the average villi widths for Efficient (79.0 μm) and Non-efficient hens (71.7 μm). However, the Efficient hens (0.130 $mm^2$) had significantly greater duodenal villus surface area/villi than in Non-efficient hens (0.105 $mm^2$) (P=0.013). The increased villus length provides more mucosal surface area for nutrient absorption (Onderci et al., 2006). In addition, the villus length to crypt depth ratio, which is considered as a useful criterion for estimating the digestive capacity the small intestine (Montgane, 2003) was significantly higher in Efficient birds than the Non-efficient birds.

Results for the gut morphometric clearly indicate that Efficient birds were suited for increased nutrient absorption than the Non-efficient birds due to their greater duodenal surface area/villi and higher villus length/crypt depth ratio. In addition, crypt depth was deeper in Non-efficient hens (58.33) than in Efficient hens (49.15; P=0.039). The deeper crypts indicate more rapid tissue turnover and

is linked to a higher energy and protein demand for gut maintenance (Yason et al., 1987). Therefore, a shallow crypt in Efficient hens possibly indicates a lower turnover rate of the intestinal epithelium, which results in a lower maintenance requirement and favours higher growth efficiency (Yang et al., 2001).

The volume of feed in the gut can influence the intestinal structural integrity (Savory and Gentle, 1976). In addition, the White Leghorn has greater ability than broiler and turkey to increase feed intake to compensate for the nutrient deficiency associated with the poor absorption at gut (Summers and Leeson, 1986). However, the villus length can be adversely affected by increased feed intake and villus atrophy will then initiate the deepening of crypt (Van Beers-Schreurs et al., 1998). In the present study, the villus length was not affected by feed intake, but an increase in the crypt depth with the High feed intake was observed ($r = 0.41$; $P = 0.043$). However, High feed intake was not associated with a reduced villi length. Birds with deeper crypts would need to increase their feed intake to make up for the high nutrient demand of the higher cell turn over associated with the deeper crypt. The birds with longer villi likely had better nutrient absorption which could subsequently be linked to a lower feed intake. Specific rates of nutrient transfer could be tested using an indigestible marker such as, d-xylose, which is a pentose sugar that is absorbed from the upper small intestinal tract, similar to the glucose and amino acids (Eberts et al, 1979; Goodwin et al., 1984). The transfer of this sugar to the blood is indicative of gut absorption potential in birds (Doerfler et al., 2000), but does not indicate if this is related to villi structure, gut capacity or any potential concentration among nutrients.

Small intestine length was positively correlated with the efficiency scores ($r = 0.29$, $P = 0.01$) and indicated that the Efficient birds had a longer gut than Non-efficient ones. The results suggest that birds with Long and Short guts had different histological measures associated with their villi and that feed intake affected this. The passage rate of digesta, although not measured in this study, could also be a factor affecting the availability of nutrients. An increase in digesta

transit time leads to increased opportunity for nutrients to be digested and absorbed (Pym, 2005). Ultimately these traits were not directly associated with egg fatty acid levels or ω-3 PUFA enrichment, suggesting that gut morphology and hen energetic efficiency have less influence on egg lipid composition and concentration than post-absorptive lipid metabolism does.

In this study, we examined birds at a specific age. However, gut length is known to be very plastic and varies with age, BW, feed type and body demands (Weaver et al., 1991; Nahashon et al., 1995; Batal and Parsons, 2002). It is therefore possible that the post mortem gut length relationships we found with total ω-3 PUFA may not be true representation of what may have happened in the bird over a longer time period.

The dietary energetic efficiency of the bird depends largely on the ingredient digestibility by gut (Flock, 1998), however the bird's metabolic efficiency and the energy distribution for the maintenance requirement and reproductive functions also plays important role in differentiating laying hens on efficiency score (Luiting, 1990). The birds selected on the basis of the RMEm methodology provide an unbiased estimate of energetic efficiency by adjusting the maintenance requirement for the effect of dietary thermogenesis (Romero et al., 2009). These highly energetic efficient birds, when used for the purpose of value-added enrichment, could contribute to reducing the variability in transfer of enrichment to the egg end product. Moreover, the uniformity in end product can be achieved by targeting the absorption efficiency at gut level. The inclusion of feed additives in the poultry ration to potentially increase the nutrient absorption either by providing protection of gut epithelium from irritants or by feeding ingredients developing macro and micro structure of gut might be helpful to achieve the target of uniform enrichment in table eggs. In further trials, it will be of value to examine how the enriched feed ingredients along with gut developing feed additives will affect the histomorphometric parameters of absorptive surface of the small intestine with time and ultimately how the transfer efficiency to the table eggs is affected.

## 3.4 ACKNOWLEDGEMENTS

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**Table 3.1: Diet composition and nutrient content of experimental diets[1].**

| Treatment | Pre-trial diet | Experimental enriched diet |
|---|---|---|
| Ingredients | | |
| Canola meal | 3.7 | 6.0 |
| Corn, yellow,grain | 15.0 | 28.0 |
| Oats, grain | 18.0 | 18.0 |
| Soybean meal | 15.7 | 13.7 |
| Wheat, hard, grain | 33.3 | 7.8 |
| Calcium carbonate | 8.7 | 8.5 |
| Dicalcium phosphate. | 1.3 | 1.5 |
| Salt, plain (NaCl) | 0.3 | 0.3 |
| D,L – methionine | 0.2 | 0.1 |
| LinPRO[2] | -- | 15.0 |
| Layer Vit/Min Premix[3] | 0.5 | 0.5 |
| Choline Chloride | 0.5 | 0.5 |
| Enzyme[4] | 0.1 | 0.1 |
| Canola oil | 2.8 | 0.5 |
| | | |
| Calculated Nutrient Analysis: | | |
| M.E. kcal/kg | 2,750.0 | 2,750.0 |
| Dry matter % | 90.0 | 90.0 |
| Protein, crude % | 17.0 | 17.5 |
| Fat, crude % | 5.0 | 5.3 |
| Fiber, crude % | 4.0 | 4.5 |
| Calcium % | 3.7 | 3.7 |
| Phos. Total % | 0.6 | 0.6 |
| Phos., available % | 0.4 | 0.4 |
| Met + cys % | 0.7 | 0.7 |
| Lysine % | 0.9 | 0.9 |

[1]Diets are: Pre-trial diet = Standard layer ration; Experimental enriched diet= standard ration formulated with inclusion of 17% linPRO was fed for 14 days to laying hens (56 wks).
[2]LinPRO is extruded flaxseed with peas in 1:1 ratio. (O & T farms, Regina, SK, Canada).
[3]The layer Vit/Min premix contained per kilogram of diet: vitamin A (retinyl acetate), 12,000 IU; cholecalciferol, 3,000 IU; vitamin E (DL-α-tocopheryl acetate), 40 IU; vitamin K, 2.0 mg; pantothenic acid, 14 mg; riboflavin, 6.5 mg; folacin, 1.0 mg; niacin, 40 mg; thiamine, 3.3 mg; pyridoxine, 6.0 mg; vitamin B12, 0.02 mg; biotin, 0.2 mg; iodine, 0.5 mg; Mn, 75 mg; Cu, 15 mg; Zn, 80 mg, Se, 0.1 mg; and Fe, 100 mg; choline chloride, 100 mg.
[4]Enzyme: Avizyme 1302, Xylanase enzyme, Danisco Animal Nutrition, Marlborough, Wiltshire, UK

**Table 3.2: Egg traits in laying hens (56 wks) scored on energetic efficiency[1] fed ω-3 PUFA diet[2] for 14 days.**

| Treatment | Duration | Egg Weight (g) | Yolk Weight (g) | Yolk % | Shell Thickness (mm) |
|---|---|---|---|---|---|
| Efficient | | 64.25[a] | 19.08 | 29.70 | 0.326 |
| Non-efficient | | 61.33[b] | 18.80 | 30.70 | 0.327 |
| SEM | | 0.713 | 0.431 | 0.500 | 0.007 |
| | 0 d | 62.84 | 19.46[a] | 31.10[a] | 0.321 |
| | 14 d | 62.74 | 18.42[b] | 29.40[b] | 0.332 |
| SEM | | 0.763 | 0.241 | 0.400 | 0.005 |
| Efficient | 0 d | 64.23 | 19.52 | 30.50 | 0.323 |
| | 14 d | 61.46 | 19.39 | 31.70 | 0.320 |
| Non-efficient | 0 d | 64.27 | 18.63 | 29.00 | 0.330 |
| | 14 d | 61.21 | 18.21 | 29.80 | 0.335 |
| SEM | | 0.983 | 0.426 | 0.600 | 0.008 |
| Source of Variation | | ----------P-value---------- | | | |
| Treatment | | 0.0102 | 0.4261 | 0.0839 | 0.8658 |
| Duration | | 0.9226 | 0.0040 | 0.0039 | 0.1195 |
| Treatment * Duration | | 0.3856 | 0.8934 | 0.6745 | 0.6683 |

[1]The birds were scored as Efficient and Non-efficient on the basis of the regression analysis of maintenance requirement (MEm) and metabolizable energy intake per day of each individual birds during the experiment.
[2]Enriched diet was a 17% inclusion of LinPRO in the basal layer diet fed to laying hen (56 wks).
[a-b] Means within a column and within a source with no common superscript are significant different (P<0.05).

**Table 3.3: Fatty acid composition of experimental diets[1].**

| Fatty acid | Pre-trial diet | Experimental enriched diet |
|---|---|---|
| | ----------% Composition---------- | |
| 14:0 | 0.209±0.01 | 0.111±0.01 |
| 14:1ω-7 | 0.151±0.01 | 0.001±0.01 |
| 16:0 | 14.540±0.03 | 8.703±0.11 |
| 16:1ω-7 | 0.243±0.01 | 0.227±0.01 |
| 17:1ω-7 | 0.084±0.01 | 0.120±0.01 |
| 18:0 | 2.621±0.01 | 2.174±0.01 |
| 18:1ω-7 | 2.472±0.05 | 2.180±0.03 |
| 18:1ω-9 | 41.380±0.12 | 28.630±0.22 |
| 18:2ω-6 | 23.590±0.15 | 29.330±0.15 |
| 18:3ω-3 | 3.717±0.10 | 26.440±0.01 |
| 18:3ω-6 | 0.029±0.00 | 0.026±0.00 |
| 20:0 | 0.639±0.01 | 0.269±0.01 |
| 20:1ω-7 | 2.787±0.05 | 0.561±0.01 |
| 20:1ω-9 | 1.401±0.01 | 0.059±0.01 |
| 20:2ω-6 | 0.107±0.00 | 0.064±0.01 |
| 20:3ω-3 | 0.099±0.07 | 0.031±0.01 |
| 20:5ω-3 | 0.095±0.02 | 0.048±0.01 |
| 22:0 | 0.537±0.04 | 0.275±0.03 |
| 22:1ω-7 | 0.213±0.01 | 0.100±0.01 |
| 22:2ω-6 | 0.170±0.24 | 0.134±0.01 |
| 22:6ω-3 | 1.073±0.01 | 1.032±0.01 |
| 24:0 | 0.640±0.01 | 0.105±0.01 |
| 24:1ω-7 | 0.180±0.00 | 0.120±0.00 |
| SFA[2] | 18.650±0.07 | 11.360±0.01 |
| MUFA[3] | 48.760±0.20 | 32.000±0.25 |
| PUFA[4] | 28.910±0.23 | 56.120±0.17 |
| LC ω-3 PUFA[5] | 1.267±0.09 | 1.111±0.08 |
| Total ω-3 PUFA[6] | 4.985±0.16 | 26.550±0.01 |
| Total ω-6 PUFA[7] | 23.920±0.40 | 29.570±0.17 |
| Ratio[8] | 4.803±0.58 | 1.114±0.01 |

[1]Diets are: Pre-trial diet = Standard layer ration; Experimental enriched diet= standard ration formulated with inclusion of 17% linPRO was fed for 14 days to laying hens (56 wks).
[2]SFA = saturated fatty acids; SFA levels were calculated as 14:0 + 16:0 + 18:0 + 20:0 + 22:0 + 24:0.
[3]MUFA = monounsaturated fatty acids; MUFA levels were calculated as 16:1 ω-7 + 18:1 ω-7 + 18:1 ω-9 + 22:1 ω-9 + 24:1 ω-7.
[4]PUFA = polyunsaturated fatty acids; PUFA levels were calculated as 18:2 ω-6 + 18:3 ω-3 +18:3 ω-6 + 20:2ω-6 + 20:3ω-3 + 20:3ω-6 + 20:4 ω-6 + 20:2 ω-6. + 20:5 ω-3 + 22:2 ω-6 + 22:6 ω-3.
[5]LC ω-3 PUFA was calculated as 20:3 ω-3 + 20:5 ω-3 + 22:6 ω-3.
[6]Total ω-3 PUFA levels were calculated as 18:3 ω-3 + 20:3 ω-3 + 20:5 ω-3 + 22:6 ω-3.
[7]Total ω-6 PUFA levels were calculated as 18:2 ω-6 + 18:3 ω-6 + 20:2ω-6 + 20:3ω-6 + 20:4 ω-6 + 22:4 ω-6.
[8]Ratio= Total ω-6/ Total ω-3

**Table 3.4: The egg yolk fatty acid (mg/g yolk) profile of hens scored for energetic efficiency[1] fed ω-3 PUFA[2] diet for 14 d.**

| Source | Duration | | Efficiency | | | 0 d | | 14 d | | | Probability | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fatty Acid | 0 d | 14 d | NE[1] | E[2] | SEM | NE[1] | E[2] | NE[1] | E[2] | SEM | D | E | D*E |
| 14:0 | 13.58[a] | 9.29[b] | 10.88 | 11.99 | 0.42 | 12.28[b] | 14.88[a] | 9.48[c] | 9.10[c] | 0.56 | 0.0001 | 0.0516 | 0.0093 |
| 16:0 | 1,253[a] | 894[b] | 1,070 | 1,077 | 19.8 | 1,235 | 1,271 | 905 | 883 | 28.00 | 0.0001 | 0.8097 | 0.3090 |
| 16:1ω-7 | 101.7[a] | 59.6[b] | 77.8 | 83.5 | 3.35 | 94.0 | 109.5 | 61.7 | 57.5 | 4.74 | 0.0001 | 0.2357 | 0.0514 |
| 18:0 | 502.0[a] | 440.6[b] | 470.4 | 472.2 | 9.43 | 500.5 | 503.4 | 440.3 | 440.9 | 13.34 | 0.0001 | 0.8951 | 0.9309 |
| 18:1ω-7 | 113.3[a] | 69.5[b] | 90.6 | 91.8 | 1.87 | 112.1 | 114.4 | 69.0 | 69.1 | 2.65 | 0.0001 | 0.6480 | 0.6848 |
| 18:1ω-9 | 2,065[a] | 1,426[b] | 1,739 | 1,752 | 35.53 | 2,055 | 2,075 | 1,424 | 1,429 | 50.24 | 0.0001 | 0.8001 | 0.8873 |
| 18:2ω-6 | 696.9[a] | 625.6[b] | 677.5 | 645 | 13.44 | 721.6 | 672.2 | 633.4 | 617.7 | 19.01 | 0.0003 | 0.0909 | 0.3780 |
| 18:3ω-3 | 45.8[b] | 188.3[a] | 123.9[a] | 110.2[b] | 4.17 | 49.6 | 42.0 | 198.2 | 178.4 | 5.90 | 0.0001 | 0.0226 | 0.2995 |
| 18:3ω-6 | 6.264[a] | 4.734[b] | 5.355 | 5.643 | 0.22 | 5.832 | 6.696 | 4.878 | 4.589 | 0.31 | 0.0001 | 0.3589 | 0.0678 |
| 20:1 | 9.54[a] | 6.37[b] | 8.19 | 7.73 | 0.47 | 9.61 | 9.48 | 6.78 | 5.97 | 0.66 | 0.0001 | 0.4776 | 0.6100 |
| 20:3ω-3 | 3.251 | 3.929 | 3.824 | 3.356 | 0.39 | 3.546 | 2.956 | 4.101 | 3.756 | 0.55 | 0.2212 | 0.3969 | 0.8242 |
| 20:3ω-6 | 9.17[b] | 11.37[a] | 10.01 | 10.54 | 0.43 | 8.56 | 9.79 | 11.46 | 11.29 | 0.60 | 0.0005 | 0.3863 | 0.2475 |
| 20:4ω-6 | 140.7[a] | 84.5[b] | 108.2[b] | 117.0[a] | 2.56 | 134.0 | 147.4 | 82.4 | 86.6 | 3.62 | 0.0001 | 0.0171 | 0.2060 |
| 20:5ω-3 | 3.459[b] | 8.533[a] | 5.842 | 6.150 | 0.58 | 3.031 | 3.886 | 8.652 | 8.413 | 0.82 | 0.0001 | 0.7085 | 0.5070 |
| 22:2ω-6 | 56.42 | 61.73 | 59.27 | 58.88 | 2.21 | 57.60 | 55.24 | 60.94 | 62.52 | 3.13 | 0.0933 | 0.9005 | 0.5310 |
| 22:5ω-3 | 6.95[b] | 9.92[a] | 7.64[b] | 9.23[a] | 0.26 | 6.35[c] | 7.56[bc] | 8.93[b] | 10.90[a] | 6.30 | 0.0001 | 0.0001 | 0.3132 |
| 22:6ω-3 | 99.1[b] | 115.0[a] | 104.2 | 109.9 | 2.33 | 98.1 | 100.0 | 110.4 | 119.7 | 3.29 | 0.0001 | 0.0902 | 0.2631 |
| SFA[3] | 1,768[a] | 1,344[b] | 1,551 | 1,561 | 27.06 | 1,748 | 1,789 | 1,355 | 1,333 | 38.26 | 0.0001 | 0.8017 | 0.4155 |
| MUFA[4] | 2,294[a] | 1,569[b] | 1,922 | 1,941 | 38.89 | 2,274 | 2,314 | 1,570 | 1,568 | 55.00 | 0.0001 | 0.7292 | 0.7100 |
| PUFA[5] | 1,078 | 1,120 | 1,114 | 1,084 | 19.21 | 1,098 | 1,057 | 1,130 | 1,110 | 27.16 | 0.1222 | 0.2660 | 0.7112 |
| LC ω-3 PUFA[6] | 112.7[b] | 137.4[a] | 121.5 | 128.6 | 2.82 | 111.0 | 114.4 | 132.0 | 142.8 | 3.99 | 0.0001 | 0.0800 | 0.3610 |
| Total ω-3 PUFA[7] | 151.6[b] | 315.8[a] | 237.8 | 229.6 | 5.69 | 154.3 | 148.9 | 321.4 | 310.2 | 8.05 | 0.0001 | 0.3091 | 0.7213 |
| Total ω-6 PUFA[8] | 919.0[a] | 794.3[b] | 868.5 | 844.8 | 14.97 | 937.2 | 900.8 | 799.8 | 788.7 | 21.17 | 0.0001 | 0.2648 | 0.5535 |
| Ratio[9] | 6.137[a] | 2.619[b] | 4.473 | 4.283 | 0.10 | 6.279 | 5.995 | 2.667 | 2.572 | 0.14 | 0.0001 | 0.1728 | 0.4961 |
| Egg lipid (g) | 6.120[a] | 5.566[b] | 5.773 | 5.912 | 0.14 | 6.095 | 6.145 | 5.452 | 5.680 | 0.19 | 0.0054 | 0.4769 | 0.6465 |

*SEM = standard error mean; SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids. D= Duration; E= Efficiency score.

[1]The birds were scored as Efficient and Non-efficient on the basis of the regression analysis of maintenance requirement (MEm) and metabolizable energy intake per day of each individual birds during the experiment. NE = Non-efficient Hen, E = Efficient Hen.

[2]Enriched diet was a 17% inclusion of LinPRO in the basal layer diet fed to laying hen (56 wks).

[3]SFA = saturated fatty acids; SFA levels were calculated as 14:0 + 16:0 + 18:0 + 20:0 + 22:0 + 24:0.

[4]MUFA = monounsaturated fatty acids; MUFA levels were calculated as 16:1 ω-7 + 18:1 ω-7 + 18:1 ω-9 + 22:1 ω-9 + 24:1 ω-7.

2848 [5]PUFA = polyunsaturated fatty acids; PUFA levels were calculated as 18:2 ω-6 + 18:3 ω-3 +18:3 ω-6 + 20:2ω-6 + 20:3ω-3 + 20:3ω-6 + 20:4 ω-6 + 20:2 ω-
2849 6. + 20:5 ω-3 + 22:2 ω-6 + 22:6 ω-3.
2850 [6]LC ω-3 PUFA was calculated as 20:3 ω-3 + 20:5 ω-3 + 22:6 ω-3.
2851 [7]Total ω-3 PUFA levels were calculated as 18:3 ω-3 + 20:3 ω-3 + 20:5 ω-3 + 22:6 ω-3.
2852 [8]Total ω-6 PUFA levels were calculated as 18:2 ω-6 + 18:3 ω-6 + 20:2ω-6 + 20:3ω-6 + 20:4 ω-6 + 22:4 ω-6.
2853 [9]Ratio= Total ω-6/ Total ω-3
2854 [a-c] Means within row and within source with no common superscript are significant different (P<0.05)

**Table 3.5: The gut morphometric analysis of hens scored for energetic efficiency[1] and fed ω-3 PUFA[2] diet for 14 d.**

| Source | Villus length (µm) (V) | Villus width (µm) (D) | Crypt depth (µm) (C) | VSA[3] ($mm^2$) | Duodenum (cm) | Ratio (V/C) |
|---|---|---|---|---|---|---|
| Efficient | 529.1[a] | 79.02 | 49.15[b] | 0.130[a] | 25.50 | 10.94[a] |
| Non-efficient | 466.3[b] | 71.70 | 58.33[a] | 0.105[b] | 26.59 | 08.36[b] |
| SEM | 15.43 | 3.776 | 2.936 | 0.006 | 0.578 | 0.640 |
| Probability | 0.019 | 0.186 | 0.039 | 0.013 | 0.197 | 0.010 |

[1]The birds were scored as Efficient and Non-efficient on the basis of the regression analysis of maintenance requirement (MEm) and metabolizable energy intake per day of each individual birds during the experiment.
[2]ω-3 PUFA diet was a 17% inclusion of LinPRO in the basal layer diet fed to laying hen (56 wks) for 14 d.
[3]Villus Surface Area (VSA) calculated with average villus length (V) and width (D)= $2\pi * (D/2) * V/10^6$
[a-b] Means within a column and within a source with no common superscript are significant different ($P<0.05$).

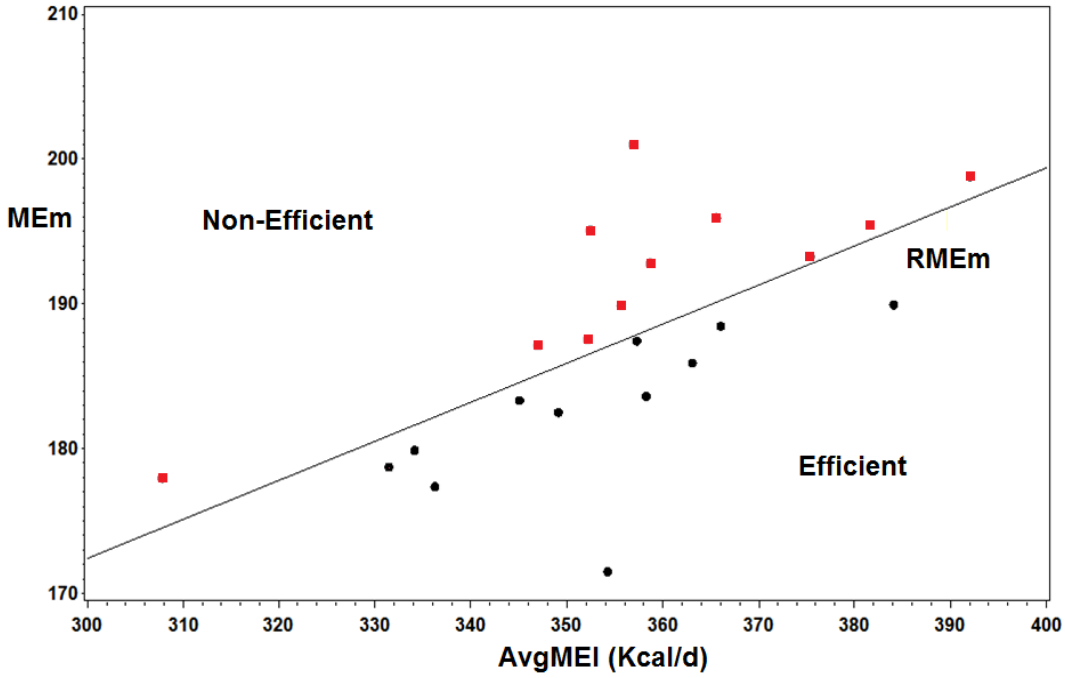


**Figure 3.1: Estimates of the metabolizable energy for maintenance (MEm) (kcal ME/BW$^{0.67}$) for individual hens with respect to average daily metabolizable energy intake (AvgMEI) (kcal/d) during the duration of feeding enriched diet (14 d). The regression of MEm and ME intake was plotted. The vertical distance between every point (birds) and the regression line corresponded to the residual metabolizable energy for maintenance (RMEm) value. The birds below the regression line (Negative RMEm) were scored as Efficient and above the lines (Positive RMEm) were scored as Non-efficient.**

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# CHAPTER 4

# Characterization of Omega-3 PUFA Enrichment Process from Diet to Blood Plasma and Egg Yolk in Hens

## 4.1 INTRODUCTION

Table eggs enriched with omega-3 polyunsaturated fatty acids (**ω-3 PUFA**) can be used to increase the ω-3 PUFA content of the human diet. Linolenic acid (18:3 ω-3; **LNA**), and linoleic acid (18:2 ω-6; **LA**) are essential fatty acids that are not synthesized in the body and can only be supplied through food (Bezard et al., 1994). Following absorption, LA and LNA are further metabolized to long chain omega-6 polyunsaturated fatty acids (**ω-6 PUFA**) and ω-3 PUFA respectively (Emken, 1994). The physiologically important long chain metabolites of LNA are eicosapentaenoic acid (20:5 ω-3; **EPA**), docosapentaenoic acid (22:5 ω-3; **DPA**) and docosahexaenoic acid (22:6 ω-3; **DHA**) (Mohrhauer et al., 1967). The long chain ω-3 PUFAs, with EPA and DHA in particular, have been demonstrated to contribute to the prevention of cardiovascular diseases, atherosclerosis, cancer, diabetes and development of fetal brain and neural system in humans (Yashodhara et al., 2009; Marik and Veron, 2009).

Birds lack the required delta-12 (Δ-**12**) and delta-15 (Δ-**15**) desaturase enzymes for *de novo* endogenous synthesis of metabolites of 22 or more carbons from LNA (Hulbert et al., 2002). However, birds are capable of biosynthesis of up to 22 or more carbons from LNA and LA with the help of delta-5 (Δ-**5**) and delta-6 (Δ-**6**) Δ-6 desaturase and elongase enzymes (Goyens et al., 2006; Holman, 1998). LNA and LA compete for same enzymatic machinery for the bioconversion into longer chain PUFAs (Holman, 1998). When present in equal amounts, LNA is metabolized preferentially compared to LA by desaturase and elongase enzymes (Kinsella, 1991). However, when a higher amount of LA is present, it can have a suppressive effect on LNA biosynthesis (Watkins, 1992; Shimizu et al., 2001). LNA is 10 times stronger at suppressing LA metabolism

compared to the effect of LA on LNA metabolism (Holman, 1998). Therefore, inclusion of more LNA in poultry diets could partially ameliorate the limited but desirable up-conversion of LNA to long chain ω-3 PUFA. A higher amount of LNA would also contribute to the achievement of a more balanced ratio of ω-6 to ω-3 fatty acids. The optimum dietary ratio of ω-6 to ω-3 in humans is between 1:1 and 1:4, which is in contrast to most Western diets, where the ratio is between 10:1 and 20:1 (Eaton et al., 1992; Simopoulos, 2004). A lower ratio can contribute to a reduction of 20:4 ω-6 (AA) associated pro-inflammatory eicosanoid products (Garg et al., 1990; Cherian, 2007).

The ω-3 PUFA sources used for enrichment of the hen diet includes fish oil, marine algae (Herber and Van Elswyk, 1996), and plant sources such as flaxseed (Jiang et al., 1991;Van Elswyk, 1997), canola oil (Jia et al., 2008), perilla oils (Kim et al., 1997), and echium oil (Kitessa and Young, 2009). Flaxseed is a popular plant source for ω-3 PUFA enrichment because it has a high amount of ω-3 PUFA, with LNA accounting for 48 to 58% of the total fatty acid present (Gonzalez-Esquerra and Leeson, 2000; Jia et al., 2008). However, inclusion of ground flaxseed in the hen ration at a level greater than 10% had a negative effect on egg production parameters (Bean and Leeson, 2003; Leeson et al., 2000). This has been attributed to reduction in nutrient utilization in birds due to antinutritional factors such as mucilage, cyanogenic glycosides, or trypsin inhibitors (Bhatty, 1993). An alternate form of flaxseed in the hen diet in an extruded product that disrupts many of the antinutritional factors associated with whole and ground flaxseed (Alzueta et. al., 2003). The combination of shearing and thermal effects of the extrusion process significantly degrades the mucilage (Wu et al., 2010) associated with the hull of flax that is responsible for increased viscosity at gut level of intestinal contents which will reduce digestibility (Bhatty, 1993).

The Canadian Food Inspection Agency permits to label a product as a "source of ω-3" if the total ω-3 PUFA is at least 0.3 g per reference amount (50 g egg) (CFIA, 2003). Whereas the lipid content of the egg varies with age and

genetics, the type and composition of dietary fatty acid source and duration of feeding are important considerations in designing an effective enrichment program (Milinsk et al., 2003). An understanding of the effects of duration of feeding and concentration of dietary enrichment ingredients is required to ensure that minimum enrichment levels in the end product are reached. Sim and Cherian (1994) have reported that ω-3 PUFA levels in the eggs stabilize between 9 and 12 days of flax-feeding. In a previous trial, there was stabilization in the level of total ω-3 PUFA in egg yolk within 14 days of feeding the enriched diet (Chapter 3). Therefore, in the present study we collected blood and egg yolk samples at an interval of six days to more closely examine the change in fatty acid profile. The objective of this study was to optimize the level and duration of flaxseed feeding to prevent the saturation in the ω-3 PUFA absorption capability of the bird. This experiment was designed to characterize changes in the fatty acid profile of the blood plasma and eggs of hens fed an extruded flaxseed product to develop a better understanding of the process of ω-3 PUFA transfer from the hen diet to the egg. The time required for the ω-3 PUFA enriched feed to generate eggs that has reached a minimum level of 300 mg ω-3 PUFA/egg was evaluated. In addition, the impact of manipulation of fatty acid composition on estimated enzyme activities for enzymes involved in biosynthesis of the long chain ω-3 PUFA was evaluated.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Study Design

An Institutional Animal Policy and Welfare Committee approved all experimental animal protocols. The experiment was a 3 × 4 factorial design including three level of ω-3 PUFA enrichment: **Control**; **Moderate,** with 7.5% LinPRO® (O & T Farms, Regina, Saskatchewan, Canada; flaxseed: peas, 1:1 wt/wt; ground-extruded); or **High**, with 15% LinPRO fed for one of four durations 0, 6, 12 or 18 d. The Moderate diet was a 50:50 mix of the Control and

the High diets (Table 4.1). All the birds were fed the layer Control diet without supplemental ω-3 PUFA prior to the start of experiment.

### 4.2.2 Sampling

This study used a group of 65 wk old, Lohmann White Leghorn laying hens. At the start of the dietary treatments (day 0), 25 birds per treatment were placed randomly into individual cages. Birds were weighed, and blood and egg samples taken to establish baseline values for BW, fatty acid composition in blood plasma and egg yolk. The birds were then provided the Control, Moderate or High ω-3 PUFA enriched diets for 18 d. All three diets provided 16% CP and 2750 Kcal/ kg. The birds had ad libitum access to water. A lighting program of 16 h of light and 8 h of dark was used for the entire experiment.

Eggs were collected at 0 d, 6 d, 12 d and 18 d for yolk lipid analysis. The egg production data were recorded daily to monitor rate of lay of individual birds. Egg weight, yolk weight and shell thickness were determined for all eggs sampled. The BW was measured and blood samples and egg yolk samples were collected for lipid composition on day 6, 12 and 18 of the study. Feed intake was calculated for each bird in each period. The blood samples were collected in vaccutainers with EDTA as the anticoagulant. Blood samples were centrifuged at 1,200 x g for 15 min at 4 C to separate the plasma layer and stored at -20 C prior to analysis. Plasma samples and egg yolk samples were subjected to GC to determine fatty acid profile for the samples from different periods in trial. Representative feed samples from all diets were collected to assess the dietary fatty acid profile.

Estimated enzyme activities can be calculated as the ratio of daughter fatty acid to the parent fatty acid (Kinesella, 1991; Warensjo et al., 2008). In the current study, the Δ-9 desaturase activity involved in MUFA was calculated as the ratio of 18:1 ω-9/18:0. The LNA will metabolize to EPA with stearidonic acid (18:4 ω-3) and ω-3 arachidonic acid (20:4ω-3) as intermidates following a series of desaturation and elongation steps involving Δ-6 desaturase, elongase and Δ-5 desaturase enzymes (Holman, 1998). Similarly, the same set of enzymatic action

is invoved in upconversion of dietary LA via γ- linolenic (20:3n-6) to arachidonic acid (20:4n-6) (Holman, 1998; Kinsella, 1991). To analyze the enzyme activity of ω-6 PUFA upconversion (Δ-6 desaturase +elongase + Δ-5 desaturase activity) the ratio of 20:4 ω-6/18:2ω-6 was calculated (Betti et al., 2009). Similarly, the enzyme activity for ω-3 PUFA upconversion (Δ-6 desaturase +elongase + Δ-5 desaturase activity) was calculated as 20:5 ω-3/18:2 ω-3.

### 4.2.3 Fatty Acid Analysis

#### *4.2.3.1 Feed*

Representative samples of the Control diet and the Moderate and High enriched diets were analyzed in triplicate for dietary fatty acid composition. Following grinding for 1 min, a 50 mg of fine powdered feed sample was placed in a 10 mL, teflon lined screw capped, tubes and kept overnight with 100 µl of chloroform at 21 C. After shaking well, 2 mL of methylating reagent (1 N Methanolic HCl, Sigma, Oakville, ON, Canada) was added to the mixture and kept in a water bath at 60 C for 120 min for derivatization of fat. Water was added to make methanol/water (95/5 v/v). A known amount of internal standard (500 µL) (heptadecanoic acid, 17:0, Sigma, Oakville, ON, Canada) and 3 mL of hexane was added, mixed thoroughly, and centrifuged at 1,500 x g for 5 min. 1 mL of clear hexane layer was transferred to GC vial after adjusting fat content to around 0.3 to 0.5 mg/mL.

#### *4.2.3.2 Blood Plasma and Egg Yolk*

From blood plasma and yolk samples collected from individual birds, either the 1 mL plasma or 1g of raw yolk was mixed at a 1:16 ratio with Folch solution (Chloroform: Methanol; 2:1 v/v) and stored overnight at room temperature to extract fat. The dried fat was resolubilized in 1 mL of chloroform and 50 µl of the reconstituted mixture (extracted fat and chloroform) was then derivatized using 2 mL of methylating reagent (1 N Methanolic HCl, Sigma, Oakville, ON, Canada) in a water bath at 60 C for 60 min. Following the derivatization, the fatty acid methyl esters were injected to GC for assessment of fatty acid composition. The GC operative condition and calculation for

quantification of the individual fatty acid were same as described previously (Chapter 2).

### 4.2.4 Statistical Analysis

Egg traits, fatty acid composition of plasma and egg yolk, and calculated enzyme activity pathway data were analyzed as a two way ANOVA using the MIXED procedure of SAS with three levels of dietary treatment (Control, Moderate and High) and four durations (0 d, 6 d, 12 d and 18 d) as fixed effects. Least squares means were adjusted using Tukey's honest test, and were reported as significant at $P < 0.05$ level (SAS System, 2002). The model used:

$$Y_{ijk} = \mu + D_i + T_j + D_iT_j + \varepsilon_{ijk}$$

where $Y_{ijk}$ = dependent variable for $K^{th}$ bird, $\mu$= overall mean, $D_i$ = $i^{th}$ dietary treatment effect, $T_j$ = $j^{th}$ duration of feeding effect, $D_iT_j$ = the interaction effect between the $i^{th}$ dietary treatment and $j^{th}$ duration of feeding and $\varepsilon_{ijk}$= the residual error.

The feed intake and BW data were analyzed as a two way ANOVA using the MIXED procedure of SAS with three levels of dietary treatment (Control, Moderate and High) and three durations (6d, 12d and 18d) as fixed effects. Least squares means were adjusted using Tukey's honest test, and were reported as significant at $P < 0.05$ level (SAS System, 2002). The model used:

$$Y_{ijk} = \mu + D_i + T_j + D_iT_j + \varepsilon_{ijk}$$

where $Y_{ijk}$ = dependent variable for $K^{th}$ bird, $\mu$= overall mean, $D_i$ = $i^{th}$ dietary treatment effect, $T_j$ = $j^{th}$ duration of feeding effect, $D_iT_j$ = the interaction effect between the $i^{th}$ dietary treatment and $j^{th}$ duration of feeding and $\varepsilon_{ijk}$= the residual error.

The dietary fatty acid composition data were analyzed as a one way ANOVA using the MIXED procedure of SAS with three levels of dietary treatments (Control, Moderate and High) as fixed effects. Least squares means were adjusted using Tukey's honest test, and were reported as significant at $P < 0.05$ level (SAS System, 2002). The model used:

$$Y_{ik} = \mu + D_i + \varepsilon_{ik}$$

where $Y_{ik}$ = dependent variable for $K^{th}$ bird, $\mu$= overall mean, $D_i$ = $i^{th}$ dietary treatment effect and $\varepsilon_{ijk}$= the residual error.

The broken stick analysis (Piecewise regression) was used to predict the threshold value (plateau) and the time duration required to reach ω-3 PUFA enrichment from the diets to the plasma and egg yolk using the segmented model for each dietary flaxseed level using NLIN procedure of SAS (Toms and Lesperance, 2003). In this model, breakpoints are used to estimate the duration required to reach a threshold at which the response variable (ω-PUFA in egg yolk or plasma) became constant to further increase in ω-PUFA with Moderate and High diets with the independent variable. The model uses the following equation:

$$\mathbf{Y}= \begin{cases} \alpha + \beta x & x_i < X_0 \\ \mathbf{C} & x_i > X_0 \end{cases}$$

where **Y** is the response variable (ω-3 PUFA concentration in Egg yolk (mg/egg) or plasma (mg/mL); **C** is the plateau or threshold value of ω-PUFA (mg/egg enrichment) or (mg/mL of plasma); ***x*** is the duration for dietary treatment; $\mathbf{X_0}$ is the break point or time to reach stationary phase. **α** is constant and **β** is a linear estimate of the rate of increment in ω-3 PUFA concentration in egg (mg/egg per d) or plasma (mg/mL).

## 4.3 RESULTS

### 4.3.1 Performance and production

The feed intake was similar in all dietary treatment throughout the experiment. Whereas dietary treatment did not significantly affect BW, there was a drop in BW from 6 d and 12 d of the three experimental periods (Table 4.2). The egg traits including, egg weight and yolk weight were unaffected by the dietary treatments or the duration of feeding (Table 4.3). The shell thickness was neither affected by the interaction of feeding treatment and duration of feeding the experimental diet nor any dietary effect. However, there was a duration of feeding

effect, with a reduction in shell thickness at 18 d compared to 0, 6 and 12 d. Egg production was also similar in hens from all dietary treatment (Control = 91.6±9.5%, Moderate = 92.8±11.4%, High = 92.1±10.4%) (P=0.77).

### 4.3.2 Dietary Fat Analysis

The fatty acid composition of the feed differed with the level of incorporation of extruded flax (Table 4.4). LNA (C18:3 ω-3), the primary fatty acid found in flax, differed most among rations, with content increased from 5.6% in the Control diet to 17.5% and 28.9% in the Moderate and High diets, respectively (Table 4.4). The 3-fold and 6-fold increase in LNA in the Moderate and High diets was the basis for the elevated total ω-3 PUFA concentration in these diets. With the greater proportion of ω-3 PUFA, the proportion of ω-6 PUFA was reduced in the diets, and therefore the ratio of ω-6 PUFA to the ω-3 PUFA in Moderate and High diets was also reduced. All diets delivered a similar low amount of total LC ω-3 PUFA. This was due to the plant source of ω-3 PUFA used in the experimental diets not containing these fatty acids. The amount of monounsaturated fatty acid and saturated fatty acids was the highest in the Control diet, followed by Moderate and High diets, respectively.

### 4.3.2 Blood Plasma Fatty Acid Profile

The fatty acid profile of blood plasma differed among the hens on experimental diets (Table 4.5, 4.6). The pattern of differences in total ω-3 PUFA concentration in plasma corresponded with what was being supplied in the diet. The mean CV for ω-3 PUFA in blood plasma of birds fed on Control, Moderate or High diet was 25.2, 29.6 and 30.8 respectively. Plasma LNA in birds fed the High diet was highest at 12 d, whereas the LNA from birds fed the Moderate diet was stabilized after 6 d. A similar pattern was observed for the total ω-3 PUFA content of plasma from birds fed High and Moderate diets (Table 4.6). The amount of LC ω-3 PUFA was similar among hens on dietary treatment. However, birds on the High diet at 6 d had higher LC ω-PUFA compared to initial 0 d. Among the important LC ω-PUFA, DHA amount was similar in all diets throughout experiment. The plasma DPA amount in Moderate and High diet was

higher compared to Control at all duration tested. However, there was no difference in plasma DPA amount in hens fed either a High or a Moderate level of enriched diet at 6 d, 12 d and 18 d. Further, the plasma EPA concentrations only differed in hens fed the High diet compared to the Control diet at 6 d.

The SFA, MUFA and total ω-6 PUFA content of plasma were not affected by the dietary treatments or their interaction with the duration of feeding (Table 4.5). However, with duration of dietary treatment SFA, MUFA, PUFA and total ω-6 PUFA content significantly increased by 12 d and the decreased again by 18 d. The predominant SFA and MUFA in plasma 16:0 and 18:1 followed a similar pattern and were neither affected by the diets nor the interaction of diets with duration. However, the AA level in plasma of hens fed the Moderate diet was reduced at 6 d compared to the Control diet but did not differ from Control birds at 12 d and 18 d (Table 4.6). The AA content in plasma of birds fed the High diet was lower at 6 d, 12 d and 18 d compared to that of from Control birds.

***4.3.2.1 Broken Stick Analysis in Blood Plasma:***

The ω-3 PUFA concentration in plasma reached a plateau phase during the course of experiment. The ω-3 PUFA concentration at saturation was 0.933 mg/mL of plasma, which was reached in 7.3 d in birds on the High treatment (Figure 4.1). Moderate treatment birds reached a plateau of 0.669 mg/mL plasma in 7.2 d (Figure 4.2). The equations to describe these changes are as follows:

High: $Y = 0.286 + 0.089 \times D$; $C = 0.933$; *Break point @ 7.26d* ($P = 0.0001$)

Moderate: $Y = 0.303 + 0.051 \times D$; $C = 0.669$; *Break point @ 7.16d.* ($P = 0.0001$)

where **Y** is ω-3 PUFA concentration in plasma (mg/mL), **D** is duration of feeding the dietary treatment and **C** is level of saturation of plasma ω-3 PUFA amount.

This assessment indicated that total plasma ω-3 PUFA concentration increased by 0.089 mg/mL plasma per day in High birds and by 0.051 mg/mL plasma per day in Moderate birds.

### 4.3.3 Egg yolk Fatty Acid Profile

The amount of SFA and total ω-6 in egg yolk were similar in all dietary treatments (Table 4.7). There were specific treatment effects, however there were no interactions of dietary treatment and duration of feeding on the amount of SFA, MUFA, PUFA and total ω-6 in egg yolk. The amount of MUFA was higher in egg yolks from hens on a Moderate diet compared to a High diet. However, the MUFA amount in High and Moderate eggs was similar to that of the Control diet eggs. The amount of PUFA in egg yolks from the Moderate and High diets was higher than that of Control diet yolks. In addition, there was a significant negative correlation of PUFA with MUFA in the egg yolk ($r = -0.836$; $P = 0.001$).

With only the Moderate and High birds receiving an ω-3 PUFA enriched ration, significant treatment by duration interactions were expected. Both total ω-3 PUFA and LC ω-3 PUFA in egg yolk has increased significantly in the Moderate and High birds during the course of the experiment, while they remained stable in the Control group (Table 4.8). The mean CV for egg yolk ω-3 PUFA content was 18.1, 17.8 and 13.5 in birds fed the Control, Moderate and High diets respectively. The total ω-3 PUFA amount in egg yolk from hens at 6 d of feeding Moderate and High diet was higher than from Control diet. However, at 6 d there was no difference in total ω-3 PUFA amount among the Moderate and High diets. A difference in the total ω-3 PUFA concentration in egg yolks for Moderate and High was evident at 12 d and 18 d on dietary treatment. The LC ω-3 PUFA (EPA, DPA and DHA) were significantly higher in egg yolk from hens on Moderate and High enriched diets compared to those from Control diet at 12 d and 18 d. Interestingly, among the LC ω-3 PUFA, there was no statistical difference for DHA and DPA amount in egg yolk from hens on High or Moderate diet throughout the experiment at 6 d, 12 d and 18 d. However, the EPA amount was higher in egg yolk from hens on High diet compared to Moderate diet at 6 d, 12 d and 18 d.

#### *4.3.3.1 Broken Stick Analysis in Egg Yolk:*

Based on the sampling interval used, the ω-3 PUFA enrichment pattern was linear prior to reaching saturation. The equations to describe the changes are in egg yolk are as follows:

High: Y =156.2+28.56×D; C=343.67; *Break point @ 6.56d. P=0.0001*

Moderate: Y=159.2+20.70×D; C=272.92; *Break point @ 5.91d. P=0.0001*

where **Y** is ω-3 PUFA concentration in egg (mg/egg), **D** is duration of feeding the dietary treatment and **C** is level of saturation of ω-3 PUFA amount in egg yolk.

The broken stick analysis indicates that total ω-3 PUFA concentration in egg yolk increased at a rate of 28.56 mg/egg per d in High birds while in Moderate birds increased at a rate of 20.70 mg/egg per d. The ω-3 PUFA content in egg yolk from High group hens was calculated to reach saturation phase at 343.67 mg/egg in 6.6 d (Figure 4.3). The total ω-3 PUFA content in egg yolk from Moderate group hens reached a saturation level at 272.92 mg/egg in 5.9 d (Figure 4.4).

### 4.3.4 Calculated Enzymatic Activity

An increase in LC ω-3 PUFA content can decrease the activities of the Δ5, Δ6 and Δ9 desaturase enzymes (Christiansen et al., 1991; Cherian and Sim, 2001). The calculated enzymatic activity associated with Δ9 desaturase activity for conversion of C16:0 to C16:1 was not affected by interaction of diets and duration of feeding (Table 4.9). However, the anticipated drop in Δ9 desaturase may have been present in birds on enriched diets compared to the Control diet (P=0.078). The calculated ω-3 PUFA and ω-6 PUFA biosynthesis pathway enzyme activity significantly varied with the feeding enriched diet for 18 days. The calculated ω-3 PUFA biosynthesis pathway enzyme activity for the hens fed the High and Moderate diet was consistant throughout the experiment. However, in birds fed Control diets the calculated ω-3 PUFA biosynthesis pathway activity was higher at 12 d and 18 d compared to 0 d and 6 d. The calculated ω-6 PUFA biosynthesis pathway activity significantly lower in birds fed enriched diets (High and Moderate) compared to the Control diet at 6 d, 12 d and 18 d. At 0 d, the

calculated enzyme activity for the ω-6 PUFA bioconversion was similar in birds from all diets.

## 4.4 DISCUSSION

The extruded flax product fed in the enriched diets has been demonstrated to have greater digestibility than ground flax (Bean and leeson, 2003; Htoo et al., 2008) because of the disruption of anti-nutritional factors normally associated with feeding ground flaxseed such as mucilage that would normally increase the viscosity of digesta at gut level leading to reduced nutrient absorption (Bhatty, 1993; Alzueta et al., 2003). Jia et al. (2008) reported a greater rate of egg production and lower feed conversion ratio in laying hens fed a diet with an extruded flax product than those fed the regular ground flaxseed diet in an 84 d experiment. In the current results, however, there was no change in feed efficiency. These results substantiate the hypothesis that feeding of the extruded flaxseed in linPRO (at up to 15% of the diet) will not negatively affect the performance of laying hens. This is an important result, as one the main concerns with the use of flax in poultry diets is the potential for negative impact on growth and gut condition.

The egg and yolk weights were unaffected by either dietary treatment or duration of feeding. Shell thickness was expected to gradually decline with hen age (Nys, 1986). Therefore, the decline in shell thickness noted at 18 d of the experiment appears age appropriate, suggesting the enriched diets did not alter the natural occurrence.

The Moderate and High diets had 206% and 402% higher total ω-3 PUFA concentration respectively, than the Control diet (Table 4.4). Similar patterns of increase in total ω-3 PUFA concentration were found in the blood plasma and egg yolk. The plasma total ω-3 PUFA increased by about 97% and 157% in hens fed Moderate and High diets compared to those on the Control diet for 18 d (Table 4.6). In addition, the increase in egg yolk was about 96% and 154% from hens fed Moderate and High diet, respectively compared to Control diet for 18 d (Table

4.8). These results suggest that the transfer efficiency of total ω-3 PUFA from diet to plasma or egg yolk was about 50% in laying hens.

The ω-6 to ω-3 ratio considered ideal for the human diet in between 4:1 to 1:1 (Simopolous, 2006), as the more balanced ω-6 to ω-3 ratio is associated with many benefits for human health (Watkins et al., 2000; Liu et al., 2003). In the current study, the ratio of ω-6 to ω-3 PUFA in egg yolk was reduced by approximately a 50% from the 5.51 in Control to 2.98 and 2.25 in Moderate and High groups at 18 d, respectively (Table 4.8). A similar trend was observed in the ω-6 to ω-3 ratio in blood plasma. The decrease in ω-6 to ω-3 ratio in egg yolk and blood plasma was a primarily due to increased LNA and reduced AA concentration. Because the reduction in ω-6 to ω-3 ratio occurred in both the blood plasma and the egg yolk, it suggests that this is a significant dietary effect.

The competition between the ω-6 PUFA and ω-3 PUFA substrates, LA and LNA, to utilize the same desaturase and elongase enzymes is well known (Watkins, 1991). An excess amount of one will suppress the metabolic activity of the other (Watkins, 1995; Shimizu et al., 2001). Therefore, a higher amount of LNA is not only increases the bioconversion of the LC ω-3 PUFA but it also acts to suppress the synthesis of pro-inflammatory eicosanoids production AA (Cherian, 2007). Being essential fatty acids, both LNA and AA contributes to important biological functions in humans. However, since the dietary levels of ω-6 PUFA in human diets are typically in excess of nutritional requirements, strategies to increase ω-3 PUFA uptake have received considerable attention.

The flax enrichment of experimental diets does not directly provide LC ω-3 PUFA, although concentration of LC ω-3 PUFA amount in the resulting egg product can still be increased (Kralik et al., 2008). The same was observed in present study, where the feeding an enriched diet increased both, DPA and DHA in egg yolk compared to the eggs from the Control diets. Interestingly, there was no additional benefit of using the High compared to a Moderate diet in hen ration. The exception to this was increased amount of EPA in egg yolks from birds fed the High compared to the Moderate diet at 6 d, 12 d and 18 d. The amount of LC

ω-3 PUFA in blood plasma also increased with similar intensity to that of egg yolk. The increase in the amount of LC ω-3 PUFA in blood plasma was 43.6% and 52.1% at 6 d in hens on the Moderate and High diets, respectively, compared to 0 d (4.6). In egg yolks, the increase in LC ω-3 PUFA was 42.5% and 42.4% in Moderate and High group respectively at 6 d compared to at 0 d (Table. 4.8). However, in the blood plasma the EPA, DHA and LC ω-3 PUFA amount lack clear contrast among dietary treatment. The only exception was the DPA amount in blood plasma; which was significantly higher in birds fed High and Moderate diets at 6 d, 12 d and 18 d compared to birds fed Control diet.

Blood plasma samples had the greatest amount of bird: bird variation for the total ω-3 PUFA. In contrast, the final egg yolk ω-3 PUFA concentrations were more stable, with the CV of this measure being 16.5% in the yolk compared to 28.5% in the blood plasma. The higher variability in the blood plasma may be associated with dilution of plasma fatty acid. The fatty acid measures from blood plasma would include both the ω-3 PUFA travelling from the gut to the liver, as well as the ω-3 PUFA that had been repackaged into the very low density lipoprotein (VLDL) particles travelling to the yolk for deposition (Walzem et al., 1994; Walzem, 1996), which could contribute to variability in blood ω-3 PUFA concentration. The egg yolk is a depository site of lipids to support embryo growth and therefore has a different, more specific type of fatty acid profile (and therefore the specific fatty acids like the ω-3 PUFA are concentrated (Cherian and Sim, 1993; 2001) compared to blood plasma, where there is a greater mix of fat types (Hodzic et al., 2008).

The dietary MUFA and SFA were highest in the Control feed, followed by the Moderate and High feeds (Table 4.4). However, the dietary difference in amount of the SFA and MUFA in plasma and SFA in egg yolk was diluted by the large influx of ω-3 PUFA in eggs from hens fed the enriched diets. The MUFA concentration in egg yolk from hens on High diet was lower than that of Moderate diet egg yolk (Table 4.6). Overall, the dietary differences in MUFA content were

more closely mirrored in the fatty acid profile of egg yolk than in blood plasma (Tables 4.4 to 4.6).

The amoung of SFA and MUFA in the egg yolk decreased throughout the course of the experiment, whereas PUFA rose initially (6 d) but then returned to the original value by 18 d of feeding. This result is not surprising considering analysis is being done on a whole egg basis. Egg yolk weight dropped by nearly 1 g between 0 d and 18 d (P = 0.089; Table 3.2), with the largest changes occurring in eggs from the enriched diets. When compard on a percentage basis, PUFA increased while MUFA dropped and SFA increased slightly in the enrichment treatments. Yolk PUFA as a percentage of yolk lipid changed quicly in eggs from the Moderate and High from 18.8% at 0 d to 23.1% by 6 d and reached a maximum of 24.1% at 18 d while values averaged 20.0% in Control eggs thoughout this period (P=0.001). Yolk MUFA as a percentage of egg weight began at 47.2% at 0 d and fell to 40.1% by 18 d in Moderage and High eggs compared to a final value of 43.0 in Control eggs (P=0.001). The yolk SFA values were not affected by feeding treatment but did still change in time, beginning at 34.0% and eventually reaching a high of 35.3% at 18 d (P=0.001). The percent increase PUFA and percent decrease in MUFA are expected to maintain a constant unsaturated fatty acid to saturated fatty acid required for a relatively constant of membrane fluidity (McMurchie et al., 1986).

The significant reduction in egg yolk AA amount from birds fed enriched diets (High and Moderate) was noted at 6 d, 12 d and 18 d compared to those fed the Control diet (Table 4.8), whereas the significant reduction in plasma AA amount was noted only at 12 d and 18 d (Table 4.6). Jiang et al. (1991) has reported a negative relationship between LNA and AA and suggested that higher amount of LNA at egg yolk or its metabolites decrease the production of AA. The reduction of AA at 12 d in egg yolk and no distinction for DPA and DHA among the birds fed Moderate and High diet in egg yolk and in blood plasma can be due to liver lipid enzyme systems requiring more than nine days responding to supplemental ω-3 PUFA (Van Elswyk, 1997). However, the significant effect of

enriched diets as increase in LNA amount was observed within the 6 d suggesting that this process is well underway prior to 9 d of dietary enrichment.

The increased concentration of LC ω-3 PUFA in the egg yolk from the High diet compared to the Control diet suggests that the higher amount of LNA in feed promotes the elongation and desaturation of LNA to LC ω-3 PUFA (Griffin, 2008). However, the extent of bioconversion efficiency of LNA to DHA in flax fed laying hens is debatable (Jiang et al., 1991, Cherian and Sim, 1991; Hargis and Van Elswyk, 1993). The enrichment level in hen ration may be an important factor to consider prior to egg enrichment. For example, Cachaldora et al. (2006) had reported that excessive intake of dietary LC ω-3 PUFA might reduce the retention efficiency of LC ω-3 PUFA in egg yolk. Therefore, it is suggested that the proper knowledge of duration and level of feeding enriched diets may avoid the negative feedback resulting from saturating the any individual fatty acid level in egg yolk.

Hen age can affect the deposition of LC ω-3 PUFA into the yolk (Scheideler et al., 1998). The bioconversion from the LNA to DHA was reported to be 18 to 109% greater in 58 wk old hens compared to 36 wk hens (Scheideler et al., 1998). Birds in the current study are 65 wk old, indicating an increased potential for bioconversion of LC ω-3 PUFA from LNA is possible.

The LC ω-3 PUFA bioconversion can also vary with bird type (Poureslami et al., 2010). For example, in a recent trial enriching the breast meat of young broilers, the upconversion to LC ω-3 PUFA from LNA ceased at DPA ranther than continuing on to DHA (Betti et al., 2009). Similar findings were reported by Zelenka et al. (2008) that broiler chickens had a restricted ability to desaturate and elongate LNA. Another difference that gives advantage to laying hens over broilers is that they are sexually mature. Hormones related to growth and metabolism interacts with estrogen in aspects of lipid metabolism. Growth hormone and thyroid stimulating hormone can act together to modulate the lipid synthesis in the liver by directing lipids away from ovarian deposition to support other growth and metabolic processes (Walzem, 1996). In the current study, there

was a significantly higher upconversion of LC ω-3 PUFA upto DHA in the egg yolk and only up to DPA in blood plasma in layers fed High diet compared to those fed Control diets. Earler, Jia et al. (2008), and Celebi & Mucit (2009), had reporeted that DHA is predominant LC ω-3 PUFA in the egg yolk.

The enrichment with LC ω-3 PUFA content can reduce the activities of Δ5, Δ6 and Δ9 desaturases in the liver microsome (Christiansen et al., 1991). The ω-6 PUFA biosynthesis pathway enzyme activity decreased in birds fed High diets over the experimental period. The decrease in this calculated ω-6 PUFA enzyme activity is likely due to competition with LNA for the Δ-6 desaturase enzyme for the LC ω-3 PUFA bioconversion (Watkins, 1995; Shimizu et al., 2001). Furthermore, the calculated ω-6 PUFA biosynthesis pathway enzyme activity for LC ω-6 PUFA biosynthesis was negatively correlated with increased presence of LNA ($r=-0.59$; $P=0.001$). Interestingly, the calculated ω-6 PUFA biosynthesis pathway enzyme activities were affected by the level of ω-3 PUFA enrichment in the diet. The calculated enzyme activity for ω-6 PUFA was significantly reduced already by 6 d and was further reduced at 18 d in egg yolks from birds fed High diet compared to Control birds. In contrast, in Moderate diet egg yolks, a significant reduction in calculated enzymatic activity was reached only after 12 d of feeding compared to in the Control birds. This inhibition is also evident from the reduced content of AA in blood plasma and egg yolk from birds on enriched diets (Table 4.6 and 4.8).

The ω-3 PUFA biosynthesis pathway enzyme activity for ω-3 PUFA bioconversion in High and Moderate diet fed hens was stable for the duration of the experiment (Table 4.9). However, the increased calculated enzyme activity for ω-3 PUFA bioconversion in birds fed Control diet at 12 d and 18 d compared to 0 d and 6 d may be related to the individual bird to bird variability in Control birds due to lower EPA content. The mean CV of EPA content for the 6 to 18 d durations in Control birds (57.2) was higher compared to High (26.5) and Moderate birds (28.5). This variation in EPA may weaken the value of the enzyme activity calculation. Previous work with these calculations has been based

on muscle tissue, where the distribution of EPA and DPA is greater (Betti et al., 2009). The significant increase in EPA, DPA, and DHA content in egg yolk and reduction in AA concentration in egg yolks from hens fed enriched diets compared to the Control diet substantiates the impact of competition for enzymes during post absorptive modification of these fatty acids.

Laying hens have the ability to synthesize EPA and DHA from LNA provided they have higher supply of LNA than LA through their diets (Kralik et al., 2008). The improvement in the enzymatic action involved in biosynthesis of LC ω-3 PUFAs may help to create enriched eggs with a greater potential biological benefit. However, if future labelling requirements for ω-3 PUFA enriched eggs follow the model of what is done in some European countries and is limited to only listing the LC ω-3 PUFAs, then revised methods to enrich these eggs will likely need to include increased use of dietary LC ω-3 PUFA sources such as fish oil or marine algae along with flax. However, the addition of direct source of LC ω-3 PUFA in hen ration would reduce the bioconversion of LNA to LC ω-3 PUFA (Whelan et al., 1991; Bezard et al., 1994).

In the present study, eggs from birds fed the Moderate ω-3 PUFA enriched diet did not reach the 300 mg/egg minimum concentration required for labelling the egg as a source of ω-3 PUFA (CFIA, 2003). Use of the High diet led to enrichment up to 343.7 mg/egg in 6.6 d. From the broken stick analysis of yolk ω-3 PUFA enrichment, it was estimated that the High birds reached the labelling threshold level of 300 mg/egg in 5.0 d. How the experimental conditions affect the fatty acid profile depend on how they are being compared. Traditionally, much of this type of work focused on percentage-based changes in fatty acid composition. However, this does not reflect what the consumer will be eating. By comparing treatment effects on a whole egg basis, the changes in fatty acid class in time are presented within the context of the treatment effect on yolk size.

Further research is needed to further characterize the physiological mechanism by which transition of fatty acid from diet to the egg occurs and to

identify the optimum method of inclusion of omega-3 sources in diets to prevent overloading of ω-3 PUFA absorption capability of laying hens.

**Table 4.1: Diet composition and nutrient content of experimental diets[1]**

| Treatment | Control (0%) | Moderate (7.5%) | High (15%) |
|---|---|---|---|
| Ingredients | | | |
| Canola meal | 2.5 | 4.3 | 6.0 |
| Corn, yellow,grain | 15.0 | 21.5 | 28.0 |
| Oats, grain | 17.0 | 17.5 | 18.0 |
| Oat hulls | -- | 1.5 | 3.0 |
| Soybean meal | 13.6 | 12.2 | 10.7 |
| Wheat, hard, grain | 37.8 | 22.8 | 7.8 |
| Calcium carbonate | 9.1 | 8.8 | 8.5 |
| Dicalcium phosphate. | 1.1 | 1.3 | 1.5 |
| Salt, plain (NaCl) | 0.3 | 0.3 | 0.3 |
| D,L – methionine | 0.1 | 0.1 | 0.1 |
| LinPRO[2] | - | 7.5 | 15.0 |
| Layer Vit/Min Premix[3] | 0.5 | 0.5 | 0.5 |
| Choline Chloride | 0.5 | 0.5 | 0.5 |
| Enzyme[4] | 0.1 | 0.1 | 0.1 |
| Canola oil | 2.4 | 1.2 | 0.1 |
| Calculated Nutrient Analysis: | | | |
| M.E. kcal/kg | 2,750.0 | 2,750.0 | 2,750.0 |
| Dry matter % | 90.0 | 90.0 | 90.0 |
| Protein, crude % | 16.1 | 16.1 | 16.1 |
| Fat, crude % | 4.5 | 4.9 | 5.3 |
| Fiber, crude % | 3.9 | 4.6 | 5.4 |
| Calcium % | 3.6 | 3.6 | 3.6 |
| Phos. Total % | 0.6 | 0.6 | 0.6 |
| Phos., available % | 0.4 | 0.4 | 0.4 |
| Met + cys % | 0.7 | 0.7 | 0.7 |
| Lysine % | 0.8 | 0.8 | 0.8 |

[1]Control with no LinPRO, Moderate diet with 7.5% of LinPRO and a High diet with 15% inclusion of LinPRO in the basal layer diet was fed for 18 days to laying hens (65 wks).
[2]LinPRO is extruded flaxseed with peas in 1:1 ratio. (O & T farms, Regina, SK, Canada).
[3]The layer Vit/Min premix contained per kilogram of diet: vitamin A (retinyl acetate), 12,000 IU; cholecalciferol, 3,000 IU; vitamin E (DL-α-tocopheryl acetate), 40 IU; vitamin K, 2.0 mg; pantothenic acid, 14 mg; riboflavin, 6.5 mg; folacin, 1.0 mg; niacin, 40 mg; thiamine, 3.3 mg; pyridoxine, 6.0 mg; vitamin B12, 0.02 mg; biotin, 0.2 mg; iodine, 0.5 mg; Mn, 75 mg; Cu, 15 mg; Zn, 80 mg, Se, 0.1 mg; and Fe, 100 mg; choline chloride, 100 mg.
[4]Enzyme: Avizyme 1302, Xylanase enzyme, Danisco Animal Nutrition, Marlborough, Wiltshire, UK.

**Table 4.2: The feed intake and change in body weight in laying hens fed experimental diets[1]**

| Treatment | Duration | Feed Intake (g) | Change in BW (g) |
|---|---|---|---|
| Control | | 690.3 | -21.2 |
| Moderate | | 697.2 | -11.8 |
| High | | 702.0 | -16.0 |
| SEM | | 17.1 | 4.6 |
| | 6 d | 695.3 | 2.7[a] |
| | 12 d | 695.2 | -36.4[c] |
| | 18 d | 699.9 | -15.3[b] |
| SEM | | 16.9 | 4.6 |
| Control | 6 d | 701.3 | -5.6 |
| | 12 d | 682.5 | 3.6 |
| | 18 d | 687.3 | 10.2 |
| High | 6 d | 696.9 | -37.7 |
| | 12 d | 699.7 | -35.7 |
| | 18 d | 709.4 | -35.7 |
| Moderate | 6 d | 688.5 | -20.2 |
| | 12 d | 701.8 | -15.8 |
| | 18 d | 701.3 | -9.8 |
| SEM | | 17.0 | 4.6 |
| Source of Variation | | ----------Probability---------- | |
| Treatment | | 0.7370 | 0.9501 |
| Duration | | 0.9370 | 0.0001 |
| Treatment*duration | | 0.9020 | 0.3788 |

[1]Control with no LinPRO, Moderate diet with 7.5% of LinPRO and a High diet with 15% inclusion of LinPRO in the basal layer diet was fed for 18 days to laying hens (65 wks).
SEM = Standard error of mean.
[a-c]Means within dietary treatment, within duration of dietary phase and interaction with no common superscript are significant different (P<0.05).

**Table 4.3: Egg traits in laying hens fed experimental diets[1]**

| Treatment | Duration | Egg wt (g) | Yolk Wt (g) | Shell Thickness (mm) |
|---|---|---|---|---|
| Control | | 63.09 | 18.03 | 0.295 |
| Moderate | | 62.40 | 17.89 | 0.286 |
| High | | 63.13 | 17.77 | 0.294 |
| SEM | | 0.525 | 1.30 | 0.006 |
| | 0 d | 63.33 | 18.13 | 0.290[a] |
| | 6 d | 62.95 | 17.99 | 0.299[a] |
| | 12 d | 62.42 | 17.80 | 0.293[a] |
| | 18 d | 62.77 | 17.55 | 0.284[b] |
| SEM | | 0.607 | 1.32 | 0.003 |
| Control | 0 d | 63.50 | 18.13 | 0.296 |
| | 6 d | 63.20 | 17.89 | 0.297 |
| | 12 d | 63.29 | 18.20 | 0.295 |
| | 18 d | 62.87 | 17.87 | 0.288 |
| Moderate | 0 d | 61.59 | 18.05 | 0.289 |
| | 6 d | 63.21 | 18.33 | 0.292 |
| | 12 d | 63.22 | 17.61 | 0.287 |
| | 18 d | 61.90 | 17.25 | 0.275 |
| High | 0 d | 63.19 | 18.21 | 0.286 |
| | 6 d | 62.80 | 17.75 | 0.307 |
| | 12 d | 62.77 | 17.58 | 0.297 |
| | 18 d | 62.91 | 17.55 | 0.290 |
| SEM | | 0.606 | 1.290 | 0.006 |
| Source of Variation | | ----------Probability---------- | | |
| Treatment | | 0.1099 | 0.4234 | 0.0901 |
| Duration | | 0.8630 | 0.0896 | 0.0150 |
| Treatment*duration | | 0.9790 | 0.4695 | 0.3666 |

[1]Control with no LinPRO, Moderate diet with 7.5% of LinPRO and a High diet with 15% inclusion of LinPRO in the basal layer diet was fed for 18 days to laying hens (65 wks).
SEM = Standard error of mean.
[a-b]Means within dietary treatment, within duration of dietary phase and interaction with no common superscript are significant different (P<0.05).

**Table 4.4: Fatty acid composition of experimental diets[1]**

| Treatment | Control | Moderate | High |
|---|---|---|---|
| Fatty acid | ----------% composition---------- | | |
| 14:0 | 0.232±0.01 | 0.185±0.02 | 0.186±0.01 |
| 15:0 | 0.081±0.01 | 0.116±0.03 | 0.107±0.02 |
| 16:0 | 12.96±0.15 | 11.73±0.21 | 10.37±0.01 |
| 16:1 ω-7 | 0.446±0.01 | 0.324±0.02 | 0.204±0.04 |
| 17:1 ω-9 | 0.140±0.04 | 0.090±0.01 | 0.035±0.05 |
| 18:0 | 3.223±0.05 | 2.637±0.01 | 2.299±0.06 |
| 18:1 ω-7 | 37.97±0.48 | 31.56±0.47 | 25.58±0.29 |
| 18:2 ω-6 | 36.92±0.31 | 34.70±0.01 | 31.28±0.55 |
| 18:3 ω-3 | 5.627±0.01 | 17.55±0.05 | 28.93±0.01 |
| 18:3 ω-6 | 0.088±0.05 | 0.087±0.01 | 0.103±0.25 |
| 20:0 | 0.448±0.01 | 0.390±0.02 | 0.326±0.01 |
| 20:1 ω-9 | 0.846±0.01 | 0.369±0.02 | 0.341±0.01 |
| 20:4 ω-6 | 0.069±0.01 | 0.071±0.03 | 0.044±0.01 |
| 20:5 ω-3 | 0.017±0.02 | 0.020±0.01 | 0.024±0.01 |
| 22:1 ω-9 | 0.348±0.06 | 0.273±0.02 | 0.332±0.01 |
| 22:2 ω-6 | 0.913±0.89 | 0.156±0.03 | 0.149±0.01 |
| 22:5 ω-3 | 0.017±0.01 | 0.007±0.01 | 0.007±0.01 |
| 22:6 ω-3 | 0.124±0.06 | 0.102±0.02 | 0.082±0.01 |
| SFA[2] | 16.94±0.20 | 15.06±0.24 | 13.22±0.02 |
| MUFA[3] | 39.75±0.61 | 32.62±0.55 | 26.50±0.29 |
| PUFA[4] | 44.62±0.40 | 53.06±1.35 | 60.96±0.27 |
| LC ω-3 PUFA[5] | 0.158±0.02 | 0.129±0.02 | 0.114±0.02 |
| Total ω-3 PUFA[6] | 5.785±0.59 | 17.68±1.07 | 29.05±0.27 |
| Total ω-6 PUFA[7] | 37.99±0.73 | 35.01±0.28 | 31.57±0.54 |
| Ratio ω-6/ ω-3[8] | 6.566±0.07 | 1.984±0.29 | 1.087±0.17 |

*LC = Long Chain, SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

[1]Control with no LinPRO, Moderate diet with 7.5% of LinPRO and a High diet with 15% inclusion of LinPRO in the basal layer diet was fed for 18 days to laying hens (65 wks).

[2]SFA levels were calculated as 14:0 + 15:0 + 16:0 + 18:0 + 20:0.

[3]MUFA levels were calculated as 16:1ω-7 + 18:1ω-7 + 20:1ω-9 + 22:1ω-9.

[4]PUFA levels were calculated as 18:2ω-6 + 18:3ω-3 + 18:3ω-6 +20:4ω-6 + 20:5ω-3 + 22:5 ω-3 +22:2ω-6 + 22:6ω-3.

[5]LC ω-3 PUFA was calculated as 20:5ω-3 + 22:5ω-3 + 22:6ω-3.

[6]Total ω-3 PUFA was calculated as 18:3ω-3 + 20:5ω-3 + 22:5ω-3 + 22:6ω-3.

[7]Total ω-6 PUFA was calculated as 18:2ω-6 + 20:4ω-6 + 22:2ω-6.

[8]Ratio ω-6/ ω-3 were calculated as Total ω-6 PUFA / Total ω-3 PUFA.

**Table 4.5: Diet and duration effects of feeding experimental diets[1] on fatty acid composition of blood plasma (mg/ mL plasma) of laying hens.**

| Fatty | Treatment | | | | Duration | | | | | Probability | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Acids | Control | Moderate | High | SEM | 0d | 6d | 12d | 18d | SEM | Treat | Duration | Treat*Duration |
| 14:0 | 0.026$^{a}$ | 0.023$^{b}$ | 0.021$^{b}$ | 0.001 | 0.020$^{b}$ | 0.020$^{b}$ | 0.027$^{a}$ | 0.025$^{a}$ | 0.001 | 0.0001 | 0.0001 | 0.8715 |
| 16:0 | 2.468 | 2.462 | 2.253 | 0.070 | 2.069$^{c}$ | 2.187$^{bc}$ | 2.853$^{a}$ | 2.468$^{b}$ | 0.081 | 0.0485 | 0.0001 | 0.6662 |
| 16:1 ω-7 | 0.179 | 0.175 | 0.160 | 0.006 | 0.139$^{b}$ | 0.159$^{b}$ | 0.194$^{a}$ | 0.193$^{a}$ | 0.007 | 0.0549 | 0.0001 | 0.3805 |
| 18:0 | 0.018 | 0.018 | 0.018 | 0.001 | 0.016$^{b}$ | 0.020$^{a}$ | 0.020$^{a}$ | 0.015$^{b}$ | 0.001 | 0.9307 | 0.0001 | 0.0358 |
| 18:1 ω-7 | 1.025 | 1.065 | 1.049 | 0.036 | 0.891$^{c}$ | 0.988$^{bc}$ | 1.235$^{a}$ | 1.072$^{b}$ | 0.042 | 0.7244 | 0.0001 | 0.8864 |
| 18:1 ω-9 | 4.094 | 4.227 | 3.859 | 0.125 | 3.827$^{bc}$ | 3.504$^{c}$ | 4.806$^{a}$ | 4.102$^{b}$ | 0.144 | 0.1093 | 0.0001 | 0.4425 |
| 18:2 ω-6 | 1.329 | 1.387 | 1.456 | 0.040 | 1.154$^{c}$ | 1.300$^{bc}$ | 1.647$^{a}$ | 1.463$^{b}$ | 0.046 | 0.0771 | 0.0001 | 0.3372 |
| 18:3 ω-3 | 0.077$^{c}$ | 0.236$^{b}$ | 0.418$^{a}$ | 0.010 | 0.058$^{c}$ | 0.268$^{b}$ | 0.336$^{a}$ | 0.314$^{a}$ | 0.011 | 0.0001 | 0.0001 | 0.0001 |
| 18:3 ω-6 | 0.021 | 0.018 | 0.018 | 0.001 | 0.019$^{ab}$ | 0.016$^{b}$ | 0.020$^{ab}$ | 0.021$^{a}$ | 0.001 | 0.0460 | 0.0063 | 0.7906 |
| 20:1 ω-9 | 0.028$^{a}$ | 0.025$^{ab}$ | 0.023$^{b}$ | 0.001 | 0.021$^{b}$ | 0.022$^{b}$ | 0.034$^{a}$ | 0.025$^{b}$ | 0.001 | 0.0062 | 0.0001 | 0.1114 |
| 20:2 ω-6 | 0.018 | 0.017 | 0.016 | 0.001 | 0.015$^{b}$ | 0.016$^{b}$ | 0.021$^{a}$ | 0.015$^{b}$ | 0.001 | 0.2259 | 0.0001 | 0.7111 |
| 20:3 ω-6 | 0.023 | 0.024 | 0.024 | 0.001 | 0.023$^{a}$ | 0.025$^{a}$ | 0.022$^{a}$ | 0.025$^{a}$ | 0.001 | 0.3480 | 0.1836 | 0.7184 |
| 20:4 ω-6 | 0.347$^{a}$ | 0.292$^{b}$ | 0.248$^{c}$ | 0.010 | 0.311$^{a}$ | 0.264$^{b}$ | 0.344$^{a}$ | 0.263$^{b}$ | 0.012 | 0.0001 | 0.0001 | 0.0146 |
| 20:5 ω-3 | 0.022$^{b}$ | 0.020$^{b}$ | 0.032$^{a}$ | 0.003 | 0.024$^{ab}$ | 0.028$^{a}$ | 0.029$^{a}$ | 0.017$^{b}$ | 0.003 | 0.0018 | 0.0210 | 0.0018 |
| 22:2 ω-6 | 0.111 | 0.120 | 0.088 | 0.012 | 0.082$^{b}$ | 0.115$^{ab}$ | 0.150$^{a}$ | 0.078$^{b}$ | 0.014 | 0.1384 | 0.0007 | 0.1587 |
| 22:4 ω-6 | 0.019 | 0.018 | 0.013 | 0.002 | 0.014$^{b}$ | 0.012$^{b}$ | 0.018$^{ab}$ | 0.023$^{a}$ | 0.002 | 0.0493 | 0.0002 | 0.0124 |
| 22:5 ω-3 | 0.016$^{b}$ | 0.022$^{a}$ | 0.023$^{a}$ | 0.001 | 0.013$^{c}$ | 0.022$^{b}$ | 0.026$^{a}$ | 0.021$^{b}$ | 0.001 | 0.0001 | 0.0001 | 0.0047 |
| 22:6 ω-3 | 0.249 | 0.285 | 0.271 | 0.011 | 0.210$^{b}$ | 0.277$^{a}$ | 0.313$^{a}$ | 0.273$^{a}$ | 0.013 | 0.0848 | 0.0001 | 0.1102 |
| SFA[2] | 2.519 | 2.509 | 2.297 | 0.072 | 2.108$^{c}$ | 2.235$^{bc}$ | 2.910$^{a}$ | 2.513$^{b}$ | 0.082 | 0.0479 | 0.0001 | 0.6907 |
| MUFA[3] | 5.334 | 5.500 | 5.098 | 0.161 | 4.886$^{bc}$ | 4.682$^{c}$ | 6.276$^{a}$ | 5.398$^{b}$ | 0.186 | 0.2086 | 0.0001 | 0.6249 |
| PUFA[4] | 2.231$^{b}$ | 2.439$^{ab}$ | 2.608$^{a}$ | 0.065 | 1.921$^{c}$ | 2.343$^{b}$ | 2.927$^{a}$ | 2.513$^{b}$ | 0.076 | 0.0001 | 0.0001 | 0.0935 |
| LC ω-3 PUFA[5] | 0.287 | 0.327 | 0.326 | 0.012 | 0.246$^{c}$ | 0.328$^{ab}$ | 0.369$^{a}$ | 0.311$^{b}$ | 0.014 | 0.0539 | 0.0001 | 0.0429 |
| Total ω-3 PUFA[6] | 0.364$^{c}$ | 0.563$^{b}$ | 0.745$^{a}$ | 0.019 | 0.304$^{c}$ | 0.595$^{b}$ | 0.705$^{a}$ | 0.624$^{b}$ | 0.022 | 0.0001 | 0.0001 | 0.0001 |
| Total ω-6 PUFA[7] | 1.849 | 1.860 | 1.847 | 0.051 | 1.602$^{c}$ | 1.732$^{bc}$ | 2.200$^{a}$ | 1.874$^{b}$ | 0.059 | 0.9828 | 0.0001 | 0.8043 |
| Ratio ω-6/ ω-3[8] | 5.165$^{a}$ | 3.822$^{b}$ | 3.044$^{c}$ | 0.089 | 5.474$^{a}$ | 3.334$^{b}$ | 3.699$^{b}$ | 3.534$^{b}$ | 0.103 | 0.0001 | 0.0001 | 0.0001 |

* SEM = Standard error of mean,

*LC = Long Chain, SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

[1]Control with no LinPRO, Moderate diet with 7.5% of LinPRO and a High diet with 15% inclusion of LinPRO in the basal layer diet was fed for 18 days to laying hens (65 wks).

[2]SFA levels were calculated as 14:0 + 16:0 + 18:0.

[3]MUFA levels were calculated as 16:1ω-7 + 18:1ω-7 + 18:1ω-9 + 20:1ω-9.

[4]PUFA levels were calculated as 18:2ω-6 + 18:3ω-3 + 18:3ω-6 +20:2 ω-6 + 20:3 ω-6 + 20:4 ω-6+ 20:5 ω-3 + 22:2 ω-6 + 22:5 ω-3 +22:4ω-6 + 22:6ω-3.

[5]LC ω-3 PUFA was calculated as 20:5ω-3 + 22:5ω-3 + 22:6ω-3.

[6]Total ω-3 PUFA was calculated as 18:3ω-3 + 20:5ω-3 + 22:5ω-3 + 22:6ω-3.

[7]Total ω-6 PUFA was calculated as 18:2ω-6 + 18:3ω-6 +20:2 ω-6 + 20:3 ω-6 + 20:4 ω-6+ 22:2ω-6.

[8]Ratio ω-6/ ω-3 were calculated as Total ω-6 PUFA / Total ω-3 PUFA.

[a-c] Means within dietary treatments and within the duration with no common superscript are significant different ($P<0.05$)

**Table 4.6: Effects of interaction of diet and duration of feeding experimental diets[1] on fatty acid composition of blood plasma (mg/ mL plasma) of laying hens.**

| Fatty | 0 d | | | 6 d | | | 12 d | | | 18 d | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Acid | Control | Moderate | High | Control | Moderate | High | Control | Moderate | High | Control | Moderate | High | SEM | Probability |
| 14:0 | 0.022 | 0.020 | 0.019 | 0.024 | 0.019 | 0.018 | 0.029 | 0.027 | 0.024 | 0.029 | 0.025 | 0.022 | 0.02 | 0.8715 |
| 16:0 | 2.133 | 2.114 | 1.961 | 2.399 | 2.097 | 2.064 | 2.817 | 3.029 | 2.714 | 2.524 | 2.609 | 2.27 | 0.14 | 0.6662 |
| 16:1 ω-7 | 0.147 | 0.138 | 0.133 | 0.178 | 0.150 | 0.150 | 0.189 | 0.215 | 0.176 | 0.203 | 0.196 | 0.180 | 0.01 | 0.3805 |
| 18:0 | $0.017^{bcd}$ | $0.017^{bcd}$ | $0.015^{cd}$ | $0.020^{abc}$ | $0.018^{abcd}$ | $0.023^{a}$ | $0.020^{abc}$ | $0.021^{ab}$ | $0.019^{abc}$ | $0.014^{d}$ | $0.016^{bcd}$ | $0.014^{d}$ | 0.01 | 0.0358 |
| 18:1 ω-7 | 0.894 | 0.930 | 0.849 | 0.997 | 0.959 | 1.009 | 1.184 | 1.240 | 1.281 | 1.024 | 1.133 | 1.059 | 0.07 | 0.8864 |
| 18:1 ω-9 | 3.755 | 3.985 | 3.742 | 3.816 | 3.360 | 3.336 | 4.593 | 5.231 | 4.594 | 4.211 | 4.330 | 3.765 | 0.25 | 0.4425 |
| 18:2 ω-6 | 1.179 | 1.152 | 1.130 | 1.281 | 1.250 | 1.370 | 1.491 | 1.632 | 1.816 | 1.366 | 1.516 | 1.509 | 0.08 | 0.3372 |
| 18:3 ω-3 | $0.059^{d}$ | $0.058^{d}$ | $0.057^{d}$ | $0.076^{d}$ | $0.257^{c}$ | $0.470^{b}$ | $0.087^{d}$ | $0.334^{c}$ | $0.587^{a}$ | $0.085^{d}$ | $0.295^{c}$ | $0.561^{a}$ | 0.02 | 0.0001 |
| 18:3 ω-6 | 0.019 | 0.019 | 0.018 | 0.019 | 0.014 | 0.014 | 0.021 | 0.018 | 0.019 | 0.024 | 0.020 | 0.019 | 0.02 | 0.7906 |
| 20:1 ω-9 | 0.021 | 0.022 | 0.019 | 0.023 | 0.023 | 0.021 | 0.036 | 0.033 | 0.033 | 0.033 | 0.023 | 0.019 | 0.02 | 0.1114 |
| 20:2 ω-6 | 0.014 | 0.016 | 0.015 | 0.017 | 0.016 | 0.016 | 0.022 | 0.021 | 0.020 | 0.017 | 0.015 | 0.014 | 0.01 | 0.7111 |
| 20:3 ω-6 | 0.022 | 0.024 | 0.023 | 0.023 | 0.025 | 0.026 | 0.023 | 0.021 | 0.022 | 0.022 | 0.025 | 0.026 | 0.02 | 0.7184 |
| 20:4 ω-6 | $0.307^{bcd}$ | $0.329^{abc}$ | $0.295^{bcd}$ | $0.340^{ab}$ | $0.239^{cde}$ | $0.214^{de}$ | $0.416^{a}$ | $0.337^{ab}$ | $0.281^{bcde}$ | $0.325^{abc}$ | $0.263^{bcde}$ | $0.200^{e}$ | 0.02 | 0.0146 |
| 20:5 ω-3 | $0.036^{ab}$ | $0.007^{c}$ | $0.028^{abc}$ | $0.012^{bc}$ | $0.031^{abc}$ | $0.042^{a}$ | $0.025^{abc}$ | $0.027^{abc}$ | $0.036^{ab}$ | $0.014^{bc}$ | $0.014^{bc}$ | $0.023^{abc}$ | 0.05 | 0.0018 |
| 22:2 ω-6 | 0.052 | 0.134 | 0.060 | 0.109 | 0.120 | 0.115 | 0.191 | 0.144 | 0.114 | 0.090 | 0.083 | 0.062 | 0.02 | 0.1587 |
| 22:4 ω-6 | $0.015^{bc}$ | $0.015^{bc}$ | $0.013^{bc}$ | $0.018^{abc}$ | $0.010^{bc}$ | $0.007^{c}$ | $0.023^{ab}$ | $0.014^{bc}$ | $0.016^{bc}$ | $0.019^{abc}$ | $0.033^{a}$ | $0.017^{bc}$ | 0.03 | 0.0124 |
| 22:5 ω-3 | $0.013^{de}$ | $0.013^{de}$ | $0.011^{e}$ | $0.017^{cde}$ | $0.024^{ab}$ | $0.027^{ab}$ | $0.021^{bcd}$ | $0.028^{a}$ | $0.030^{a}$ | $0.015^{de}$ | $0.025^{ab}$ | $0.023^{abc}$ | 0.02 | 0.0047 |
| 22:6 ω-3 | 0.215 | 0.225 | 0.190 | 0.251 | 0.298 | 0.281 | 0.303 | 0.292 | 0.345 | 0.227 | 0.324 | 0.268 | 0.02 | 0.1102 |
| SFA[2] | $1.593^{c}$ | $2.154^{cd}$ | $1.997^{d}$ | $2.448^{abcd}$ | $2.144^{cd}$ | $2.113^{cd}$ | $2.878^{ab}$ | $3.084^{a}$ | $2.767^{abc}$ | $2.576^{abcd}$ | $2.653^{abcd}$ | $2.310^{bcd}$ | 0.14 | 0.6907 |
| MUFA[3] | 4.825 | 5.083 | 4.751 | 5.023 | 4.500 | 4.524 | 6.012 | 6.728 | 6.088 | 5.476 | 5.689 | 5.028 | 0.32 | 0.6249 |
| PUFA[4] | 1.930 | 1.991 | 1.841 | 2.164 | 2.283 | 2.583 | 2.624 | 2.870 | 3.286 | 2.206 | 2.613 | 2.722 | 0.13 | 0.0935 |
| LC ω-3 PUFA[5] | $0.263^{bcd}$ | $0.245^{cd}$ | $0.230^{d}$ | $0.280^{bcd}$ | $0.352^{abc}$ | $0.350^{abc}$ | $0.349^{abc}$ | $0.347^{abc}$ | $0.411^{a}$ | $0.256^{bcd}$ | $0.362^{ab}$ | $0.314^{abcd}$ | 0.03 | 0.0429 |
| Total ω-3 PUFA[6] | $0.323^{e}$ | $0.303^{e}$ | $0.287^{e}$ | $0.356^{e}$ | $0.610^{d}$ | $0.820^{bc}$ | $0.436^{e}$ | $0.681^{cd}$ | $0.998^{a}$ | $0.341^{e}$ | $0.657^{cd}$ | $0.875^{ab}$ | 0.04 | 0.0001 |
| Total ω-6 PUFA[7] | 1.581 | 1.673 | 1.540 | 1.791 | 1.658 | 1.746 | 2.166 | 2.168 | 2.268 | 1.847 | 1.940 | 1.834 | 0.10 | 0.8043 |
| Ratio ω-6/ω-3[8] | $5.055^{a}$ | $5.793^{a}$ | $5.575^{a}$ | $5.065^{a}$ | $2.765^{cd}$ | $2.172^{cd}$ | $5.051^{a}$ | $3.752^{b}$ | $2.293^{cd}$ | $5.490^{a}$ | $2.975^{bc}$ | $2.135^{bcd}$ | 0.18 | 0.0001 |

*SEM = Standard error of mean

*LC = Long Chain, SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

[1]Control with no LinPRO, Moderate diet with 7.5% of LinPRO and a High diet with 15% inclusion of LinPRO in the basal layer diet was fed for 18 days to laying hens (65 wks).

[2]SFA levels were calculated as 14:0 + 16:0 + 18:0.
[3]MUFA levels were calculated as 16:1ω-7 + 18:1ω-7 + 18:1ω-9 + 20:1ω-9.
[4]PUFA levels were calculated as 18:2ω-6 + 18:3ω-3 + 18:3ω-6 +20:2 ω-6 + 20:3 ω-6 + 20:4 ω-6+ 20:5 ω-3 + 22:2 ω-6 + 22:5 ω-3 +22:4ω-6 + 22:6ω-3.
[5]LC ω-3 PUFA was calculated as 20:5ω-3 + 22:5ω-3 + 22:6ω-3.
[6]Total ω-3 PUFA was calculated as 18:3ω-3 + 20:5ω-3 + 22:5ω-3 + 22:6ω-3.
[7]Total ω-6 PUFA was calculated as 18:2ω-6 + 18:3ω-6 +20:2 ω-6 + 20:3 ω-6 + 20:4 ω-6+ 22:2ω-6.
[8]Ratio ω-6/ ω-3 were calculated as Total ω-6 PUFA / Total ω-3 PUFA.
[a-e]Means within the interaction of dietary treatment and duration of feeding across the rows with no common superscript are significant different ($P<0.05$).

**Table 4.7: Diet and duration effects of feeding experimental diets[1] on fatty acid composition of egg yolk (mg/egg) from laying hens.**

| Fatty | Treatment | | | | Duration | | | | | Probability | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Acids | Control | Moderate | High | SEM | 0 d | 6 d | 12 d | 18 d | SEM | Treatment | Duration | Treat*Duration |
| 14:0 | 13.50$^{a}$ | 12.77$^{a}$ | 11.35$^{b}$ | 0.311 | 15.88$^{a}$ | 12.76$^{b}$ | 10.77$^{c}$ | 10.75$^{c}$ | 0.361 | 0.0001 | 0.0001 | 0.1710 |
| 16:0 | 1228 | 1226 | 1170 | 21.41 | 1350$^{a}$ | 1265$^{a}$ | 1134$^{b}$ | 1083$^{b}$ | 24.71 | 0.0960 | 0.0001 | 0.2440 |
| 16:1 ω-7 | 104.0$^{a}$ | 100.1$^{ab}$ | 94.77$^{b}$ | 2.582 | 117.7$^{a}$ | 102.3$^{b}$ | 88.12$^{c}$ | 90.39$^{c}$ | 2.980 | 0.0420 | 0.0001 | 0.3800 |
| 18:0 | 487.6 | 513.3 | 516.3 | 9.212 | 521.2$^{a}$ | 529.5$^{a}$ | 507.5$^{a}$ | 464.7$^{b}$ | 10.64 | 0.0570 | 0.0001 | 0.7710 |
| 18:1 ω-7 | 2023$^{ab}$ | 2099$^{a}$ | 1952$^{b}$ | 37.61 | 2409$^{a}$ | 2112$^{b}$ | 1798$^{c}$ | 1780$^{c}$ | 43.42 | 0.0230 | 0.0001 | 0.3210 |
| 18:1 ω-9 | 84.81$^{a}$ | 83.53$^{a}$ | 74.94$^{b}$ | 1.791 | 98.25$^{a}$ | 82.67$^{b}$ | 71.89$^{c}$ | 71.56$^{c}$ | 2.068 | 0.0001 | 0.0001 | 0.1560 |
| 18:2 ω-6 | 626.2$^{b}$ | 665.1$^{ab}$ | 685.1$^{a}$ | 13.24 | 678.6$^{ab}$ | 714.4$^{a}$ | 637.8$^{bc}$ | 604.5$^{c}$ | 15.29 | 0.0070 | 0.0001 | 0.8560 |
| 18:3 ω-3 | 34.07$^{c}$ | 97.49$^{b}$ | 148.3$^{a}$ | 3.230 | 43.83$^{b}$ | 108.3$^{a}$ | 106.6$^{a}$ | 114.5$^{a}$ | 3.730 | 0.0001 | 0.0001 | 0.0001 |
| 18:3 ω-6 | 4.424$^{a}$ | 3.728$^{b}$ | 4.014$^{ab}$ | 0.180 | 4.574$^{a}$ | 4.716$^{a}$ | 3.365$^{b}$ | 3.567$^{b}$ | 0.210 | 0.0260 | 0.0001 | 0.0050 |
| 20:1 ω-9 | 10.98$^{a}$ | 10.93$^{a}$ | 9.812$^{b}$ | 0.240 | 12.66$^{a}$ | 10.61$^{b}$ | 9.421$^{c}$ | 9.602$^{bc}$ | 0.278 | 0.0010 | 0.0001 | 0.0020 |
| 20:3 ω-6 | 12.78 | 12.46 | 12.65 | 0.420 | 12.18$^{b}$ | 12.7$^{ab}$ | 14.02$^{a}$ | 11.61$^{b}$ | 0.487 | 0.8600 | 0.0060 | 0.5780 |
| 20:4 ω-6 | 153.0$^{a}$ | 127.0$^{b}$ | 121.2$^{b}$ | 2.480 | 146.8$^{a}$ | 144.4$^{a}$ | 131.7$^{b}$ | 111.9$^{c}$ | 2.866 | 0.0001 | 0.0001 | 0.0001 |
| 20:5 ω-3 | 2.593$^{c}$ | 7.196$^{b}$ | 8.670$^{a}$ | 0.240 | 3.341$^{b}$ | 7.003$^{a}$ | 7.348$^{a}$ | 6.919$^{a}$ | 0.275 | 0.0001 | 0.0001 | 0.0001 |
| 22:2 ω-6 | 39.24 | 39.43 | 38.66 | 0.990 | 31.40$^{c}$ | 41.36$^{b}$ | 48.81$^{a}$ | 34.86$^{c}$ | 1.139 | 0.8450 | 0.0001 | 0.0001 |
| 22:5 ω-3 | 6.499$^{b}$ | 9.598$^{a}$ | 10.51$^{a}$ | 0.290 | 5.994$^{b}$ | 10.32$^{a}$ | 9.599$^{a}$ | 9.562$^{a}$ | 0.340 | 0.0001 | 0.0001 | 0.0001 |
| 22:6 ω-3 | 107.7$^{b}$ | 130.2$^{a}$ | 125.2$^{a}$ | 2.460 | 103.4$^{c}$ | 132.7$^{a}$ | 130.5$^{a}$ | 117.5$^{b}$ | 2.838 | 0.0001 | 0.0001 | 0.0020 |
| SFA[2] | 1,739 | 1,762 | 1,707 | 29.84 | 1,896$^{a}$ | 1,817$^{a}$ | 1,662$^{b}$ | 1,568$^{b}$ | 34.45 | 0.4120 | 0.0001 | 0.3810 |
| MUFA[3] | 2,223$^{ab}$ | 2,293$^{a}$ | 2,132$^{b}$ | 41.00 | 2,638$^{a}$ | 2,307$^{b}$ | 1,968$^{c}$ | 1,952$^{c}$ | 47.34 | 0.0210 | 0.0001 | 0.2990 |
| PUFA[4] | 986.5$^{b}$ | 1092$^{a}$ | 1,154$^{a}$ | 20.33 | 1030$^{b}$ | 1176$^{a}$ | 1090$^{ab}$ | 1015$^{b}$ | 23.46 | 0.0001 | 0.0001 | 0.1510 |
| LC ω-3 PUFA[5] | 116.8$^{b}$ | 147.0$^{a}$ | 144.4$^{a}$ | 2.780 | 112.7$^{c}$ | 150.1$^{a}$ | 147.5$^{a}$ | 134.0$^{b}$ | 3.210 | 0.0001 | 0.0001 | 0.0001 |
| Total ω-3 PUFA[6] | 150.9$^{c}$ | 244.5$^{b}$ | 292.8$^{a}$ | 5.248 | 156.6$^{b}$ | 258.4$^{a}$ | 254.0$^{a}$ | 248.5$^{a}$ | 6.059 | 0.0001 | 0.0001 | 0.0001 |
| Total ω-6 PUFA[7] | 835.7 | 847.7 | 861.6 | 15.82 | 873.5$^{ab}$ | 917.6$^{a}$ | 835.6$^{b}$ | 766.5$^{c}$ | 18.26 | 0.5130 | 0.0001 | 0.9270 |
| Ratio ω-6/ ω-3[8] | 5.564$^{a}$ | 3.683$^{b}$ | 3.348$^{c}$ | 0.043 | 5.604$^{a}$ | 3.906$^{b}$ | 3.703$^{c}$ | 3.581$^{c}$ | 0.050 | 0.0001 | 0.0001 | 0.0001 |

*SE = standard error of mean

*LC = Long Chain, SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

[1]Control with no LinPRO, Moderate diet with 7.5% of LinPRO and a High diet with 15% inclusion of LinPRO in the basal layer diet was fed for 18 days to laying hens (65 wks).
[2]SFA levels were calculated as 14:0 + 16:0 + 18:0.
[3]MUFA levels were calculated as 16:1ω-7 + 18:1ω-7 + 18:1ω-9 + 20:1ω-9.
[4]PUFA levels were calculated as 18:2ω-6 + 18:3ω-3 + 18:3ω-6 + 20:3 ω-6 + 20:4ω-6 + 20:5ω-3 + 22:5 ω-3 +22:4ω-6 + 22:6ω-3.
[5]LC ω-3 PUFA was calculated as 20:5ω-3 + 22:5ω-3 + 22:6ω-3.
[6]Total ω-3 PUFA was calculated as 18:3ω-3 + 20:5ω-3 + 22:5ω-3 + 22:6ω-3.
[7]Total ω-6 PUFA was calculated as 18:2ω-6 + 20:3 ω-6 + 20:4ω-6 + 22:2ω-6.
[8]Ratio ω-6/ ω-3 were calculated as Total ω-6 PUFA / Total ω-3 PUFA
[a-c] Means within dietary treatments and within duration with no common superscript are significant different ($P<0.05$)

**Table 4.8: Effects of interaction of diet and duration of feeding experimental diets[1] on fatty acid composition of the egg yolk (mg/ egg) from laying hens.**

| | 0 d | | | 6 d | | | 12 d | | | 18 d | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Fatty acid | Control | Moderate | High | Control | Moderate | High | Control | Moderate | High | Control | Moderate | High | SEM | Probability |
| 14:0 | 15.73 | 16.19 | 15.72 | 14.02 | 13.01 | 11.24 | 12.45 | 10.95 | 8.89 | 11.82 | 10.91 | 9.53 | 0.63 | 0.1710 |
| 16:0 | 1318 | 1355 | 1378 | 1324 | 1293 | 1178 | 1175 | 1125 | 1101 | 1094 | 1133 | 1023 | 42.79 | 0.2440 |
| 16:1 ω-7 | 117.8 | 116.0 | 119.3 | 112.6 | 102.7 | 91.7 | 94.7 | 89.4 | 80.3 | 91.0 | 92.5 | 87.7 | 5.16 | 0.3800 |
| 18:0 | 496.7 | 529.2 | 537.8 | 523.6 | 538.2 | 526.7 | 489.0 | 500.3 | 533.2 | 441.0 | 485.6 | 467.5 | 18.42 | 0.7710 |
| 18:1 ω-7 | 2316 | 2450 | 2462 | 2152 | 2210 | 1973 | 1832 | 1844 | 1719 | 1793 | 1891 | 1656 | 75.19 | 0.3210 |
| 18:1 ω-9 | 96.0 | 101.0 | 97.7 | 89.2 | 86.7 | 72.1 | 77.0 | 71.8 | 66.9 | 77.0 | 74.6 | 63.1 | 3.58 | 0.1560 |
| 18:2 ω-6 | 669.3 | 670.5 | 695.9 | 684.2 | 724.2 | 734.8 | 599.8 | 637.6 | 675.8 | 551.7 | 628.1 | 633.8 | 26.48 | 0.8560 |
| 18:3 ω-3 | $43.5^{d}$ | $43.0^{d}$ | $45.0^{d}$ | $37.8^{d}$ | $118.0^{c}$ | $169.2^{b}$ | $28.4^{d}$ | $115.9^{c}$ | $175.3^{ab}$ | $26.5^{d}$ | $113.1^{c}$ | $203.9^{a}$ | 6.46 | 0.0001 |
| 18:3 ω-6 | $4.40^{abc}$ | $4.14^{abcd}$ | $5.18^{a}$ | $5.36^{a}$ | $5.02^{ab}$ | $3.76^{abcd}$ | $3.84^{abcd}$ | $2.58^{d}$ | $3.68^{abcd}$ | $4.09^{abcd}$ | $3.17^{cd}$ | $3.43^{bcd}$ | 0.36 | 0.0050 |
| 20:1 ω-9 | $12.0^{ab}$ | $12.9^{a}$ | $13.0^{a}$ | $11.2^{abc}$ | $11.0^{abc}$ | $9.59^{cd}$ | $10.8^{abc}$ | $10.0^{bc}$ | $7.49^{d}$ | $9.91^{bc}$ | $9.75^{cd}$ | $9.15^{cd}$ | 0.48 | 0.0020 |
| 20:3 ω-6 | 12.04 | 12.34 | 12.17 | 13.25 | 12.62 | 12.24 | 14.15 | 12.82 | 15.10 | 11.69 | 12.05 | 11.09 | 0.84 | 0.5780 |
| 20:4 ω-6 | $145.2^{abc}$ | $145.6^{abc}$ | $149.6^{abc}$ | $166.0^{a}$ | $136.0^{cd}$ | $131.3^{cde}$ | $161.5^{ab}$ | $116.9^{de}$ | $116.6^{de}$ | $139.2^{bcd}$ | $109.4^{ef}$ | $87.31^{f}$ | 4.96 | 0.0001 |
| 20:5 ω-3 | $1.66^{fg}$ | $5.51^{de}$ | $2.85^{fg}$ | $1.47^{g}$ | $8.65^{bc}$ | $10.89^{a}$ | $3.86^{ef}$ | $7.59^{cd}$ | $10.62^{ab}$ | $3.39^{efg}$ | $7.06^{cd}$ | $10.32^{ab}$ | 0.48 | 0.0001 |
| 22:2 ω-6 | $28.98^{f}$ | $34.64^{def}$ | $30.58^{ef}$ | $46.31^{abc}$ | $40.17^{bcd}$ | $37.61^{cdef}$ | $47.54^{ab}$ | $44.93^{abc}$ | $53.96^{a}$ | $34.13^{def}$ | $37.99^{cde}$ | $32.47^{def}$ | 1.97 | 0.0001 |
| 22:5 ω-3 | $5.91^{b}$ | $6.30^{b}$ | $5.77^{b}$ | $7.09^{b}$ | $11.50^{a}$ | $12.38^{a}$ | $6.15^{b}$ | $10.16^{a}$ | $12.48^{a}$ | $6.84^{b}$ | $10.44^{a}$ | $11.41^{a}$ | 0.59 | 0.0001 |
| 22:6 ω-3 | $103.1^{c}$ | $104.4^{c}$ | $102.6^{c}$ | $117.6^{bc}$ | $145.3^{a}$ | $135.2^{ab}$ | $111.4^{c}$ | $136.0^{ab}$ | $144.3^{a}$ | $98.8^{c}$ | $135.0^{ab}$ | $118.9^{bc}$ | 4.92 | 0.0020 |
| SFA[2] | 1,839 | 1,909 | 1,941 | 1,873 | 1,855 | 1,725 | 1,687 | 1,646 | 1,652 | 1,555 | 1,640 | 1,509 | 59.65 | 0.3810 |
| MUFA[3] | 2,542 | 2,679 | 2,692 | 2,365 | 2,411 | 2,146 | 2,014 | 2,016 | 1,873 | 1,971 | 2,068 | 1,816 | 81.97 | 0.2990 |
| PUFA[4] | 1,014 | 1,027 | 1,050 | 1,079 | 1,202 | 1,247 | 977 | 1,084 | 1,208 | 876 | 1,056 | 1,113 | 40.63 | 0.1510 |
| LC ω-3 PUFA[5] | $110.7^{d}$ | $116.2^{cd}$ | $111.2^{d}$ | $126.2^{cd}$ | $158.5^{ab}$ | $165.5^{ab}$ | $121.4^{cd}$ | $153.7^{ab}$ | $167.4^{a}$ | $108.9^{d}$ | $140.6^{bc}$ | $152.5^{ab}$ | 5.56 | 0.0001 |
| Total ω-3 PUFA[6] | $154.2^{d}$ | $159.2^{d}$ | $156.2^{d}$ | $164^{d}$ | $283.4^{bc}$ | $327.7^{ab}$ | $149.8^{d}$ | $269.6^{c}$ | $342.7^{a}$ | $135.4^{d}$ | $265.6^{c}$ | $344.5^{a}$ | 10.49 | 0.0001 |
| Total ω-6 PUFA[7] | 859.9 | 867.3 | 893.5 | 915.2 | 918.1 | 919.7 | 826.8 | 814.8 | 865.2 | 740.8 | 790.7 | 768.1 | 31.62 | 0.9270 |
| Ratio ω-6/ ω-3[8] | $5.604^{a}$ | $5.460^{a}$ | $5.746^{a}$ | $5.599^{a}$ | $3.274^{b}$ | $2.844^{cd}$ | $5.539^{a}$ | $3.023^{bc}$ | $2.548^{de}$ | $5.514^{a}$ | $2.976^{bc}$ | $2.253^{e}$ | 0.09 | 0.0001 |

*SEM = Standard error of mean

*LC = Long Chain, SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

[1]Control with no LinPRO, Moderate diet with 7.5% of LinPRO and a High diet with 15% inclusion of LinPRO in the basal layer diet was fed for 18 days to laying hens (65 wks).
[2]SFA levels were calculated as 14:0 + 16:0 + 18:0.
[3]MUFA levels were calculated as 16:1ω-7 + 18:1ω-7 + 18:1ω-9 + 20:1ω-9.
[4]PUFA levels were calculated as 18:2ω-6 + 18:3ω-3 + 18:3ω-6 + 20:3 ω-6 + 20:4ω-6 + 20:5ω-3 + 22:5 ω-3 +22:4ω-6 + 22:6ω-3.
[5]LC ω-3 PUFA was calculated as 20:5ω-3 + 22:5ω-3 + 22:6ω-3.
[6]Total ω-3 PUFA was calculated as 18:3ω-3 + 20:5ω-3 + 22:5ω-3 + 22:6ω-3.
[7]Total ω-6 PUFA was calculated as 18:2ω-6 + 20:3 ω-6 + 20:4ω-6 + 22:2ω-6.
[8]Ratio ω-6/ ω-3 were calculated as Total ω-6 PUFA / Total ω-3 PUFA
[a-c] Means within the interaction of dietary treatment and duration of feeding across the rows with no common superscript are significant different ($P<0.05$).

**Table 4.9: The enzymatic action involved in the biosynthesis of fatty acids in laying hens fed experimental diets[1].**

| Treatment | Duration | [2]ω-3 PUFA biosynthesis pathway | [3]ω-6 PUFA biosynthesis pathway | [4]Δ-9 desaturase enzyme activity |
|---|---|---|---|---|
| Control | | 0.088[a] | 0.248[a] | 0.084 |
| High | | 0.061[b] | 0.192[b] | 0.081 |
| Moderate | | 0.068[b] | 0.178[c] | 0.080 |
| SEM | | 0.006 | 0.003 | 0.001 |
| | 0 d | 0.058[b] | 0.220[a] | 0.087[a] |
| | 6 d | 0.060[b] | 0.206[b] | 0.080[bc] |
| | 12 d | 0.088[a] | 0.208[ab] | 0.077[c] |
| | 18 d | 0.082[a] | 0.190[c] | 0.083[ab] |
| SEM | | 0.004 | 0.004 | 0.001 |
| Control | 0 d | 0.042[c] | 0.222[b] | 0.089 |
| | 6 d | 0.039[c] | 0.246[ab] | 0.084 |
| | 12 d | 0.138[a] | 0.269[a] | 0.080 |
| | 18 d | 0.132[a] | 0.255[a] | 0.083 |
| High | 0 d | 0.064[bc] | 0.219[bc] | 0.086 |
| | 6 d | 0.068[bc] | 0.182[d] | 0.077 |
| | 12 d | 0.060[bc] | 0.172[d] | 0.073 |
| | 18 d | 0.051[bc] | 0.139[e] | 0.085 |
| Moderate | 0 d | 0.066[bc] | 0.219[bc] | 0.085 |
| | 6 d | 0.074[b] | 0.191[cd] | 0.079 |
| | 12 d | 0.066[bc] | 0.182[d] | 0.079 |
| | 18 d | 0.064[bc] | 0.176[d] | 0.082 |
| SEM | | 0.007 | 0.007 | 0.002 |
| Source of Variation | | ----------Probability---------- | | |
| Treatment | | 0.0001 | 0.0001 | 0.0781 |
| Duration | | 0.0001 | 0.0001 | 0.0001 |
| Treatment*duration | | 0.0001 | 0.0001 | 0.3472 |

SEM = standard error of mean.

[1]Control with no LinPRO, Moderate diet with 7.5% of LinPRO and a High diet with 15% inclusion of LinPRO in the basal layer diet was fed for 18 days to laying hens (65 wks).

[2]ω-3 PUFA biosynthesis pathway enzyme activity (Δ-6 desaturase, elongase and Δ-5 desaturase) was calculated as ratio of 20:5ω-3 to 18:3ω-3.

[3]ω-6 PUFA biosynthesis pathway enzyme activity (Δ-6 desaturase, elongase and Δ-5 desaturase) was calculated as ratio of 20:4ω-6 to 18:2ω-6.

[4]Δ-9 Steryl CoA desaturase enzyme activity for was calculated as ratio of 16:1 to 16:0.

[a-e]Means within dietary treatments, duration of feeding and within the column of interaction effect of treatment and duration with no common superscript are significant different ($P < 0.05$).

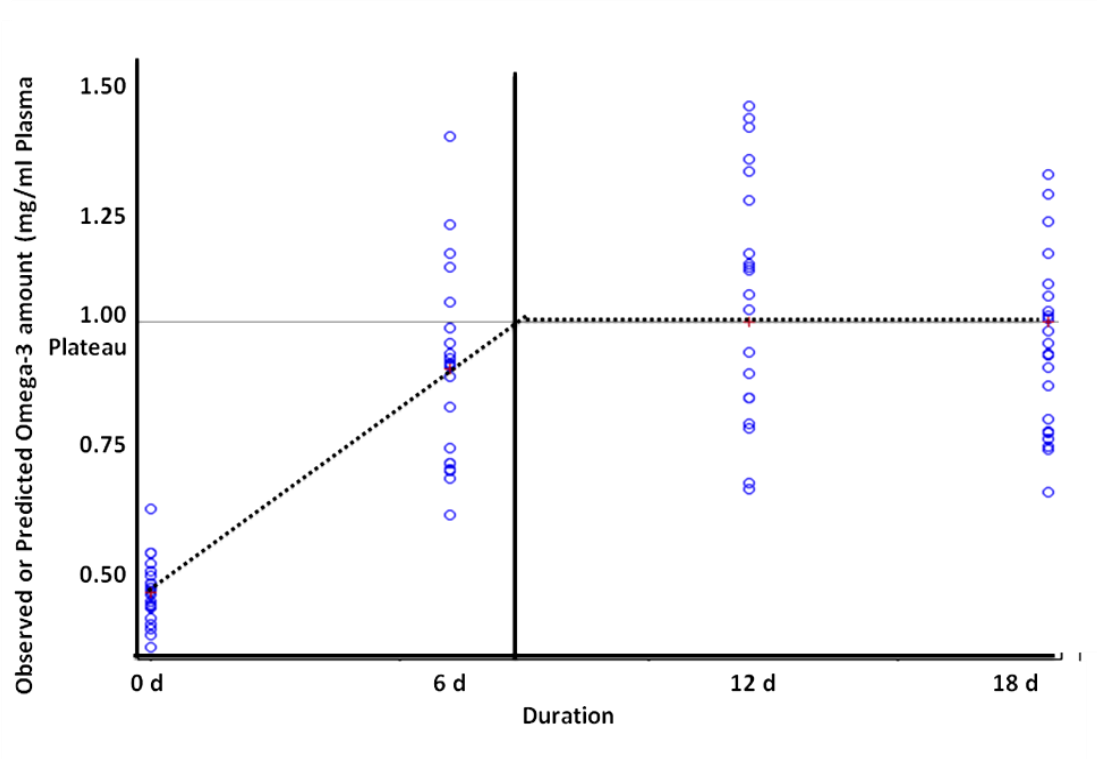


**Figure 4.1: The level of omega-3 PUFA (ω-3 PUFA) enrichment in the blood plasma from the laying hens (65 wks) fed High ω-3 PUFA enriched diets with 15% inclusion of LinPRO in the standard layer diet for 18 days. ω-3 PUFA (dotted line). Break of point or time duration required to reach plateu phase of enrichment (vertical line).**

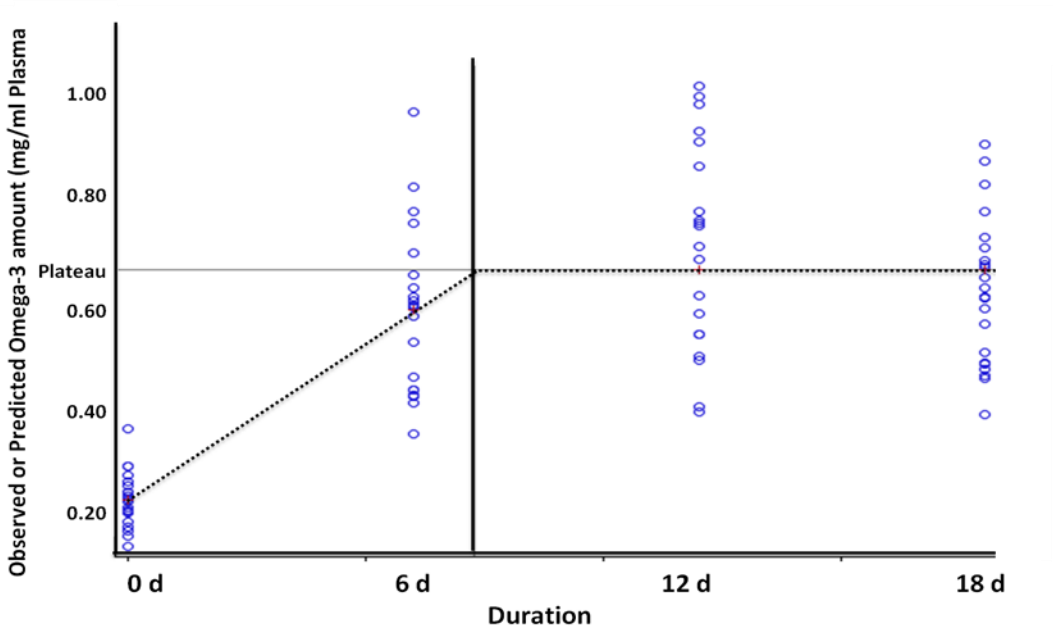


**Figure 4.2: The level of omega-3 PUFA (ω-3 PUFA) enrichment in the blood plasma from the laying hens (65 wks) fed Moderate ω-3 PUFA enriched diets with 7.5% inclusion of LinPRO in the standard layer diet for 18 days. ω-3 PUFA (dotted line). Break of point or time duration required to reach plateu phase of enrichment (vertical line).**

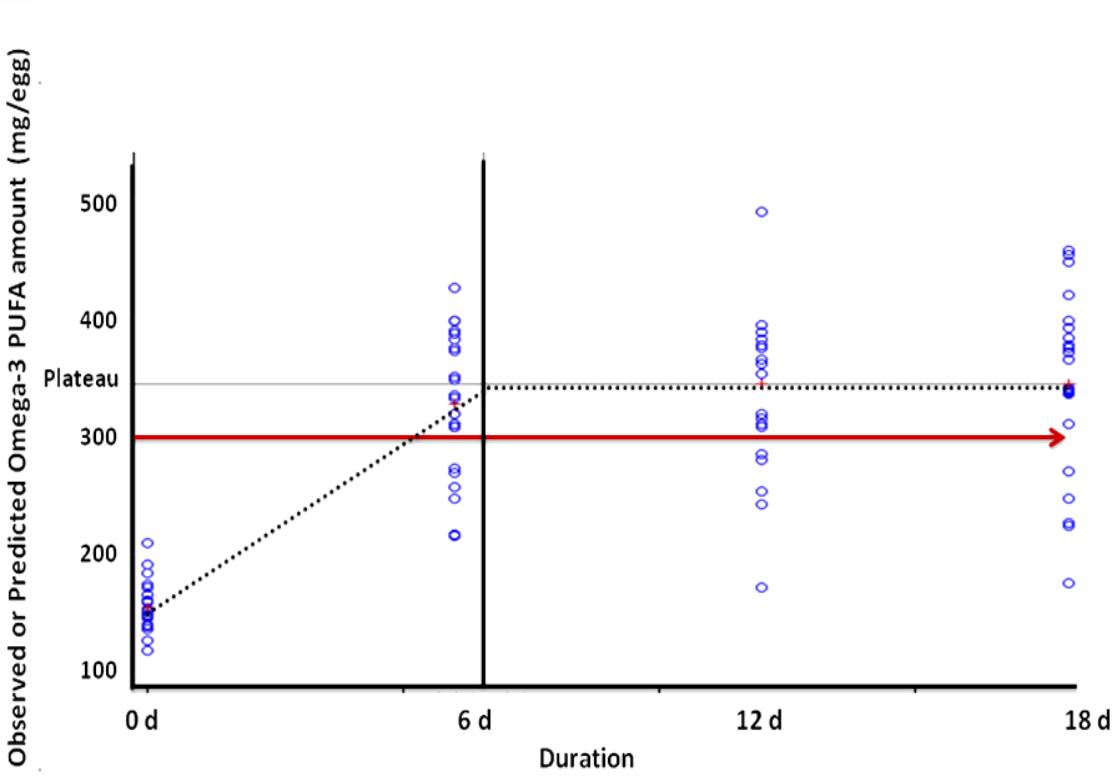


**Figure 4.3: The saturation phase of omega-3 PUFA (ω-3 PUFA) enrichment in the egg yolk from the laying hens (65 wks) fed High ω-3 PUFA enriched diets with 15% inclusion of LinPRO in the standard layer diet for 18 days. ω-3 PUFA (dotted line). Break of point or time duration required to reach plateu phase of enrichment (vertical line). Target enrichment level of ω-3 PUFA (300 mg/egg) (horizontal line).**

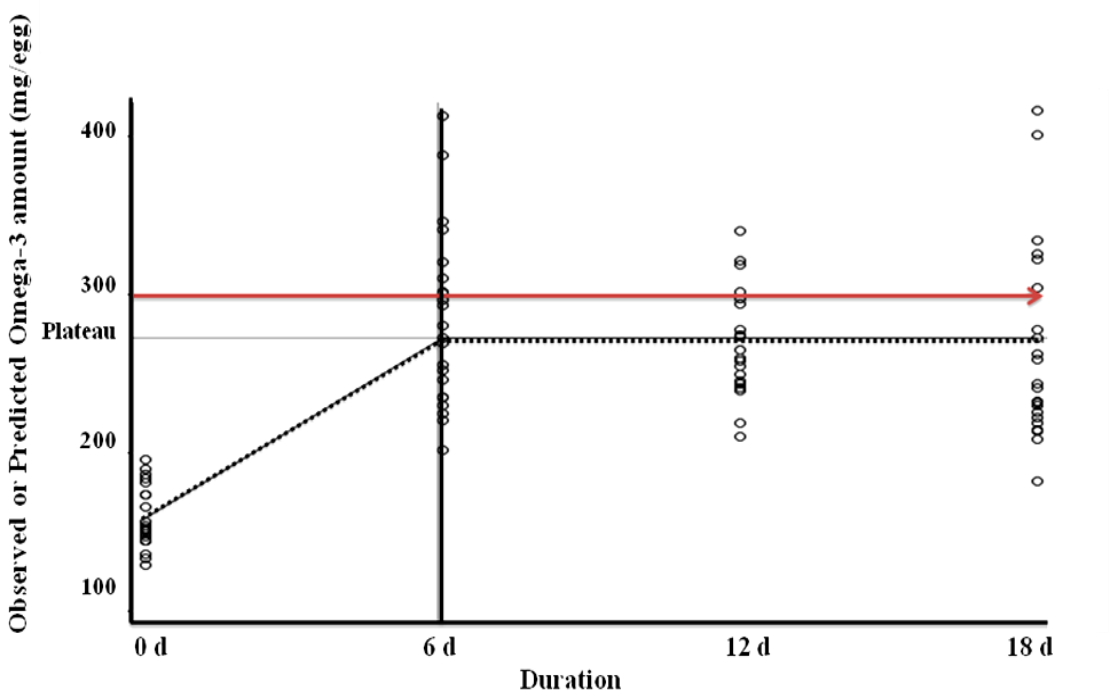


**Figure 4.4: The saturation phase of omega-3 PUFA (ω-3 PUFA) enrichment in the egg yolk from the laying hens (65 wks) fed Moderate ω-3 PUFA enriched diets with 7.5% inclusion of LinPRO in the standard layer diet for 18 days. ω-3 PUFA (dotted line). Break of point or time duration required to reach plateu phase of enrichment (vertical line). Target enrichment level of ω-3 PUFA (300 mg/egg) (horizontal line).**

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# CHAPTER 5

# Summary and Implications

## 5.1 PROJECT SUMMARY

Omega-3 polyunsaturated fatty acids (ω-3 PUFA) and lutein are important value-added ingredients that when incorporated into laying hen diets are deposited into the egg yolks. Whereas numerous varieties of enriched eggs are currently available, the mechanism by which these multiple enriched ingredients might interact during the enrichment process is not well understood. For example, due to the antinutritive factors associated with flax, either ground or whole, it has been reported that the incorporation of ω-3 PUFA in the diet may reduce the transfer of lutein to the yolk (Leeson and Caston, 2004). Strategies to improve incorporation of enrichment ingredients into the yolk include modifying the mode of delivery of the ingredients, or modifying the ingredients themselves.

Modification of dietary ingredients with digestibility issues has been shown to increase nutrient absorption (Bean and Leeson, 2002). Flax is an example of a product that can be altered through additional processing to increase digestibility (Shen et al., 2004). An extruded form of flax was the source of ω-3 PUFA enrichment used in the diets of several of the experiments. There is minimal information about this type of product. For flax, the extrusion process breaks open the seed and provides an increased exposure of the fats, carbohydrates and proteins to the gut, while disrupting the activity of the antinutritive factors (Htoo et al., 2008). The anti-nutritional factors, like mucilage and lignin (Bhatty 1993), affect the use of whole or ground flax by increasing the viscosity of digesta and causing irritation of the gut mucosal surface (Alzueta et al., 2003). If flax was extruded alone, it would be an extremely oily unmanageable mixture due to the high oil content of these seeds. The product we used was coextruded with peas, which provide carbohydrates to enhance structure and provide a dry stable product (Thacker et al., 2005). Unlike ground flaxseed, which can go rancid in a matter of months (or weeks in warm weather), the

extruded product is claimed to be stable for about a year (O & T Farms), which is well beyond the practical storage time for feed.

In the first study, I attempted to increase delivery of lutein to the egg by feeding the ω-3 PUFA diet and lutein diet on alternate days. While this may not be a practical solution at the farm level, the objective was to separate the enrichment ingredients in the gut while allowing them to still coexist in the final product.

Providing dietary ω-3 PUFA and lutein to the hen either alone or in combination were both effective methods of egg yolk enrichment. After the 56 days of experimental period, the alternate day feeding method (daily alternating diet: Alt-1 or every other day alternating diet: Alt-2) resulted in 57% enrichment of ω-3 PUFA in the egg yolks relative to the daily feeding of enriched diets (10% flaxseed (F), 500 ppm lutein (L), and a 1:1 mixed diet of F and L diet (LF). However, it is important to recognize that the hens on alternate diets received only half the amount of flax that the daily fed hens received. This allowed for direct comparison of diets containing similar amounts of flax or lutein enrichment. In order to deliver an identical egg product, the level of inclusion of flax in the alternating diets would need to approximately be doubled. The increased flax in the hen ration could have increased the level of antinutritive effects and that would have reduced the potential to compare the effects of similar amounts of ω-3 PUFA or lutein enrichment in the either together or apart.

An objective of this experiment was to test if lutein enrichment would be negatively affected by the presence of flax in the diet. The lutein levels in eggs from the L diet did not differed from that of the LF diet after 28 d on the test diet. There was no negative interaction of feeding 10% of flaxseed with lutein (500 ppm) in hen ration for the enriching eggs, suggesting that at level of 10% flax does not negatively influence the gut condition to the point of affecting the absorption of other dietary enrichment ingredients present in hen ration. This result did not agree with findings of Leeson and Caston, (2004), which suggested a decline in lutein levels when a ground-flax source of ω-3 PUFA was used. This

is an important outcome because in future work related to multiple enrichment of egg yolk. The flax can be included with less concern over absorption related problem of the other enrichment ingredients. The carotenoid analysis of eggs from this study indicated the peak of lutein enrichment in egg yolk from hens on LF and L diets occurred after 28 d on enrichment diets, with the lutein concentration in egg yolk being 7 to 8-fold higher than the C or F diets. Whereas, Leeson and caston (2004) observed 5 to 8-fold increment in egg yolk lutein amount with a similar level of dietary enrichment (500 ppm) with corn based diet compared to wheat based diet in present study. In addition, the storage of eggs for 30 d had no affect on the stability of lutein and β-carotene in egg yolk.

The lowest values for shell thickness and for specific gravity in the eggs were observed in the Alt-1 diet. This suggests that the daily switching of enriched diet might have created an issue with acclimatization of the gut microflora and villous structures due to rapid dietary changes (Dibner et al., 1996), and thus ultimately affecting the calcium absorption and shell quality of egg. Further, results for egg shell thickness for the hens on the Alt-2 diet did not differ statistically from the other treatments, suggesting that keeping hens on enriched diets for two consecutive days was less disruptive to the absorptive ability of birds than a diet is switched every day. However, further research is needed to provide proof of this theory.

The sole inclusion of lutein in the hen diet did not affect the yolk fatty acid composition compared to eggs from birds fed the control diet. The amount of saturated fatty acid (SFA), PUFA, total ω-3 PUFA, ω-6 PUFA in eggs from hens on the lutein alone diet was similar to that of control eggs at all times tested along the duration of the experiment. This result was expected considering that the lutein diet had no additional dietary fatty acid source. In contrast, the inclusion of flax in the diets significantly increased the total ω-3 PUFA concentration in all the flax containing diets at all times tested after the start of the experiment compared to either the control diet or the lutein alone diets.

Dietary inclusion of an antioxidant source (lutein) along with ω-3 PUFA source for the egg enrichment is also beneficial through the provision of an increased oxidative stability during storage. The lipids in instored eggs have some natural resistance to oxidative damages due to the presence of natural antioxidant fractions in the egg yolk (phosvitins, lecithin) (Pike and Peng, 1985). However, in ω-3 PUFA enriched egg, the presence of the double bonds in ω-3 PUFA results in increased susceptibility to oxidative deterioration (Frankel, 1984). This would add additional pressure on the antioxidants naturally present in the egg. Of the ω-3 PUFA, the long chain ω-3 PUFA (LC ω-3 PUFA) have multiple double bonds thus further increasing their susceptibility to lipid oxidation (Cherian et al., 2007).

In the current study, significant reductions were observed in the LC ω-3 PUFA concentration following 30 d of storage while medium chain ω-3 PUFA like LNA were not affected. These results were further substantiated through TBARS analysis of lipid stability. Low TBARS value indicates a lower level of lipid oxidation of fatty acids in the egg yolk (Pikul and Kummerow, 1991). The TBARS value in eggs from hens on lutein (L) and control (C) diets were similar to that of unstored eggs, but after 30 d of storage the TBARS number in egg from L diets were significantly lower than in C diets, suggesting a potential antioxidant role of lutein in the protection of fatty acids from oxidative damage during storage. However, since there were no significant treatment effects on the decline of LC ω-3 PUFA during storage, it appears that this antioxidant potential was not enough to protect the desirable LC ω-3 PUFA in egg yolk.

The results for ω-3 PUFA and lutein enrichment were most promising for the treatment with the combined feeding of ω-3 PUFA and lutein. There was no negative interaction of feeding 10% flaxseed with 500 ppm lutein for the multiple enrichment of eggs. However, the antioxidant role of lutein in protecting the LC ω-3 PUFAs during storage was not clear in the present study, as the LC ω-3 PUFA amount fell during storage with no significant dietary effect this reduction. The results of this experiment can be utilized as a base for further studies on the

development of feeding models targeted to enrichment of eggs with multiple ingredients.

In the next experiment, I explored the impact of other physiological factors on enrichment ingredients transfer from the hen ration to the egg. The impact of individual hen energetic efficiency was determined and compared to the amount of ω-3 PUFA that was present in the final egg product. Hens with increased energetic efficiency tend to be more effective at supporting cell turnover at gut level and are considered to have optimum absorptive conditions at gut level. The objective was to examine if these birds would have more effective egg enrichment. However, when it came to transfer of ω-3 PUFA from dietary flax to the egg, no strong relationship with energetic efficiency was found.

The effect that differences in energetic efficiency score of birds could have on enrichment may be masked due to the inclusion of an elevated amount of ω-3 PUFA over an extended time period. It is possible that by oversupplying the nutrients it could effectively neutralize the effect of bird to bird variability that might have existed with lower levels of enrichment. Since I also examined other factors that relate to the final ω-3 PUFA enrichment, I was still able to identify some interesting differences. For example, the Efficient birds had increased uniformity in transfer of enrichment to the egg yolk. At 0 d there was no difference in the variability (as measured by CV) for total ω-3 PUFA in egg yolks from Efficient and Non-efficient birds (27.7 vs. 28.2). After 14 d, the CV for total ω-3 PUFA in egg yolk from efficient hens was lower than that of Non-efficient birds (11.1 vs. 21.4), indicating a more uniform level of enrichment. In addition, the histomorphological parameters of the duodenum had significant differences among birds with different scores for energetic efficiency.

The Efficient hens had 25% longer villi, resulting in greater absorptive surface area/villi than in Non-efficient. Also, the Efficient hens had shallow crypts compared to Non-efficient hens. The shallow crypts in Efficient hens possibly indicates a lower cell turnover rate of the intestinal epithelium, which results in lower maintenance requirements and favors increased growth (Yason et

al., 1987). The ratio of villus length to crypt depth, which is considered as a useful criterion for estimating the digestive capacity of the small intestine (Montagne, 2003) was significantly higher in Efficient birds than the Non-efficient birds. The histomorphological results of this study indicated that the Efficient birds were better suited for nutrient absorption than the Non-efficient birds. However, ultimately this did not affect egg ω-3 PUFA enrichment suggesting that gut condition has a limited effect on lipid metabolism or egg fatty acid composition. Building on what has been found so far; future work could include assessment of markers of lipid metabolism, such as liver enzymes, given that 90 to 95 % of lipids in poultry are manufactured in the liver (O'Hea and Leveille, 1969). Utilizing more energetically Efficient birds for the purpose of value-added egg enrichment might help in reducing the variability in end product. In brief, the efficiency of the ω-3 PUFA enrichment process in eggs may be increased by optimizing the metabolic efficiency and improving the absorptive capacity of the birds. This can be accomplished by manipulating the gut condition through dietary treatments of the birds.

A final experiment was performed due to concerns in the previous study that the level of dietary enrichment for ω-3 PUFA was so high that it could be masking potential bird to bird variability in transfer of enrichment from diet to the egg. A time course experiment was added to examine several dietary enrichment levels. This trial demonstrated that final egg enrichment could be just as variable with Moderate (7.5% Linpro) and High (15% Linpro) levels of dietary enrichment compared to the negative control birds.

Interestingly, the enrichment process reached a plateau state in a much shorter time period than we had expected. In an earlier report, Sim and Cherian (1994), found that ω-3 PUFA levels in the eggs stabilize between days 9 to 12 of flax-feeding. However, extruded flax was used in the current work as the ω-3 PUFA source, and this form of flax has a demonstrated increased digestibility and therefore a higher AMEm (Bean and Leeson, 2002). We found that enrichment plateaued at approximately 6 d on the enrichment diet. The total ω-3 PUFA in egg

yolk achieved plateau levels of 343.7 mg/egg and 272.0 mg/egg in 6.6 and 5.9 d, respectively, on the High and Moderate diets. In the blood plasma, the ω-3 PUFA concentrations reached saturation in 7.2 d, with 0.93 mg/mL and 0.67 mg/ mL for the High and Moderate diets, respectively. Since the testing interval in this study was 6 d, it is possible that improved resolution on the enrichment plateau could be achieved by examining yolk levels at times even lesser than 6 d mark. However, considering the fact that it takes approximetely 10 d to develop a fully formed yolk in a laying hen, the dilution effect from having an unenriched yolk core will increase further if testing would have done at times below this.

The ω-3 PUFA from the diet can be measured in the feed, in the blood plasma and in the final egg product. This allows us to track the measurement of the ω-3 PUFA from the diet, through the transition into the blood and finally into the egg product. Blood samples had the greatest amount of bird: bird variation. In the final egg product, ω-3 PUFA concentrations were more stable than in blood, with uniformity being about 50% higher than what was recorded for blood. I took blood samples at the same time each day in order to reduce time effects on plasma lipid measures, but still saw marked differences in the fatty acid pattern of the egg yolks. Blood measurements have a degree on inaccuracy because they include both the ω-3 PUFA travelling from the gut to the liver as well as the ω-3 PUFA that had been repackaged into the very low density lipoprotein (VLDL) particles travelling to the yolk for deposition; which could have contributed to variability obtained in blood ω-3 PUFA concentration. The blood plasma can also contain fat that has been remobilized from lipid depot (Leclercq, 1975). However, this would be limited in older birds, when the energetic demands are reduced. When the fatty acid profiles were assessed, the level of ω-3 PUFA in the blood was much lower than that of the egg. The egg yolk is a final product that follows a ‘recipe’ that provides highly available lipids to the potential growing embryo and therefore the specific fatty acids like the ω-3 PUFA are concentrated (Cherian and Sim, 1993; 2001). The VLDL particles targeted for the yolk have a different composition than the VLDL going to the rest of the body, with the yolk VLDL containing a

higher proportion of the high-energy triglycerides than regular VLDL (Walzem, 1996). The fatty acid composition of the yolk lipid can still be manipulated. Since the fatty acids that are integrated into the various lipid classes can still differ.

One of the objectives of this study was to measure long-chain ω-3 PUFA concentration in the egg. The long-chain ω-3 PUFA (such as DHA and DPA) have more demonstrated ties to health effects in humans compared to the less studied and therefore also considered potentially less effective medium chain ω-3 PUFA found in flax (LNA). Interestingly, I did not find a statistical difference for long-chain ω-3 PUFA in the different diets, but we did find then in the eggs. Dietary LNA from flax did increase yolk LNA, and it also led to increased long-chain ω-3 PUFA in blood plasma and egg yolk.

The amount of LC ω-3 PUFA in blood plasma also increased with similar intensity to that of egg yolk. The average increase in LC ω-3 PUFA in blood plasma was average 48% from hens after 6 d on Moderate and High diets. In egg yolks, the increase in LC ω-3 PUFA was about 43% in Moderate and High hens at 6 d. However, in the blood plasma the EPA and DHA amount lack clear contrast among dietary treatment. In contrast, feeding an enriched diet significantly increased both, DPA and DHA amount in egg yolk compared to the Control diet. However, there was no statistical difference between two enriched diets (High or Moderate) for the LC ω-3 PUFA in egg yolk. The exception to this was increased amount of EPA in egg yolks from birds fed the High compared to the Moderate diet at 6 d, 12 d and 18 d. This result has implications for markets where the labeling rules limit the listing of ω-3 PUFA only to individual LC ω-3 PUFA. In such case, the inclusion of a LC ω-3 PUFA source in the hen ration would likely cause greater treatment difference for the final LC ω-3 PUFA in egg yolk.

The essential fatty acids LA (medium chain ω-6 PUFA) and LNA (medium chain ω-3 PUFA) compete with each other to utilize the same set of desaturases and elongase enzymes for their respective long chain substrate synthesis (Holman, 1986). The calculated overall desaturase and elongase enzyme activity for long chain ω-6 PUFA biosynthesis (ratio of 20:4 ω-6:18:2 ω-6)

indicated a negative correlation with the amount of LNA (r= -0.59; P<0.05) and significantly reduced with increased ω-3 PUFA amount in egg yolk. The significant increase in yolk EPA, DPA, and DHA content and reduction in AA content in egg yolk, despite having same original concentrations in the feed, substantiates the role of competition for enzymes during post absorptive modification of these fatty acids.

The target threshold of 300 mg of total ω-3 PUFA/egg (as required in order to label an egg as source of ω-3 PUFA) can be achieved in just 5 d using a 15% Linpro ration (extruded flax product). Earlier Jia et al., (2008) found that feeding extruded flax as in Linpro (equivalent to 7.5% flax) had resulted in higher ω-3 PUFA enrichment compared to feeding 10 % of ground flax in hen ration. In addition, our results support previous work suggesting the fact that feeding extruded flaxseed improves the digestibility of flax because of the disruption of anti-nutritional factors, like mucilage and lignins, which is normally associated with feeding ground flaxseed (Alzueta et al., 2003). This is an important result, as one of the main concerns with the use of flax in poultry diets is the potential for negative impact on growth and gut condition.

## 5.2 FUTURE RESEARCH

Laying hens have the ability to synthesize limited amounts of EPA and DHA from LNA, even if they receive diet enriched with higher amounts of LNA than LA through their diets (Kralik et al., 2008). However, excess dietary ω-3 PUFA in chicken ration can reduce the desaturation of LNA to the ω-3 LC-PUFA in egg yolk, especially when the diet content of ω-3 LC-PUFA is higher (Whelan et al. 1991; and Bezard et al. 1994). Hence, merely increasing the LNA concentration in the diet of the hens is not enough for increasing up-conversion to ω-3 LC-PUFAs in the yolk. To maximize the conversion efficiency, an improved activity of the rate limiting factor Δ-6 desaturase is needed (Yamazaki et al., 1992). The Δ-6 desaturase activity may be increased with elevated dietary protein levels (Narce et al., 1988), while its activity is decreased by factors such as fasting

and high temperature (Poisson and Cunnane, 1991). Recently it was reported by Tsukui et al. (2009), that supplementation of dietary fucoxanthin (brown algal carotenoid), which had been previously known to accumulate as fucoxanthinol and amarouciaxanthin in the laying hens (Strand et al. 1998), could also increase the Δ6-desaturase activity in mice. Alternatively, the use of genetically modified feed sources enriched in stearidonic acid (SDA, 18:4) might be useful to improve the biosynthesis of long chain ω-3 PUFA (Ruiz-Lopez et al., 2009). The SDA can bypass the rate-limiting step in the conversion of ALA to EPA and DHA. In order to achieve direct deposition of LC- ω-3 PUFA into egg yolk, DHA sources derived from commercial algae have also shown promising effects in increasing DHA in egg yolk (Abril and Barclay, 1998).

There is potential for future work in this area through use of alternate ω-3 PUFA sources that contain longer chain ω-3 PUFA, or that solve enzymatic limitations in the up-conversion of ω-3 PUFA into the more desirable longer forms, either by bypassing this step or increasing the ability to the next step in the ω-3 PUFA biosynthesis pathway. While there has been some success in adding this enzyme to food animals, such as pigs, it may be more likely to achieve public awareness for new products in this field by focusing on plant or bacteria based modification. In the short term, we still have LC ω-3 PUFA sources such as fish oils that could be blended into hen enrichment diets to achieve a better ω-3 PUFA fatty acid profile.

The common value added enrichments added to layer rations such as ω-3 PUFA, lutein and vitamin E are fat soluble ingredients. The digestion of fat from the diet is enhanced by emulsification with bile salts, and they are digested and absorbed mainly in the small intestine. The age of the hen, the intestinal microflora, and the gut microstructure are the main factors affecting the utilization of dietary fat (Fedde, 1960; Maisonnier et al., 2003). However, fat digestibility in chickens is greatly influenced by structural type, form, composition, and amount of dietary fat in the ration (Gerrat and Young, 1975; Krogdahl, 1985). Hence, the maintenance of gut health is essential for optimizing digestion and improving the

production potential of value added enrichment products from layers. The individual hen effects on ω-3 PUFA absorption in this project suggest further work to optimize egg enrichment through dietary strategies would be beneficial for the field of egg enrichment. In summary, future research is needed to focus on to meet following objectives:

- Exploration of diet-based treatments to improve gut condition and improve uptake of value added nutrients.
- Improve consistency of product through reducing bird: bird variation in value added enrichment deposition.
- Explore alternate sources of enrichment to capitalize on new opportunities for enrichment of eggs in ways that can deliver greater potential health benefits to the consumer.

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# CHAPTER 6: Appendix-1

## Calculation of the Temperature and Time Duration Required for Derivatization of Free Fatty Acid into Fatty Acid Methyl Esters for Gas Chromatography.

The one of common way of quantification of the fatty acids is through gas chromatography (**GC**) with a flame ionized detector. For preparing sample for GC, it is necessary to convert free fatty acid extracted from egg yolk into more volatile non-reactive derivatives of fatty acids (methyl esters or other derivatives). The fatty acid methyl esters (**FAME**) are most commonly obtained by heating free fatty acids with an excess amount of anhydrous methanol in the presence of any strong acid (HCl, $H_2SO_4$, $BF_3$). Therefore, it becomes very important to know about the optimum condition of temperature and time duration required for heating extracted free fatty acids to get fully derivitized FAME.

A short trial was conducted to assess the optimum condition required for derivatization of fatty acid. From the 1 g egg yolk samples, fat was extracted as described earlier in chapter 2. Then six sets of duplicate samples from the same egg yolk were prepared into twelve sets of Teflon lined screw caped, test tube. In the samples2 mL of methylating reagent (Methanolic HCl, 1N, Sigma, Oakville, ON, Canada) was added and tubes were heated in replicate sample in water bath at 60 C for 15, 30, 45, 60, 75 and 90 min and at 80 C for 15, 30, 45, 60, 75 and 90 min. Then, 100 µL of distilled water, a known amount of internal standard (0.5 mL) (heptadecanoic acid, 17:0, Sigma, Oakville, ON, Canada) and 4 mL of hexane was added, mixed thoroughly, and centrifuged at 1500 x g for 3 min. The top hexane layer was separated and transferred to another test tube containing about 10-20 mg anhydrous sodium sulphate to absorb any moisture, if there, from hexane. Finally, after adjusting fat to 0.2 to 0.3 mg/mL of hexane in GC vial, 1 µL was injected to GC for analysis with operative conditions as described in detail in Chapter.1.

The fatty acid composition of egg yolk derivatized at 60 C and 80 C for the different time duration was analyzed (Table A.1.1). The amount total fatty

acid was estimated by sum of all individual fatty acid. The amount of total fatty acid was highest in the samples derivitized at 60 C for 60 min compare to other samples (Figure A.1.1). In addition, the percentage recovery of fat was also calculated with the amount of total fatty acid recover from a known amount of free fatty acid (Figure A.1.2). The result indicates that heating the fat with methylating agent at 60 C had shown gradual increase in extent of derivatization with increase in time duration. The peak level was recorded at heating the sanple for derivitization at 60 C for 60 min. In contrast, heating the fat samples at 80 C did not show continuity in the extent of the derivatization. Moreover, the percentage recovery of fat after derivatization was also low in case of 80 C compared to 60 C at all the time durations.

The trends of total fatty acid recover after heating at 60 C and 80 C at different durations is described in following equations:

Equation: 1. **for 60 C**; $\mathbf{Y = -0.0038x2 + 0.4948x + 71.675;\ \ (R^2 = 0.9353)}$

Equation: 2. **for 80 C**; $\mathbf{Y = -0.0008x^2 + 0.0824x + 82.313;\ \ (R^2 = 0.0786)}$

These results indicates that heating extracted free fatty acids at 60 C for 60 min is the most appropriate temperature and time combination for derivitization, as it allows the efficient reaction of free fatty acid with methylating agent to obtain more volatile form of FAME, which in turn increases the efficiency of flame ionized detector used to quantify the hydrocarbons.

**Table A.1.1 The amount of fatty acid recovered from derivatization at 60 C and 80 C at different duration of time.**

| Time (min) | Temperature | 16:1 | 18:0 | 18:1ω-7 | 18:1 ω- | 18:2ω-6 | 18:3ω -3 | 20:4ω-6 | 22:5ω-3 | 22:6 ω-3 | Total | Unk[2] | Percent Recovery |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 15 | | 0.060 | 0.250 | 0.060 | 1.352$^{b}$ | 0.440$^{c}$ | 0.145$^{b}$ | 0.035 | 0.010$^{a}$ | 0.050$^{b}$ | 3.170$^{b}$ | 0.040 | 80.66$^{b}$ |
| 30 | | 0.058 | 0.258 | 0.060 | 1.403$^{ab}$ | 0.455$^{bc}$ | 0.148$^{ab}$ | 0.038 | 0.010$^{a}$ | 0.053$^{b}$ | 3.275$^{ab}$ | 0.043 | 83.33$^{ab}$ |
| 45 | | 0.060 | 0.265 | 0.060 | 1.450$^{a}$ | 0.475$^{a}$ | 0.153$^{ab}$ | 0.040 | 0.010$^{a}$ | 0.060$^{a}$ | 3.388$^{a}$ | 0.045 | 86.22$^{a}$ |
| 60 | | 0.060 | 0.265 | 0.060 | 1.437$^{a}$ | 0.470$^{ab}$ | 0.153$^{ab}$ | 0.045 | 0.010$^{a}$ | 0.060$^{a}$ | 3.367$^{a}$ | 0.043 | 85.69$^{a}$ |
| 75 | | 0.060 | 0.265 | 0.060 | 1.435$^{a}$ | 0.470$^{ab}$ | 0.155$^{a}$ | 0.040 | 0.010$^{a}$ | 0.060$^{a}$ | 3.362$^{a}$ | 0.045 | 85.56$^{a}$ |
| 90 | | 0.060 | 0.262 | 0.060 | 1.428$^{a}$ | 0.468$^{ab}$ | 0.150$^{ab}$ | 0.040 | 0.010$^{a}$ | 0.060$^{a}$ | 3.337$^{a}$ | 0.045 | 84.93$^{a}$ |
| SEM | | 0.001 | 0.003 | 0.000 | 0.012 | 0.004 | 0.002 | 0.002 | 0.000 | 0.001 | 0.030 | 0.002 | 0.749 |
| | 60 | 0.060 | 0.261 | 0.060 | 1.424 | 0.464 | 0.152 | 0.039 | 0.010 | 0.054$^{b}$ | 3.332 | 0.045 | 84.78 |
| | 80 | 0.059 | 0.261 | 0.060 | 1.411 | 0.462 | 0.149 | 0.040 | 0.010 | 0.060$^{a}$ | 3.302 | 0.042 | 84.01 |
| SEM | | 0.001 | 0.002 | 0.000 | 0.007 | 0.002 | 0.001 | 0.001 | 0.000 | 0.001 | 0.017 | 0.001 | 0.434 |
| 15 | 60 | 0.060 | 0.240 | 0.060 | 1.300$^{b}$ | 0.420$^{b}$ | 0.140 | 0.030 | 0.010 | 0.040$^{b}$ | 3.060$^{b}$ | 0.040 | 77.86$^{b}$ |
| | 80 | 0.060 | 0.260 | 0.060 | 1.405$^{ab}$ | 0.460$^{a}$ | 0.150 | 0.040 | 0.010 | 0.060$^{a}$ | 3.280$^{ab}$ | 0.040 | 83.46$^{ab}$ |
| 30 | 60 | 0.060 | 0.255 | 0.060 | 1.400$^{ab}$ | 0.455$^{ab}$ | 0.150 | 0.035 | 0.010 | 0.045$^{b}$ | 3.270$^{ab}$ | 0.040 | 83.21$^{ab}$ |
| | 80 | 0.055 | 0.260 | 0.060 | 1.405$^{ab}$ | 0.455$^{ab}$ | 0.145 | 0.040 | 0.010 | 0.060$^{a}$ | 3.280$^{ab}$ | 0.045 | 83.46$^{ab}$ |
| 45 | 60 | 0.060 | 0.265 | 0.060 | 1.465$^{a}$ | 0.475$^{a}$ | 0.155 | 0.040 | 0.010 | 0.060$^{a}$ | 3.410$^{a}$ | 0.045 | 86.83$^{a}$ |
| | 80 | 0.060 | 0.265 | 0.060 | 1.435$^{a}$ | 0.475$^{a}$ | 0.150 | 0.040 | 0.010 | 0.060$^{a}$ | 3.365$^{a}$ | 0.045 | 85.62$^{a}$ |
| 60 | 60 | 0.060 | 0.270 | 0.060 | 1.465$^{a}$ | 0.480$^{a}$ | 0.155 | 0.050 | 0.010 | 0.060$^{a}$ | 3.435$^{a}$ | 0.045 | 87.41$^{a}$ |
| | 80 | 0.060 | 0.260 | 0.060 | 1.410$^{ab}$ | 0.460$^{a}$ | 0.150 | 0.040 | 0.010 | 0.060$^{a}$ | 3.300$^{ab}$ | 0.040 | 83.97$^{ab}$ |
| 75 | 60 | 0.060 | 0.270 | 0.060 | 1.465$^{a}$ | 0.480$^{a}$ | 0.160 | 0.040 | 0.010 | 0.060$^{a}$ | 3.430$^{a}$ | 0.050 | 87.28$^{a}$ |
| | 80 | 0.060 | 0.260 | 0.060 | 1.405$^{ab}$ | 0.460$^{a}$ | 0.150 | 0.040 | 0.010 | 0.060$^{a}$ | 3.295$^{ab}$ | 0.040 | 83.84$^{ab}$ |
| 90 | 60 | 0.060 | 0.265 | 0.060 | 1.450$^{a}$ | 0.475$^{a}$ | 0.150 | 0.040 | 0.010 | 0.060$^{a}$ | 3.385$^{a}$ | 0.050 | 86.14$^{a}$ |
| | 80 | 0.060 | 0.260 | 0.060 | 1.405$^{ab}$ | 0.460$^{a}$ | 0.150 | 0.040 | 0.010 | 0.060$^{a}$ | 3.290$^{ab}$ | 0.040 | 83.72$^{ab}$ |
| SEM | | 0.002 | 0.004 | 0.000 | 0.017 | 0.006 | 0.003 | 0.003 | 0.000 | 0.002 | 0.042 | 0.003 | 1.056 |
| Source of Variation | | ----------Probability---------- | | | | | | | | | | | |
| Time | | 0.5110 | 0.0537 | - | 0.0035 | 0.0013 | 0.0435 | 0.2192 | - | 0.0001 | 0.0051 | 0.6067 | 0.0049 |
| Temperature | | 0.3774 | 1.0000 | - | 0.2062 | 0.4602 | 0.1394 | 0.6887 | - | 0.0001 | 0.2404 | 0.0929 | 0.2337 |
| Time*Temperature | | 0.5110 | 0.0720 | - | 0.0098 | 0.0045 | 0.0808 | 0.2192 | - | 0.0001 | 0.0216 | 0.1614 | 0.0212 |

[a-c] Means across the column within time duration, within temperatures and within their interaction with no common superscript are significant different (P<0.05).
Temp[1] = Temperature (C), Total = Total fat recover after derivatization at different time and temperature combination, Unk[2] = Sum of Unidentified fatty acids

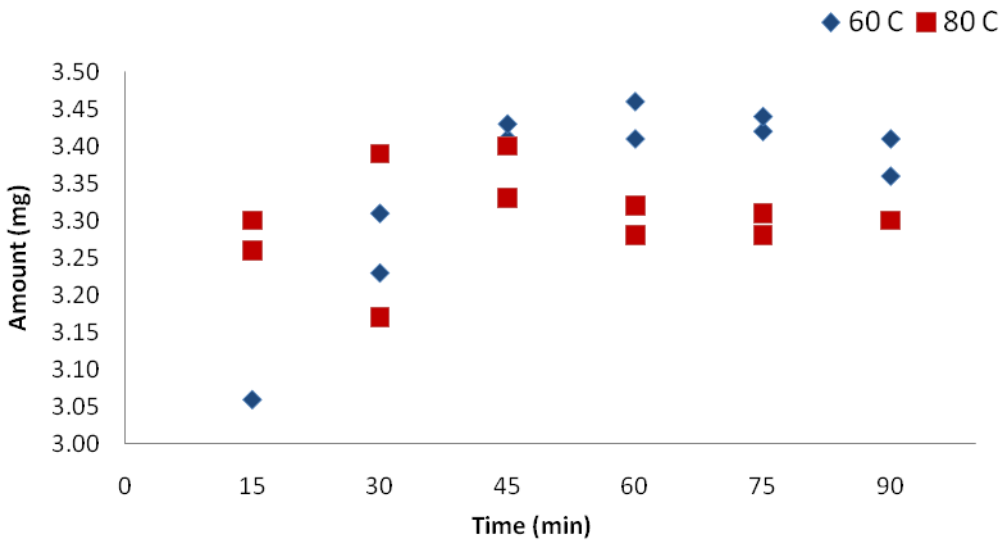


**Figure A.1.1: "Total fatty acids (mg)" quantified after a known amount of fat (0.39 mg) was subjected to derivatization at 60 C and 80 C for 15, 30, 45, 60, 75 and 90 min respectively.**

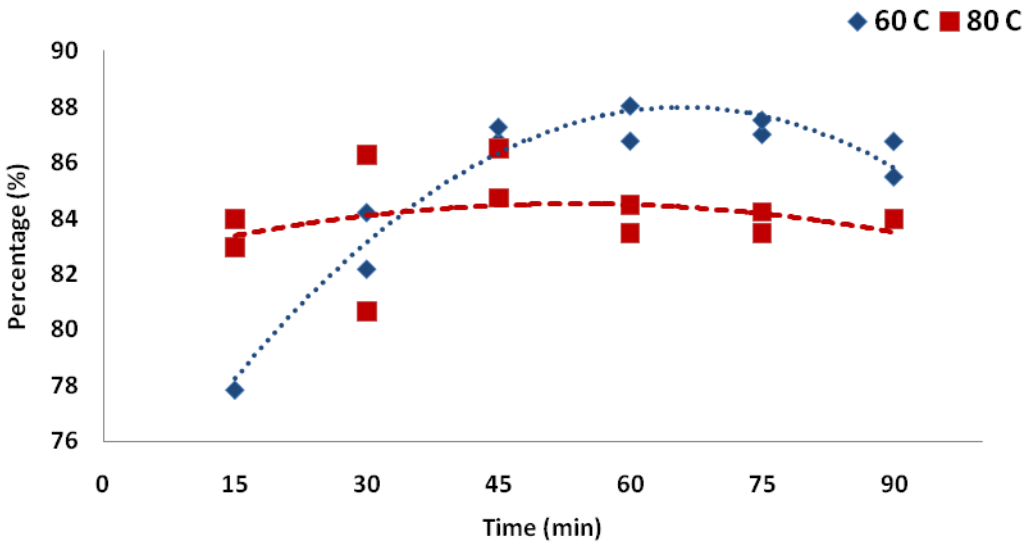


**Figure A.1.2: "Percentage Recovery" of fat after a known amount of fat (0.39 mg) was subjected to derivatization at 60 C and 80 C for 15, 30, 45, 60, 75 and 90 min respectively.**

# CHAPTER 6: Appendix-2

# Comparison of the Fatty Acid Composition of Organic Farm Eggs, Conventional White Table Egg, and Several Forms of Enriched Eggs

The egg is one of nature's most compact energy sources in the animal kingdom, with essential vitamins and minerals and containing all the essential amino acids required for embryonic growth. By extension, eggs are a highly digestible part of the human diet, with superior bioavailability of many of its ingredients. The advent of value-added eggs had further added variety to the consumers. In the Canadian egg market, ω-3 PUFA eggs contribute up to 12% and organic free-range and free-run, all add up to 3.5% (Egg farmers of Canada, 2008). The classic white eggs still holds 84.5% of egg market share largely due to its cheaper cost. However, some Canadian companies are currently marketing over 20% of their eggs as ω-3 enriched eggs in some markets. The Scientific Review Committee of Health Canada recommends that a men and women aged 19 to 70+ should daily consume 1.6 g and 1.1 g of ω-3 PUFA, respectively. The additional health benefits associated with the value added eggs are motivating more and more people to choose the enriched eggs.

The fatty acid composition of the commonly available table eggs in the market was assessed. Four different types of eggs were analyzed. The conventional white table egg **(C)**, ω-3 PUFA enriched egg **(N)**, an egg with multiple enrichments **(NL)**, and organically free run farm egg **(F)** from backyard raised flock of brown laying hens. The three market egg types, C, N, and NL egg, had a nutritional label indicating the fatty acid composition. The label from C stated that there was 0.05 grams of ω-3 PUFA in a 50 gram serving, N stated to have 0.4 grams of ω-3 PUFA with 75 mg of docosahexaenoic acid (DHA, C22:6 ω-3) per 50 gram serving and lastly, NL stated to have 0.4 grams of ω-3 PUFA with 125 mg of DHA plus 1 mg of lutein. A total of 6 samples of each type with

two pooled egg were analyzed in replicate. The modified folch method as described in Chapter 2 was used to analyze the fatty acid composition.

The C and F egg had almost same fatty acid composition except for the significant difference in ω-6 concentration (Table.1). Moreover, C eggs had the highest proportion of saturated fatty acid (**SFA**) compared to the other three egg types (Table 2). However, the absolute amount of SFA per gram yolk was same in all treatments (Table 2). The F eggs had highest amount of total ω-6 PUFA compared to other three types. There was significant difference in the total fatty acid amount for all the four types of egg. The C eggs had lowest total fatty acid while the F type egg had highest amount of total fatty acids followed by NL and N. Among value added eggs, NL eggs had the highest amount of total ω-3 PUFA along with DHA. The NL and N eggs contained approximately 8-fold more C18:3ω-3 (**LNA**) than the C and F eggs. When all of the ω-3 PUFA were combined to calculated total ω-3 PUFA, the NL and N still contained about 4-fold more ω-3 PUFA than that of the C and F eggs.

The ratio of ω-6 PUFA to ω-3 PUFA in case of C and F eggs (Ratio = 6) was about 3 fold higher than the NL and N eggs (Ratio = 2). A lower ratio of ω-6 PUFA to ω-3 PUFA (1 to 4) is recommended for prevention chronic heart disease (Simopolous, 2006).

The normal egg serving for commercial eggs is based on a 50 g of egg that contains approximately 17 g of yolk. The calculated amounts of total ω-3 PUFA, DHA, LNA and total fatty acids considering a 17 g yolk in each egg are shown in Figures 1 to 4. The amount of ω-3 PUFA in the case of N was found to be less than the claim while in case of NL it was about 50 mg higher than the stated nutritional claim. If the N eggs were actually larger (data not collected), they could have met their label claims and the NL eggs could have been significantly greater than their label claims. The range of total ω-3 PUFA in individual eggs was from 319 to 524 mg/egg in case of NL and N type's egg. Although, we had fairly small sample sizes, it is evident that there is very high variability in the enrichment of the table eggs. Feeding or management methods to increase

uniformity of enrichment among eggs would be beneficial, as it could reduce the cost of enrichment if such a high margin of protection did not have to be used.

The value added eggs, NL and N are good source of ω-3 PUFA with significantly higher amount of DHA compared to traditional white egg (C) and organic egg (F). The nutritional claims mentioned on the labels of value added eggs are stated correctly except the N type suffered from variability in total ω-3 PUFA amount among eggs tested (means were on target). Furthermore, the multiple enriched eggs contain lutein, a fat soluble Carotenoid, which helps in preventing oxidative damage to the ω-3 PUFA. Hence, the protective anti-oxidant role of lutein in NL eggs may be reason for significant higher ω-3 PUFA amount compared to N (ω-3 PUFA) eggs. In research occurring concurrently with this study, it was noted that there is much more stability of ω-3 PUFA in eggs where lutein was also included in the hen diet (Chapter 2).

The ω-3 PUFA enriched eggs - particularly those also enriched with DHA are associated with lots of health benefits including the reduction in the chronic heart diseases, stroke, diabetes, cancer, and arthritis in humans (Yashodhara et al., 2009; Marik et al., 2009). Moreover, the multiple enriched eggs with Lutein not only provide the additional health advantages particularly to the elderly people, in eyes diseases like cataract and age-related macular degeneration (Bone & Landrum, 2001) but also aids in protecting the enriched eggs from oxidative damage. Despite having additional health benefits for humans in enriched eggs, the value-added enrichments provide health benefits to the hens such as improved immune system and reduced liver hemorrhages. The higher cost of production for enriched egg is a challenge for this segment of the egg industry. This hurdle can be overcome by achieving target of uniformity in absorption and uniformity of enrichment in end product through reducing bird: bird variation in enrichment. The higher cost of value added feed ingredients added in poultry feed in order to enrich the eggs can be compensated for by improving the absorptive capability of through optimizing the intestinal efficiency, or changes to feed ingredients to enhance absorption and deposition of the value-added ingredients.

**Table A.2.1: The Fatty acids profile of the different table eggs available in market**

| Fatty Acids | C[1] | F[2] | NL[3] | N[4] | SEM | Probability |
|---|---|---|---|---|---|---|
| 16:0 | 24.69$^{a}$ | 22.46$^{b}$ | 22.60$^{b}$ | 21.90$^{b}$ | 0.22 | 0.0001 |
| 16:1 ω-9 | 2.068$^{c}$ | 1.891$^{d}$ | 2.268$^{b}$ | 2.831$^{a}$ | 0.04 | 0.0001 |
| 18:0 | 9.546$^{a}$ | 9.453$^{a}$ | 9.544$^{a}$ | 8.706$^{b}$ | 0.16 | 0.0027 |
| 18:1ω-7 | 1.898$^{c}$ | 2.194$^{a}$ | 1.983$^{b}$ | 1.866$^{c}$ | 0.02 | 0.0001 |
| 18:1ω-9 | 42.12$^{a}$ | 42.44$^{a}$ | 35.14$^{c}$ | 37.59$^{b}$ | 0.59 | 0.0001 |
| 18:2ω-6 | 13.63$^{b}$ | 15.64$^{a}$ | 15.87$^{a}$ | 16.12$^{a}$ | 0.47 | 0.0052 |
| 18:3ω-3 | 0.806$^{c}$ | 0.875$^{c}$ | 6.288$^{a}$ | 5.265$^{b}$ | 0.14 | 0.0001 |
| 20:1ω-9 | 0.224$^{a}$ | 0.196$^{ab}$ | 0.163$^{c}$ | 0.179$^{bc}$ | 0.01 | 0.0001 |
| 20:3ω-6 | --- | --- | 0.142$^{a}$ | 0.218$^{a}$ | 0.03 | 0.0001 |
| 20:4ω-6 | 2.677$^{a}$ | 2.568$^{a}$ | 1.306$^{c}$ | 1.646$^{b}$ | 0.07 | 0.0001 |
| 20:5ω-3 | 0.064$^{b}$ | 0.341$^{ab}$ | 0.643$^{a}$ | 0.595$^{a}$ | 0.11 | 0.0051 |
| 22:5ω-3 | --- | --- | 0.291$^{a}$ | 0.405$^{a}$ | 0.03 | 0.0001 |
| 22:6ω-3 | 1.669$^{c}$ | 1.667$^{c}$ | 3.264$^{a}$ | 2.404$^{b}$ | 0.12 | 0.0001 |
| SFA[5] | 34.52$^{a}$ | 32.19$^{b}$ | 32.64$^{b}$ | 30.88$^{c}$ | 0.29 | 0.0001 |
| MUFA[6] | 46.31$^{a}$ | 46.73$^{a}$ | 39.55$^{c}$ | 42.47$^{b}$ | 0.59 | 0.0001 |
| PUFA[7] | 18.85$^{b}$ | 21.09$^{b}$ | 27.80$^{a}$ | 26.65$^{a}$ | 0.60 | 0.0001 |
| ω-3 PUFA | 2.54$^{c}$ | 2.88$^{c}$ | 10.49$^{a}$ | 8.67$^{b}$ | 0.20 | 0.0001 |
| ω-6 PUFA | 16.31$^{b}$ | 18.20$^{a}$ | 17.32$^{ab}$ | 17.98$^{ab}$ | 0.47 | 0.0435 |
| ω-3/ω-6 | 6.443$^{a}$ | 6.379$^{a}$ | 1.656$^{b}$ | 2.077$^{b}$ | 0.13 | 0.0001 |
| LC ω-3 PUFA[8] | 1.734$^{c}$ | 2.008$^{c}$ | 4.199$^{a}$ | 3.404$^{b}$ | 0.16 | 0.0001 |

SEM = standard error Mean. SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = polyunsaturated fatty acids.

[1]C= Common White Egg;

[2]F= Organically Grown Farm Egg;

[3]N= Omega-3 enriched Egg;

[4]NL= Multiple Enriched egg with Omega-3, Lutein and DHA (22:6 ω-3).

SFA[5] levels were calculated as 16:0+18:0.

MUFA[6] levels were calculated as 16:1+ 18:1ω-7+ 18:1 ω-9 + 20:1.

ω-3 levels were calculated as 18:3 ω-3+ 20:5 ω-3+ 22:5 ω-3+ 22:6 ω-3.

ω-6 levels were calculated as 18:2 ω-6+ 20:3 ω-6+ 20:4 ω-6.

PUFA[7] levels were calculated as 18:2 ω-6 + 18:3 ω-3 + 20:3 ω-6 + 20:4 ω-6 + 20:5 ω-3+ 22:5 ω-3+ 22:6 ω-3.

LC ω-3 PUFA[8] was calculated as C20:5 + C22:5 + C22:6.

Total fatty acid was calculated as SFA + MUFA + PUFA.

[a-c] Means within fatty acids and within egg type with no common superscript are significant different ($P<0.05$).

**Table A.2.2. The percentage composition of the fatty acids present in different table eggs[1] available in market.**

| Fatty Acids | C[1] | F[2] | NL[3] | N[4] | SEM | Probabilty |
|---|---|---|---|---|---|---|
| C16:0 | 50.49[a] | 57.75[a] | 57.69[a] | 50.19[a] | 2.82 | 0.0001 |
| C16:1 ω-9 | 4.226[c] | 4.836[bc] | 5.773[ab] | 6.493[a] | 0.26 | 0.0520 |
| C18:0 | 19.50[a] | 24.46[a] | 24.32[a] | 19.96[a] | 1.31 | 0.0550 |
| C18:1ω-7 | 3.884[c] | 5.677[a] | 5.060[ab] | 4.286[bc] | 0.29 | 0.0001 |
| C18:1ω-9 | 86.6[a] | 110.2[a] | 89.8[a] | 86.3[a] | 6.35 | 0.0020 |
| C18:2ω-6 | 27.84[b] | 40.12[a] | 40.36[a] | 36.99[a] | 2.02 | 0.0001 |
| C18:3ω-3 | 1.66[c] | 2.23[c] | 15.93[a] | 12.09[b] | 0.47 | 0.0001 |
| C20:1ω-9 | 0.457[a] | 0.511[a] | 0.417[a] | 0.411[a] | 0.04 | 0.0520 |
| C20:3ω-6 | --- | --- | 0.363[a] | 0.504[a] | 0.07 | 0.0300 |
| C20:4ω-6 | 5.413[b] | 6.607[a] | 3.331[c] | 3.763[c] | 0.23 | 0.0001 |
| C20:5ω-3 | 0.673[a] | 0.822[a] | 1.645[a] | 1.440[a] | 0.42 | 0.1100 |
| C22:5ω-3 | --- | --- | 0.742[a] | 0.926[a] | 0.08 | 0.0001 |
| C22:6ω-3 | 3.389[c] | 4.291[bc] | 8.338 | 5.478[b] | 0.38 | 0.0170 |
| SFA[5] | 70.57[a] | 82.93[a] | 83.28[a] | 70.77[a] | 4.11 | 0.0001 |
| MUFA[6] | 95.2[a] | 121.2[a] | 100.9[a] | 97.4[a] | 6.89 | 0.0440 |
| PUFA[7] | 38.98[c] | 54.08[b] | 70.71[a] | 61.20[ab] | 2.85 | 0.1820 |
| Total ω-3 PUFA[8] | 5.72[c] | 7.35[c] | 26.66[a] | 19.94[b] | 0.91 | 0.0010 |
| Total ω-6 PUFA[9] | 33.26[b] | 46.73[a] | 44.06[a] | 41.26[ab] | 2.16 | 0.0001 |
| Ratio ω-6:ω-3[10] | 5.984[a] | 6.379[a] | 1.656[b] | 2.077[b] | 0.26 | 0.0001 |
| LC ω-3 PUFA[11] | 4.062[c] | 5.113[c] | 10.73[a] | 7.851[b] | 0.60 | 0.0001 |
| Total Fatty Acid[12] | 204.7[b] | 258.2[a] | 254.9[ab] | 229.4[ab] | 13.01 | 0.3250 |

SEM = standard error Mean. SFA = saturated fatty acids; MUFA = monounsaturated fatty acids; PUFA = Polyunsaturated fatty acids.

[1]C= Common white egg.

[8]F= Organically grown farm egg.

[3]N= Omega-3 enriched egg.

[4]NL= Multiple enriched egg with Omega-3, Lutein and DHA (C22:6 ω-3).

[5]SFA levels were calculated as C16:0+C18:0.

[6]MUFA levels were calculated as C16:1ω-7 + C18:1ω-7+C18:1 ω-9 + C20:1ω-9.

[7]PUFA levels were calculated as C18:2 ω-6+ C18:3 ω-3 + C20:3 ω-6 + C20:4 ω-6 + C20:5 ω-3+C22:5 ω-3+C22:6 ω-3.

[8]Total ω-3 levels were calculated as C18:3 ω-3+C20:5 ω-3+C22:5 ω-3+C22:6 ω-3.

[9]Total ω-6 levels were calculated as C18:2 ω-6+C20:3 ω-6+ C20:4 ω-6.

[10]Ratio ω-6:ω-3 was calculated as Total ω-6 PUFA /Total ω-3 PUFA.

[11]LC ω-3 PUFA[8] was calculated as C20:5 ω-3+C22:5 ω-3+C22:6 ω-3.

[12]Total fatty acid was calculated as SFA + MUFA + PUFA.

[a-c] Means within fatty acids and within egg type with no common superscript are significant different ($P<0.05$).

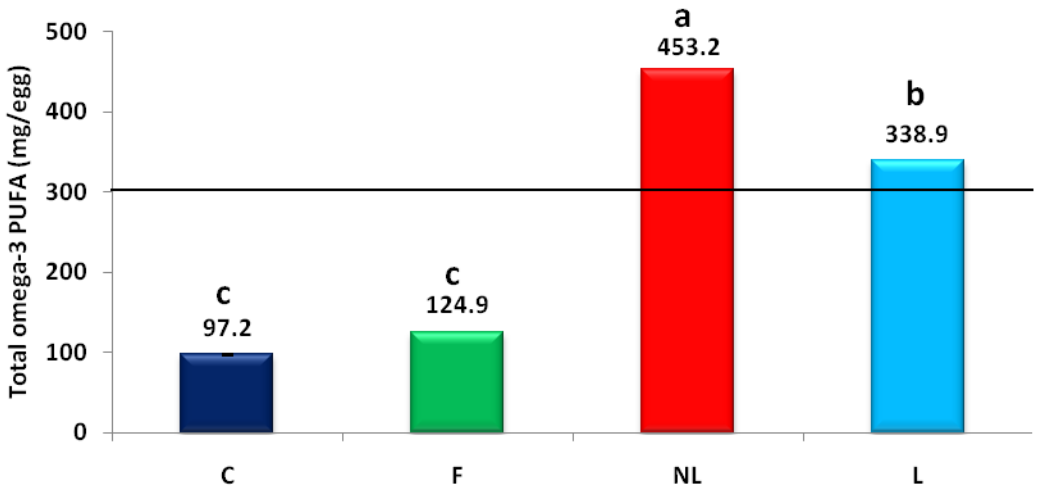


**Figure A.2.1: The graph showing the estimated amount of total Omega-3 in eggs available in the market based on an estimated yolk size of 17g per egg.The labels claims the amount of total Omega-3 to be 400 mg/50 g egg.**
**C= Common white table egg; F= Organically grown farm egg; N= Omega-3 enriched egg; NL= Multiple enriched egg with Omega-3 PUFA, lutein and DHA (C22:6ω-3).**
**a-c Means within egg types with no common superscript are significant different (P<0.05).**

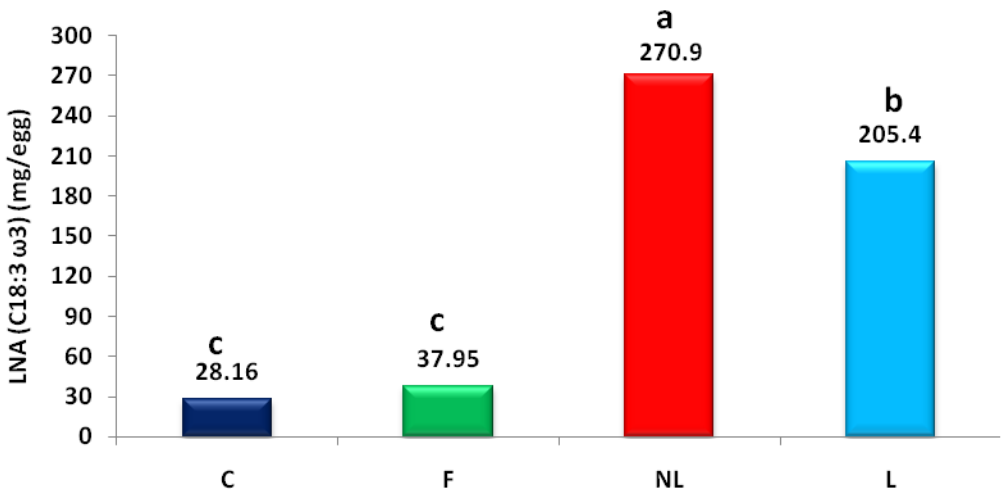


**Figure A.2.2: The estimated amount of LNA (C18:3 ω 3) in the eggs available in the market based on an estimated yolk size of 17g per egg. The enriched egg types NL and N had 8 fold higher amount of LNA than the C and F eggs.**
**C= Common white table egg; F= Organically grown farm egg; N= Omega-3 enriched egg; NL= Multiple enriched egg with Omega-3 PUFA, lutein and DHA (C22:6ω-3).**
**a-c Means within egg types with no common superscript are significant different (P<0.05).**

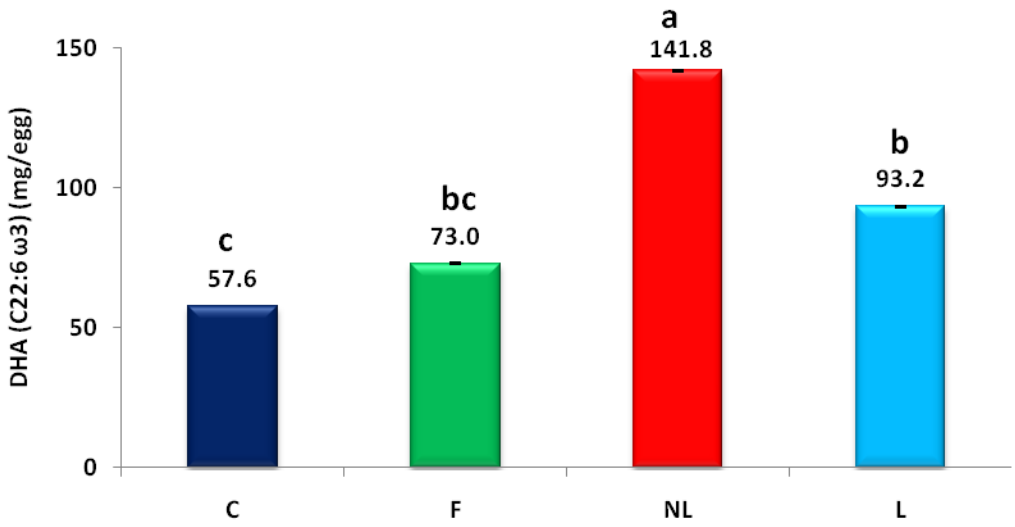


**Figure A.2.3: The estimated amount of DHA (C22:6 ω3) in eggs available in the market based on an estimated yolk size of 17g per egg. The labels claims the amount of DHA to be 75 mg and 125 mg in N and NL type eggs.**
**C= Common white table egg; F= Organically grown farm egg; N= Omega-3 enriched egg; NL= Multiple enriched egg with Omega-3 PUFA, lutein and DHA (C22:6ω-3).**
**a-c Means within egg types with no common superscript are significant different (P<0.05).**

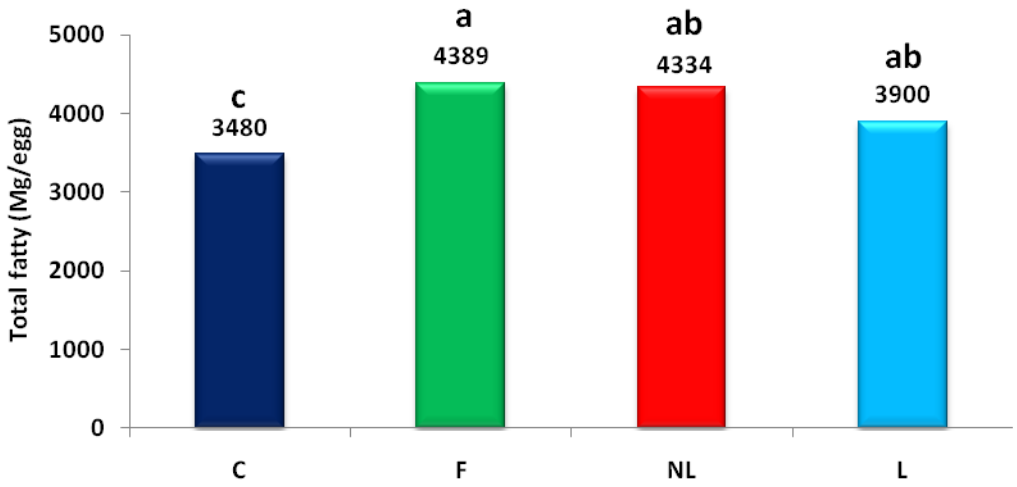


**Figure A.2.4: The graph estimated amount of total fatty acids in eggs available in market based on an estimated yolk size of 17g per egg.**
**C= Common white table egg; F= Organically grown farm egg; N= Omega-3 enriched egg; NL= Multiple enriched egg with Omega-3 PUFA, lutein and DHA (C22:6ω-3).**
**a-c Means within egg types with no common superscript are significant different (P<0.05).**

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