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**Production and health responses of laying hens and growing broilers to dietary omega-3 fatty acid supplementation**

by

**Isa J. Ehr**

A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Animal Science

Program of Study Committee:
Elizabeth Bobeck, Co-Major Professor
Hongwei Xin, Co-Major Professor
Anna K. Johnson
Brian Kerr

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University

Ames, Iowa

2017

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## DEDICATION

This dissertation is dedicated to my father, Gregory Ehr, who has taught me to always perform at the best of my abilities and to my wife, Kara Ehr, who encouraged me to never give up.

# TABLE OF CONTENTS

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## ABSTRACT

Omega-3 fatty acids, specifically alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) are known for possessing anti-inflammatory and bone modulating properties in murine and *in vitro* models. Health conscious consumers have created market demand for value-added omega-3 fatty acid enriched food, as demonstrated by the fact that specialty eggs make up 26.4% of the shell egg market. This has led producers to supplement poultry diets to create enriched eggs and meats and satisfy consumer demand. Thus far, omega-3 fatty acid research has primarily focused on creating value-added foods for the consumer with little attention paid to the effects of supplementation on the animals. The overarching goal of this dissertation research was to investigate the potential positive effects related to poultry health of dietary omega-3 fatty acid supplementation as a secondary benefit for producers. Two models were used to pursue the overarching goal: aged hens with mature immune systems at risk of osteoporosis and growing broilers with naïve immune systems prone to leg bone pathology. The experimental objectives of this dissertation were as follows: 1) Evaluate how dietary ALA sources affect the fatty acid transfer rate from laying hen diet to the egg yolk; 2) Determine if dietary supplementation of ALA or the combination of EPA and DHA improves performance and bone health of aged laying hens and whether protective anti-inflammatory effects would be exerted during acute inflammation; 3) Investigate if dietary supplementation of ALA or EPA and DHA impact bone health and performance in growing broilers, and 4) Examine the protective anti-inflammatory and bone modulating effects of ALA or EPA and DHA on broiler performance and health during a period of repeated inflammation.

To evaluate dietary ALA sources and their fatty acid transfer rate to egg yolk (Objective 1, Experiment 1), diets supplemented with flaxseed oil or ground flaxseed were fed to Hy-Line W-36 laying hens near peak production for 8 wk. Egg yolk fatty acid profiles were measured to determine the transfer rates for the ALA sources. To determine if ALA or EPA and DHA affect bone health and inflammatory status of aged hens (Objective 2, Experiment 2), near end-of-second-cycle laying hens (122 to 134 wk of age) were fed diets supplemented with flaxseed oil or fish oil for 12 wk, followed by an acute lipopolysaccharide (LPS) inflammatory challenge. During the 12 wk dietary supplementation period, feed intake (FI), egg production, and egg weight were measured; and eggs and tibia were measured for strength prior to the inflammatory challenge. Liver inflammatory gene expression was measured 12 h post-LPS challenge.

To investigate if ALA or EPA and DHA affect the bone health and performance of growing Ross 308 broilers (Objective 3, Experiment 3), diets supplemented with flaxseed oil or fish oil were fed for 28 consecutive d. FI, body weight (BW), breast yield, lameness, and bone mineral content (BMC) and density (BMD) were evaluated. Finally, to examine the protective anti-inflammatory effects of ALA or EPA and DHA on broiler performance and health (Objective 4, Experiment 4), the broilers from Experiment 3 were subjected to a 7 d repeated LPS inflammatory challenge, with the LPS dosage continually increasing by 20% every 48 h. Broiler BW and FI were measured from 35 to 42 d of age. Liver, muscle, and bone were measured for gene expression and composition.

Results of the first experiment showed that the laying hens deposited ALA into egg yolk at a rate of 1.9 times more when fed flaxseed oil compared to ground flaxseed and total omega-3 fatty acids (ALA, EPA, and DHA) at a rate of 2.0 times more at the same dietary

flaxseed oil concentrations, demonstrating the use of an extruded oil as a more efficient omega-3 fatty acid supplement than a ground seed ($P \leq 0.01$). It was also observed that it might take up to six wk for transfer efficiency to stabilize and plateau in laying hens. The results from Experiment 1 were used for predicting omega-3 fatty acid transfer to egg yolk in Experiment 2 and as the basis for using oil as an ingredient source and selecting timeframes for diet acclimation for the experiments. The second experiment revealed that feeding aged hens EPA and DHA reduced average daily FI by 0.8 ± 0.19 g/hen and egg weight by 2.39 ± 0.556 g compared to control hens ($P \leq 0.01$). Feeding hens ALA supplemented diets resulted in increased average daily FI by 1.2 ± 0.19 g/hen g compared to control hens ($P \leq 0.01$). Feeding dietary ALA or EPA and DHA did not affect keel bone conformation score, tibia bone ash, tibia breaking strength, or eggshell breaking strength in aged hens ($P \geq 0.13$). However, in response to the acute inflammatory challenge, feeding hens ALA or EPA and DHA resulted in intermediate gene expression levels of liver pro-inflammatory cytokines interleukin 1 beta (IL-1β), IL-6, and IL-18, and the adipogenic mediator peroxisome proliferator factor gamma as compared to the control hens ($P \leq 0.05$), demonstrating anti-inflammatory protection. Further testing was conducted using a broiler model with growing bones and developing immune system to determine if administration of ALA or EPA and DHA at an earlier age would result in health and performance benefits.

Results of the third experiment demonstrated that feeding broilers diets supplemented with ALA or EPA and DHA did not improve BMC ($P = 0.21$) or BMD ($P > 0.05$) compared to the control. However, ALA supplementation increased broiler BMD by 10% compared to EPA and DHA supplementation ($P \leq 0.05$), while control was intermediate. Feeding EPA and DHA decreased 34 d broiler breast muscle yield by 1.301 ± 0.3553% compared to the

control. Moreover, the broilers fed EPA and DHA decreased 34 d BW by 9% and increased feed conversion by 11% compared to the control and ALA supplement ($P \leq 0.05$). Results of the fourth experiment demonstrated that the repeated LPS challenge in broilers reduced 42 d BW by 4% and FI by 6% compared to saline injections ($P \leq 0.01$). Feeding EPA and DHA resulted in increased total lean tissue body composition in broilers by > 4% compared to the control and ALA supplemented diet ($P \leq 0.01$). A protective effect was observed in broilers fed EPA and DHA, resulting in reduced liver gene expression of pro-inflammatory cytokines IL-1β, IL-6, IL-18, and IFNγ by 0.38, 0.21, 0.62, and 0.62-fold, respectively, compared to the control ($P \leq 0.05$). Broilers fed EPA and DHA also experienced reduced liver gene expression of anti-inflammatory cytokine IL-10 by 0.41-fold compared to the control ($P \leq 0.01$). Feeding broilers ALA or EPA and DHA supplemented diets increased total BMC by ≥ 0.2 ± 0.03 g and BMC accretion by ≥ 3.625 ± 0.9263% compared to the control ($P \leq 0.01$).

Collectively, omega-3 fatty acid supplementation did not improve or mitigate performance losses during periods of inflammation regardless of poultry model. The only instance where supplementation demonstrated a benefit to bone health was at therapeutic levels (4.1% ALA or EPA and DHA) during repeated inflammation in growing broilers. However, therapeutic levels of oil (14% oil used in Experiments 3 and 4) may not be practical to use in commercial broiler diets due to the limitations of feed manufacturing. Anti-inflammatory effects were achieved in the aged laying hen and naïve broiler models, but actively suppressing the immune system may not necessarily equate to a benefit to animal health. Anti-inflammatory effects in poultry housed in research or clean environments with low bacterial loads would allow a shift in metabolic activity from the immune system to productive activity. Commercial poultry live in microbial laden environments and immune

suppression may allow opportunistic infections to occur, such as with Gram-positive bacteria or viruses; therefore anti-inflammatory effects would not be a benefit in these scenarios. Based on the experiments in this thesis, omega-3 fatty acid supplementation in poultry diets would not provide a practical secondary benefit for producers.

# CHAPTER 1. LITERATURE REVIEW

## 1.1 Introduction

Consumers concerned about personal health have created demand for value-added omega-3 fatty acid enriched eggs and meats, with the US egg market consisting of 26.4% specialty eggs (USDA, 2017b). The omega-3 fatty acids alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) possess anti-inflammatory properties and modulate bone mineralization *in vitro* and in murine models (Kruger et al., 2010; Kajarabille et al., 2013; Longo and Ward, 2016). Supplementing poultry diets with functional ingredients, such as omega-3 fatty acids, generates value-added eggs and meats for these health conscious consumers (Fraeye et al., 2012; Ribeiro et al., 2013). Thus far, omega-3 fatty acid research has primarily focused on creating value-added foods for the consumer (Fraeye et al., 2012; Goyal et al., 2014); however; dietary omega-3 fatty acid supplementation in poultry diets may be serving an overlooked second purpose.

Physical stress and bacterial challenges in commercial environments may compromise the immune system and overall health of laying hens (Lay et al., 2011; Zhao et al., 2016). Furthermore, laying hens have been genetically selected over the past 50 years for improved egg production and quality, which has contributed to increased risk of keel bone fractures and osteoporosis in commercial environments (Wilkins et al., 2011; Stratmann et al., 2016). Broilers have a naïve immune system due to their young age and are vulnerable to opportunistic infections during their short and demanding production cycle (Saif et al., 2008; Siegel, 2014). Broiler chickens have been genetically selected for rapid growth and muscle accretion, which has contributed to lameness and leg bone pathology commonly observed in

commercial flocks (Knowles et al., 2008; Grupioni et al., 2015). Therefore, two models were used to investigate dietary omega-3 fatty acid supplementation health effects on poultry in this dissertation research: aged hens with mature immune systems at risk of osteoporosis and growing broilers with naïve immune systems prone to leg bone pathology. Omega-3 fatty acid supplementation in poultry diets, used by producers to make value-added products, may be providing beneficial health effects to aged laying hens and growing broilers under production stress.

## 1.2 Omega-3 Fatty Acids

Polyunsaturated fatty acids are composed of a carboxyl group, the alpha terminus, attached to a carbon chain with a methyl end, identified as the omega terminus. Omega-3 fatty acids are part of the long chain polyunsaturated fatty acid family, characterized by the presence of more than two double bonds in an 18 to 22 carbon length tail. Omega-3 fatty acids contain an unsaturated double bond between the carbon 3 and 4 from the omega terminus (Scorletti and Byrne, 2013). Alpha-linolenic acid (ALA) is an essential omega-3 fatty acid, 18 carbons in length containing 3 double bonds, that cannot be synthesized endogenously by mammals and birds and must be consumed in order to maintain biological homeostasis because they lack the Δ-15 desaturase enzyme, which converts omega-6 fatty acid linoleic acid to omega-3 fatty acid ALA (Scorletti and Byrne, 2013). Omega-3 fatty acids are required for normal brain development, neural and immunologic cell signaling, cardiovascular regulation, and bone mineral deposition and resorption (Goyal et al., 2014; Hou et al., 2015; Kim et al., 2017).

Alpha-linolenic acid can be converted to longer chain omega-3 fatty acids EPA (20 carbon chain containing 5 double bonds) and DHA (22 carbon chain containing 6 double bonds) via liver hepatocytes (Figure 1.1), although the conversion rate is limited in mammal and avian species due to ALA oversaturation of the desaturase and elongase enzymes

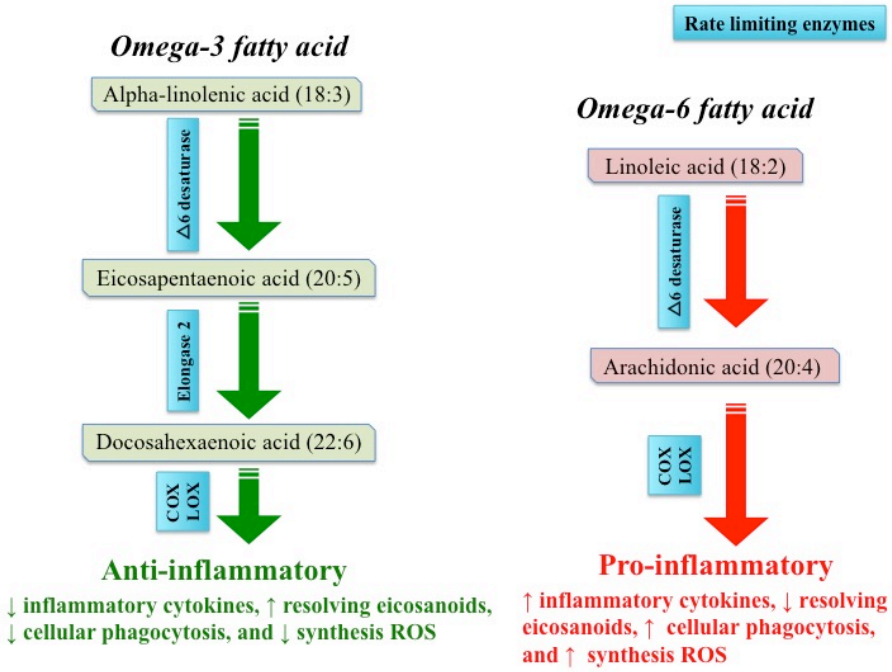


**Figure 1.1.** Fatty acid cellular metabolism of omega-3 alpha-linolenic acid and omega-6 linoleic acid by rate limiting enzymes to modulate respective anti- and pro-inflammatory pathways.

(Zivkovic et al., 2011). On average, mammal and avian species convert 5% of dietary ALA to EPA and < 1% of dietary ALA to DHA (Burdge and Calder, 2006; Zivkovic et al., 2011). In a murine model, tissue deposition of DHA remained relatively constant when fed EPA or DHA included at ≤ 0.25% of the diet (Sierra et al., 2008). Due to the limited endogenous conversion of ALA to EPA and DHA, the two longer chain omega-3 fatty acids are considered conditionally essential because normal cognitive functions require DHA along with fetal neurologic development (Horrocks and Yeo, 1999). Although recognized as essential or conditionally essential, no dietary requirements have been established for ALA,

EPA, or DHA for laying hens or broilers (Tinoco, 1982; NRC, 1994; Ross, 2007; Hy-Line, 2012).

Cell membranes throughout the body contain EPA and DHA, which influence membrane receptor organization within microdomains known as lipid rafts composed of glycolipoproteins and cell signaling molecules (Hou et al., 2015). Membrane EPA and DHA content influences membrane fluidity, which impacts cell signaling, activation, and differentiation of immune B and T cells (Hou et al., 2015). A diet containing 10.5% ALA, 3% EPA, and 2% DHA fed to mice resulted in disruption of B cell lipid raft clustering and size by 40% compared to control-fed mice ($P \leq 0.01$), demonstrating omega-3 fatty acid influence on distribution of membrane receptors located within the lipid raft microdomains (Rockett et al., 2011). It is possible that reduced lipid raft clustering and size could prevent protein recruitment into lipid rafts from non-raft regions, such as Toll-like receptor (TLR) dimerization, due to increased lipid raft spatial distribution across the membrane resulting in reduced inflammatory signaling (Rockett et al., 2011).

Membrane incorporated EPA and DHA also function as precursors for lipid-mediated anti-inflammatory signaling eicosanoids metabolized by cyclooxygenase and lipoxygenase which include prostaglandins, prostacyclins, thromboxanes, and leukotrienes (Scorletti and Byrne, 2013; Longo and Ward, 2016). Eicosanoid metabolites that resolve inflammation could not be synthesized without EPA and DHA found in the cell membrane.

Omega-3 fatty acids EPA and DHA may modulate immune system function in various ways. For example, it has been suggested that they serve an anti-inflammatory role by displacing pro-inflammatory omega-6 fatty acid arachidonic acid, which is responsible for producing pro-inflammatory cytokines including interleukin-1 (IL-1) and tumor necrosis

factor (de Pablo et al., 2002). The omega-3 fatty acids EPA and DHA are also thought to modulate inflammation through lipid peroxide production where reactive oxygen species are neutralized by oxidizing the unsaturated bonds or by inhibiting translocation of transcription factors such as nuclear factor kappa B (NF-κB) reducing pro-inflammatory stimulation (Figure 1.2; de Pablo et al., 2002; Hichami et al., 2016).

The anti-inflammatory functional properties of EPA and DHA contribute to consumer demand for niche market omega-3 fatty acid enriched poultry products (Fraeye et al., 2012;

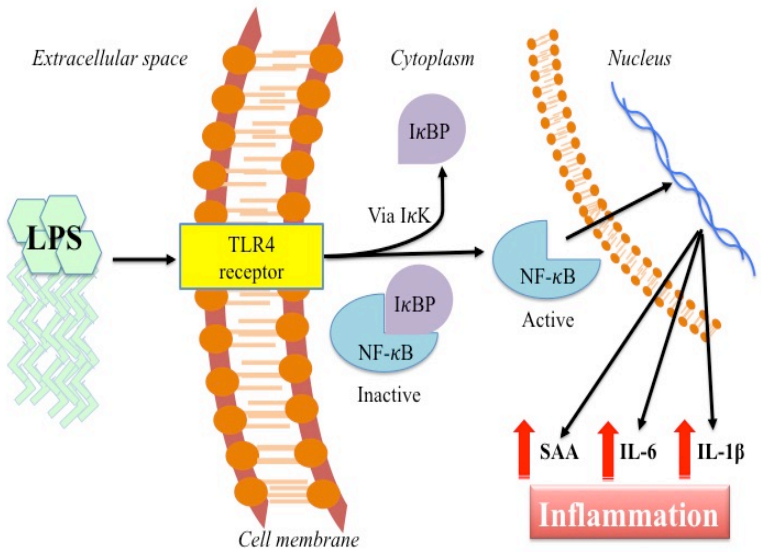


**Figure 1.2.** Lipopolysaccharide (LPS) ligand complex stimulation of cell membrane Toll-like Receptor 4 (TLR4) and Nuclear Factor kappa B (NF-κB) activation by removal of Inhibiting NF-κB Binding Protein (IκBP), which allows NF-κB translocation from the cell cytoplasm into the nucleus for pro-inflammatory cytokine transcription and translation of interleukin 1β (IL-1β), IL-6 and acute phase protein serum amyloid A (SAA).

Ribeiro et al., 2013). Human epidemiological studies and randomized clinical trials associated EPA and DHA consumption with reduced risk of coronary heart disease and sudden cardiac death (Mozaffarian and Wu, 2011). Dietary recommendations from USDA and US Department of Health and Human Services (2010) suggests ≥ 250 mg consumption of EPA and DHA per day. Other human studies indicate that dietary EPA and DHA are linked to increased bone formation, peak bone mass, and reduced bone loss with the

mechanisms connected to the receptor activator of NF-κB which controls osteoclast formation (Kajarabille et al., 2013). Attenuation of inflammation through omega-3 fatty acid dietary manipulation (ALA, EPA, and DHA) may benefit human and animal well-being, warranting further investigation into digestion, transfer, metabolic, and immunologic effects.

### 1.3 Dietary Omega-3 Fatty Acid Sources

Alpha-linolenic acid is naturally produced by terrestrial plants, such as flax, that are used as ALA sources for enriching human and animal diets (Goyal et al., 2014). Terrestrial plants do not produce or only accumulate trace amounts of EPA and DHA (Fraeye et al., 2012). Conversely, fish meal and marine oils contain primarily EPA and DHA with trace amounts of ALA (Fraeye et al., 2012; Lemahieu et al., 2013). This is because EPA and DHA are produced by marine and heterotrophic microalgae and accumulate in the tissue of fish that consume prey that feed on algae (Table 1.1; Fraeye et al., 2012).

The choice of omega-3 fatty acid source and how that ingredient source has been processed directly affects the fatty acid profile and may potentially affect the bioavailability of the fatty acids used for dietary supplementation (Gunstone, 2006; Fraeye et al., 2012). Plant sources containing ALA can be ground or milled into a meal and used as an ingredient for supplementing poultry diets. Alternatively, the concentrated oil from seeds and other plant components can be mechanically extracted by pressing and filtering to create cold pressed oil. Solvents can also be used to separate the oil from plant components, or a combination of mechanical and solvent extraction techniques can be applied to maximize oil yield (Gunstone, 2006).

Marine heterotrophic microalgae containing DHA can be fed relatively unaltered as a meal ingredient, whereas marine fish sources containing differing concentrations of EPA and DHA are processed to make oils (Gunstone, 2006; Fraeye et al., 2012). Marine fish are processed in a steam cooker at 100°C where the co-product of water, oil, and fine solids are diverted into a decanter which removes the solid particles (Gunstone, 2006). The water and oil mixture is centrifuged to separate the water fraction from the remaining oil (Gunstone, 2006). Ground meal and oil ingredients containing omega-3 fatty acids are available for poultry diet supplementation (Table 1.1; Fraeye et al., 2012; Lemahieu et al., 2013). Omega-3 fatty acid ingredients may differ in ALA, EPA, and DHA compositions based on the raw starting material and whether centrifugation or ingredient blending was involved during processing (Fraeye et al., 2012; Lemahieu et al., 2013).

**Table 1.1.** Ingredient omega-3 fatty acid composition.[1]

| Ingredient | ALA % | EPA % | DHA % |
|---|---|---|---|
| **Palm oil** | 0.2 | 0.0 | 0.0 |
| **Soybean oil**[2] | 6.5 | 0.0 | 0.0 |
| **Canola oil** | 9.1 | 0.0 | 0.0 |
| **Flaxseed oil**[3] | 53.4 | 0.0 | 0.0 |
| **Flaxseed meal** | 22.8 | 0.0 | 0.0 |
| **Menhaden fish oil** | 1.5 | 13.2 | 8.6 |
| **Menhaden fish meal**[4] | 0.1 | 1.1 | 0.7 |

[1]Adapted from USDA (2017a) Food Composition Databases, National Agricultural Library on an as-fed basis. ALA = alpha-linolenic acid; EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid.
[2]Non-hydrogenated.
[3]Cold pressed.
[4]Containing 8% fat.

Milling processes may also affect energy availability of poultry diets containing omega-3 fatty acid ingredients. Flaxseed supplemented diets with inclusions of 0, 5, 10, 15,

and 20% ground flaxseed fed to 79 week old roosters resulted in increased nitrogen corrected apparent metabolizable energy ($AME_n$) when processed in pelleted (4,578 kcal/kg) or crumbled (4,277 kcal/kg) forms when compared to mash (3,659 kcal/kg) diets ($P \leq 0.01$; Gonzalez-Esquerra and Leeson, 2000b). In addition, the heat and pressure from processing may have released trapped oil within the flaxseed cellular matrix and degraded some of the anti-nutrients that flaxseed contain (Calet, 1965; Gonzalez-Esquerra and Leeson, 2000b). Omega-3 fatty acid source and processing techniques are important factors of consideration when using omega-3 fatty acid supplementation in poultry diets.

### 1.3.1 Flaxseed Supplementation

Flaxseed, also known as linseed, is used by producers in the US as a functional feed ingredient for supplementing poultry diets with ALA. Whole flaxseed, commonly added to diets in a ground meal form, contains up to 45% fat, with 60% of the fat composition consisting of ALA and 15% omega-6 linoleic acid (Kratzer and Vohra, 1996; USDA, 2017a). Poultry eggs and meat can be enriched with ALA by supplementing ground flaxseed meal into the diets (Fraeye et al., 2012; Lopes et al., 2013).

Different sources of dietary ALA supplementation yield varying total omega-3 fatty acid incorporation into egg yolk. Without ALA supplementation in laying hen diets, a sample of 48 eggs obtained from retail outlets contained an average of 68 mg (combined ALA and DHA) per 50 g egg from conventionally managed laying hens (Samman et al., 2009). In a study investigating ALA sources fed to laying hens for 24 weeks, use of 15% dietary canola seed yielded 173 mg total omega-3 fatty acids (93 mg ALA) per 50 g egg while 15% dietary flaxseed-pea mixtures (1:1 wt/wt, ground-extruded) resulted in increased ALA deposition

averaging 355 mg total omega-3 fatty acids (254 mg ALA) per 50 g egg ($P \leq 0.01$; Jia et al., 2008). Dietary inclusion of 15% flaxseed resulted in 468 mg total omega-3 fatty acids (358 mg ALA) per 50 g egg, demonstrating the increased ALA enrichment potential of dietary flaxseed compared to the alternative supplement ingredients ($P \leq 0.01$; Jia et al., 2008). However, hens fed canola seed diets consumed 6% less feed and produced 5% more eggs, resulting in 10% improvement in feed conversion compared to hens fed flaxseed diets ($P \leq 0.01$), suggesting that diets containing 15% flaxseed may significantly reduce performance (Jia et al., 2008).

Flaxseed varieties may differ in their anti-nutrient content of cyanogenic glycosides, phytic acid, trypsin inhibitor, and gel-forming non-starch polysaccharides (Feng et al., 2003; Jia and Slominski, 2010; Goyal et al., 2014). Flaxseed supplementation may reduce the palatability of poultry diets and the presence of anti-nutrients suggests a biological limit for ration incorporation to prevent negative effects on performance and feed efficiency. One anti-nutrient factor is hydrogen cyanide, which non-competitively inhibits cellular respiration and is generated as a metabolic product from cyanogenic glycosides, which includes linustatin, neolinustatin, and linmarin (Oomah et al., 1992; Feng et al., 2003). Phytic acid is another anti-nutrient that acts as chelator, forming complexes from ions and molecules bonding to metal ions such as calcium, iron, or zinc in the digesta interfering with mineral absorption, reducing bioavailability (Erdman, 1979). Trypsin inhibitors, common in soybeans, reduce feed digestibility and are less concerning in flaxseed because they are only found in trace amounts (Bhatty, 1993; Feng et al., 2003). Mucilage is a water-soluble non-starch polysaccharide common in flaxseeds and causes increased viscosity of chicken intestinal content (Rodriguez et al., 2001; Alzueta et al., 2003). When intestinal viscosity is

increased, nutrient digestion and absorption may become impaired, thereby reducing nutrient utilization (Rodriguez et al., 2001; Alzueta et al., 2003). Although ground flaxseed may be used as an ALA supplement to produce value-added eggs and meat, it may have limitations as a functional ingredient depending on anti-nutrient content.

Dietary inclusion of flaxseed at ≤ 15%, ground or whole, did not affect hen-day egg production (HDEP) in 43 week old laying hens. However, hen body weight and egg weight were reduced in the flaxseed treatments when contrasted to the control ($P \leq 0.05$; Scheideler and Froning, 1996). The conclusions of this study regarding performance were only based on 4 experimental units, consisting of 3 hens each, fed for 8 weeks, which might have been too short in duration to detect the treatment effect on HDEP, plus the relatively low number of replicates (Scheideler and Froning, 1996).

In a 48 week experiment, feeding diets containing 20% ground flaxseed to laying hens resulted in reduced egg production by 5% and increased feed consumption by 7% compared to feeding diets containing 0 and 10% flaxseed ($P \leq 0.01$; Leeson et al., 2000). Even with increased feed consumption, the hens fed 20% flaxseed diets weighed 10% less than hens fed diets containing 0 and 10% flaxseed ($P \leq 0.01$), which might be due to anti-nutrients contained in the flaxseeds (Leeson et al., 2000).

Consumer preference also needs to be taken into consideration when evaluating dietary flaxseed supplementation and poultry performance. Sensory panels from multiple experiments detected unacceptable “fishy” flavors of value-added eggs from hens fed diets containing > 10% ground flaxseed (Leeson et al., 1998; Fraeye et al., 2012; Goyal et al., 2014). Thus, from the standpoints of performance and consumer acceptability, laying hen diets should not include > 10% ground flaxseed.

Age and breed are factors that affect how well poultry are able to digest flaxseed when included at > 10% in diets. It was observed that 81 week old, single comb white leghorn roosters fed 10% ground flaxseed diets did not have adverse reactions to dietary flaxseed inclusion (Gonzalez-Esquerra and Leeson, 2000b). In the same experiment, 9 day old broiler chicks were fed diets supplemented with 5, 10, 15, and 20% ground flaxseed and experienced diarrhea, demonstrating sensitivity to increased dietary flaxseed inclusion due to gastrointestinal immaturity and limited digestive capacity (Gonzalez-Esquerra and Leeson, 2000b). It appears that ground flaxseed can be included up to 10% in laying hen diets without performance losses; however, broilers appear sensitive to ground flaxseed even at levels below 10% of the diet (Gonzalez-Esquerra and Leeson, 2000b).

Extracted oils may be an efficient alternative to ground meal products for ALA supplementation because they do not possess anti-nutrients yet contain concentrated amounts of desired fatty acids. Compared to soybean and corn oils, flaxseed oil contains 7 times more ALA content with 3 times less linoleic acid content (NRC, 1994; Goyal et al., 2014). Seed cell wall matrix, structural components within flaxseed, remains after processing and trap portions of the lipid fraction as observed with other seed types, such as almonds (Ellis et al., 2004; Mandalari et al., 2008). Portions of oil contained within seed cell compartments, even after mastication or mechanical grinding, are not entirely liberated and pass through the gastrointestinal tract undigested preventing the oil from being absorbed and used for fatty acid transfer to tissue or egg yolk (Cassady et al., 2009). Theoretically, fatty acid transfer rate efficiency would improve if unimpeded by cellular compartmentalization.

Dietary ALA supplementation in laying hen diets yields omega-3 fatty acid (ALA, EPA, and DHA) enriched egg yolks (Scheideler and Froning, 1996; Samman et al., 2009).

However, fatty acid transfer from diet to egg yolk depends on source and dietary inclusion level. Laying hens fed 16% flaxseed diets produced eggs containing 1.4 times higher relative egg yolk total omega-3 fatty acid content compared with egg yolk from hens fed 8% flaxseed and 6.6 times higher compared to unsupplemented control hens ($P \leq 0.05$; Cherian and Sim, 1991). In another experiment, laying hens were fed 0, 7.5, and 15% ALA supplemented diets (50% extruded flaxseed and 50% alfalfa-pea carrier) for 18 d; and the dietary total omega-3 fatty acid (ALA, EPA, and DHA) transfer efficiency to egg yolk was shown to reduce by 33% when comparing the 15% treatment to control ($P \leq 0.01$; Nain et al., 2012). Broken stick analysis was used to determine dietary omega-3 fatty acid transfer efficiency to egg yolk, a linear pattern was observed prior to reaching saturation (Nain et al., 2012). The reduced transfer efficiency of the 15% treatment (22.2% transfer efficiency) compared to control (55.6% transfer efficiency) demonstrated that as dietary ALA inclusion increases, then total omega-3 fatty acid transfer efficiency to egg yolk decreases due to down regulation of enzymatic activity ($P \leq 0.01$; Nain et al., 2012). Eggs from the 15% treatment reached a plateau of total omega-3 fatty acid content within 6.2 days, achieved the target threshold of 300 mg total omega-3 fatty acid egg yolk content within 5 days, and attained blood plasma saturation within 7.2 days ($P \leq 0.01$; Nain et al., 2012). It appeared that peak transfer efficiency for laying hens required a week of diet acclimation when feeding ALA supplemented diets using ground-extruded flaxseed and pea meal. Further characterization regarding omega-3 fatty acid transfer rate in relation to oil inclusion level would be warranted for future industry practical and research applications.

### 1.3.2 Fish Supplementation

Ground fish meal and fish oils can be included in poultry diets to enhance the concentrations of EPA and DHA found in poultry eggs and meat (Hargis et al., 1991; Nash et al., 1996; Gonzalez-Esquerra and Leeson, 2000a). Fish meal contains 5 to 10% fat and 60 to 70% protein depending on the type of fish used in processing (Koning, 2005). Similar to flaxseed meal, the omega-3 fatty acids reside in the fat fraction of fish meal. Unlike ground flaxseed meal, fish meal does not contain the anti-nutrients of concern that may impair digestion, absorption, and utilization of a diet (Kumar et al., 2012). As described earlier, flaxseed-based ingredients contain ALA with trace amounts of EPA and DHA (Fraeye et al., 2012; Goyal et al., 2014). Fish oil differs from flaxseed oil because it contains up to 35% EPA and DHA with little to no ALA in its fatty acid profile (Fraeye et al., 2012; Goyal et al., 2014). Manufacturers distribute a wide range of fish oils containing < 4% ALA, 18% EPA, and 12% DHA, while a variety of custom concentrated or blended fish oils may contain > 70% DHA (Koning, 2005; Gunstone, 2006).

Plant derived ALA can be converted into EPA and DHA through rate limiting elongation, desaturation, and beta-oxidative reactions by most mammals and birds (Figure 1.1), but direct supplementation forgoes the post-absorptive modifications (Harris et al., 2008; Gregory et al., 2011; Gregory et al., 2013). Broiler chickens are able to endogenously elongate and desaturate ALA to the longer chain EPA and DHA forms in a quadratic curvilinear manner (Kartikasari et al., 2012). It was observed that broiler breast muscle tissue became saturated with EPA and DHA as dietary flaxseed oil supplementation approached 8% ($P < 0.01$; Kartikasari et al., 2012). However, direct dietary EPA and DHA supplementation,

rather than ALA supplementation, is the most efficient method to enrich muscle tissue with EPA and DHA (Ribeiro et al., 2013).

Ingredient fatty acid composition has a direct effect on fat deposition and utilization by poultry. Monogastric species limit fatty acid structural alteration post-absorptively, unlike ruminants that modify lipids via ruminal microbial enzymatic activity (Hurtaud et al., 2013; Haug et al., 2014). During post-absorptive metabolism, ALA is stored in adipocyte lipid droplets added to triglycerides for long-term energy storage (Cherian and Sim, 1991). Longer chain omega-3 fatty acids EPA and DHA, found in fish oils, are almost exclusively deposited for storage in phospholipid form, particularly phosphatidylethanolamine in egg yolk (Jiang et al., 1991). Dietary supplementation of EPA and DHA sources into laying hen rations generate value-added eggs in a similar manner as ALA supplementation.

Dietary fish oil supplementation in laying hen diets is a practical way to deposit omega-3 fatty acids into egg yolks. Dietary fish oil inclusion of 3.0% has been shown to increase egg yolk total omega-3 fatty acid content by 400% when compared to egg yolk from unsupplemented hens (Van Elswyk, 1997). In the experiment, Single Comb White Leghorn laying hens, 27 hens per treatment, were fed diets containing 0, 0.5, 1.0, 1.5, 2.0, 2.5, or 3.0% fish oil for 4 weeks and egg yolk (10 yolks per treatment) from each treatment was analyzed for total omega-3 fatty acid content weekly (Van Elswyk, 1997). Egg yolks from the 3.0% fish oil treatment reached omega-3 fatty acid saturation after 3 weeks of supplementation, containing 234 mg of total omega-3 fatty acids compared to control egg yolk which contained 45 mg total omega-3 fatty acids (Van Elswyk, 1997). However, EPA and DHA content and omega-3 fatty acid regression analysis were not reported. The study verified that

dietary fish oil supplementation was able to increase egg yolk total omega-3 fatty acid content.

Broiler meat can be directly enriched with EPA and DHA through dietary fish oil supplementation. It has been demonstrated that feeding 8% fish oil supplementation in broiler diets resulted in 450% increase in broiler breast tissue EPA and DHA content compared to unsupplemented control broiler breast tissue ($P \leq 0.05$; Rymer and Givens, 2010). In an experiment, Ross 308 broilers were fed either 0, 4, or 8% fish oil supplemented diets from 21 to 42 days of age using 4 pens per treatment with 5 broilers per experimental unit (Rymer and Givens, 2010). Skinless breast tissue from each treatment was analyzed for fatty acid composition at 42 days of age (Rymer and Givens, 2010). Breast tissue from broilers fed 4% fish oil supplemented diet contained 504 mg EPA and 1071 mg DHA per kg breast tissue and breast tissue from broilers fed 8% fish oil supplemented diets contained an average of 545 mg EPA and 1363 mg DHA per kg breast tissue (Rymer and Givens, 2010). The pattern of increasing EPA and DHA deposition from Rymer and Givens (2010) broiler breast tissue analysis was in agreement with results from Saleh et al. (2010) who used 0, 1.5, 3.0, and 6.0% dietary fish oil treatments and Navidshad et al. (2012) who used 0, 2, and 4% dietary fish oil treatments in broiler experiments. Samples from the unsupplemented control group only contained 164 mg EPA and 259 mg DHA per kg breast tissue, demonstrating the effectiveness of dietary fish oil as an ingredient for enriching broiler breast tissue with EPA and DHA ($P \leq 0.05$; Rymer and Givens, 2010).

Dietary fish oil also influences how fatty acids are incorporated into tissues throughout the body (Newman et al., 2002a; Newman et al., 2002b). Fatty acid tissue incorporation was determined in a broiler study using dietary treatments containing 8% fish

oil, 8% sunflower oil, or 8% tallow fed from 21 to 35 days of age (Newman et al., 2002b). It was discovered that feeding 8% fish oil and 8% sunflower oil treatments to broilers resulted in an average reduction of abdominal fat pad weight by 59% and compared to broilers fed the 8% tallow treatment ($P \leq 0.01$; Newman et al., 2002b). The 8% fish oil and 8% sunflower oil treatments fed to broilers resulted in an average increase in breast muscle:fat pad ratio by 180% compared to broilers fed the 8% tallow treatment ($P \leq 0.01$; Newman et al., 2002b). This study demonstrated that diets high in polyunsaturated fatty acids shift fatty acid incorporation into lean muscle and away from adipose tissue (Newman et al., 2002b).

A follow up study using the same broiler tissue confirmed that feeding the 8% fish oil treatments to broilers increased EPA and DHA deposition in muscle tissue compared to broilers fed the 8% sunflower oil and 8% tallow treatments ($P \leq 0.05$, Newman et al., 2002a). When analyzing the phospholipid content of the muscle tissue, broilers fed the 8% fish oil treatment increased EPA and DHA incorporation into phosphatidylchoine and phosphatidylethanolamine and decreased arachidonic acid phospholipid incorporation compared to the other two treatment groups ($P \leq 0.05$, Newman et al., 2002a). These findings were in agreement with Carragher et al. (2016) demonstrating EPA and DHA selective phospholipid remodeling and incorporation in broiler muscle tissues, which fundamentally alter cell membrane composition (Newman et al., 2002a).

Consumer palatability is a factor that must be considered when using fish meal or fish oil to generate value-added produce, because EPA and DHA are highly volatile polyunsaturated fatty acids that may go rancid. Sensory panels were able to detect and taste unpleasant flavors in EPA and DHA enriched eggs from laying hens fed 4 to 8% fish meal supplemented diets (Nash et al., 1996). A sensory panel from another study detected

unpleasant flavors from enriched eggs from hens fed 2 and 4% fish oil supplemented diets (Gonzalez-Esquerra and Leeson, 2000a). The flavor of value-added broiler meat may also become compromised. A study found that 23% of sensory panelists were able to detect "fishy taint" in meat from broilers fed diets containing 4 and 8% fish oil (Rymer and Givens, 2010). These findings suggest fish oil supplementation may result in off flavors when approaching 4% in poultry diets. Further sensory evaluations should be conducted in value-added industry applications to prevent production of unpalatable produce that may be rejected by consumers.

## 1.4 Omega-3 Fatty Acid Functions

### 1.4.1 Influence on Inflammation

Omega-3 fatty acids EPA and DHA are known to influence the immune system in an anti-inflammatory manner through modulation of lymphocyte proliferation, cytokine synthesis, natural killer cell activity, and mononuclear phagocytosis (de Pablo et al., 2000; de Pablo et al., 2002). Pre-clinical studies using human and rodent models have shown that EPA and DHA perform anti-inflammatory functions that are associated with reducing circulating plasma C-reactive protein, an acute phase protein produced by the liver in response to inflammation and reduction of pro-inflammatory cytokine IL-6 (Cottin et al., 2011; Skulas-Ray, 2015). However, C-reactive protein levels were not reduced *in vivo* compared to *in vitro* models (Cottin et al., 2011; Skulas-Ray, 2015). The scientific knowledgebase would benefit from further *in vivo* evaluation of omega-3 fatty acid mode of action, specifically in chicken models, during periods of inflammation based on omega-3 fatty acid form (ALA, EPA, and DHA).

***Cytokine Modulation***

Omega-3 fatty acids EPA and DHA play a direct role in cell function and signaling by altering synthesis of cytokines produced by a range of immune cells (de Pablo et al., 2002). Pro-inflammatory cytokines IL-1β, IL-6, IL-18, and interferon gamma (IFNγ) are increased during infection and acute inflammatory responses in avian species (Staeheli et al., 2001). During an infection, activated macrophages produce IL-1β, which has pleiotropic functions including initiating the acute phase response, fever induction, T cell recruitment, IL-6 synthesis, and synergistically co-stimulates lymphocytes with IL-12 (Dinarello, 1998; Tominaga et al., 2000). Natural killer cells and $T_H1$ lymphocytes stimulated by IL-18 produce IFNγ (Wawrocki et al., 2016). Circulating extracellular IFNγ is a major macrophage activating factor and drives the $T_H1$ immune response, characterized by bactericidal and virucidal clearance by macrophages and stimulated B cell antibody production (Irons and Fritsche, 2005; Spits et al., 2016). IL-18 also serves to enhance natural killer and CD8 T cell cytotoxic activity and promotes $T_H1$ and $T_H2$ differentiation (Staeheli et al., 2001; Liu et al., 2006). Cytokine modulation or suppression via EPA or DHA would reduce activation and response of phagocytic immune cells such as monocytes and macrophages.

IL-6 is critical in development of pathological pain and is one of the primary regulators of the acute phase inflammatory response in avian species along with IL- 1β (Zhou et al., 2016). During active infections or cellular trauma, monocytes, T and B lymphocytes, endothelial cells, and fibroblasts produce IL-6 (Staeheli et al., 2001). In addition to inducing the acute phase inflammatory response, IL-6 serves a multifunctional role including regulating immunoglobulin synthesis and initiating T lymphocyte activation for pathogen

clearance (Staeheli et al., 2001; Abdelsalam et al., 2011). Reduced IL-6 production in birds, influenced by dietary EPA and DHA, may result in shortened or less intense acute phase response.

One mechanism of how EPA and DHA influence cell signaling is by acting as intracellular second messengers. Fatty acids released from membrane phospholipids function as cellular second messengers or substitute for classical second messengers modulating cellular gene expression (de Pablo and de Cienfuegos, 2000). Fatty acids, such as the omega-6 fatty acid arachidonic acid, act in a reversible manner for short durations intracellularly at the level of cell membranes (Sumida et al., 1993; de Pablo and de Cienfuegos, 2000).

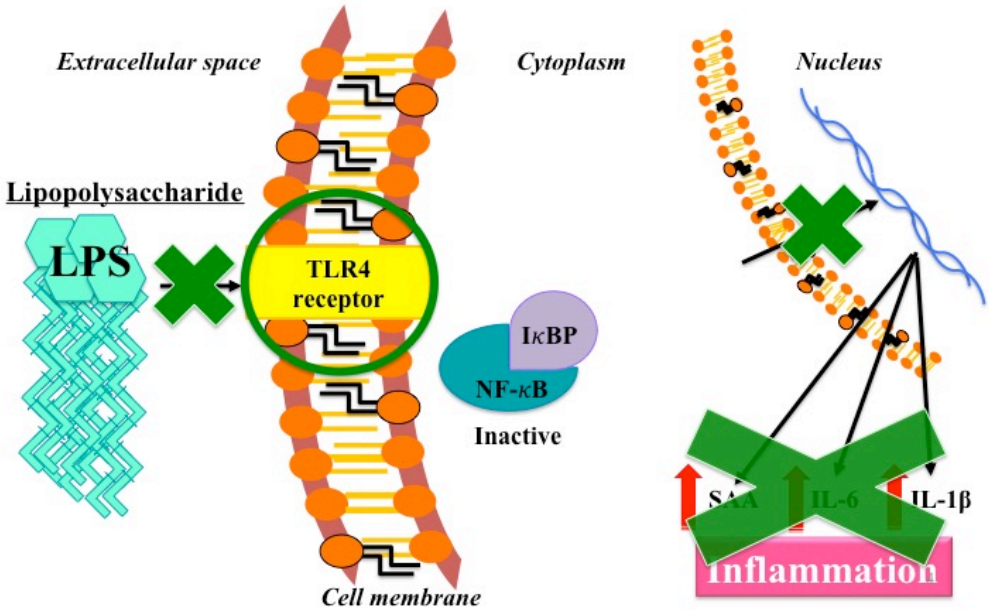


Figure 1.3. Lipopolysaccharide (LPS) ligand complex inhibited stimulation of cell membrane Toll-like Receptor 4 (TLR4) and resulting inhibition of Nuclear Factor kappa B (NF-κB) activation by Inhibiting NF-κB Binding Protein (IκBP). Inactive NF-κB cannot translocate from cell cytoplasm into the nucleus, therefore preventing pro-inflammatory cytokine transcription and translation of interleukin 1β (IL-1β), IL-6 and acute phase protein serum amyloid A (SAA).

Arachidonic acid activates enzymes and proteins from the cyclic AMP and protein kinase C cell signaling pathways, which increases intracellular protein phosphorylation shifting cell activity towards a pro-inflammatory state (Sumida et al., 1993; de Pablo and de Cienfuegos, 2000). Whether through cellular pathway alterations or lipid mediator formation, EPA and

DHA modulate inflammatory cytokine gene activation and synthesis (Figure 1.3; Serhan, 2014).

Alternatively, EPA and DHA may also be acting directly by suppressing immune cell activation and function (Shoda et al., 2015). In an experiment using mice with an autoimmune condition causing retinal inflammation, 6 week old female mice were fed EPA and DHA (1% and 1% of the diet, respectively) supplemented diets for 2 weeks (Shoda et al., 2015). Feeding EPA and DHA supplemented diets to mice resulted in reduced intraocular IFNγ production by $T_H1$ cells to undetectable levels and decreased IL-17 production by $T_H17$ cells to levels 7 times below what was observed in mice fed 2% arachidonic acid supplemented diets ($P \leq 0.01$; Shoda et al., 2015). Short-term dietary supplementation of EPA and DHA in mice resulted in reduced inflammatory signaling by immune cells at the site of inflammation (Shoda et al., 2015). In a separate study performed *in vitro*, human macrophages and hepatocytes cultured in EPA-enriched media (100 µmol/L) were exposed to prostaglandin $E_2$ (0.1 µg/mL) or lipopolysaccharide (0.1 µg/mL) to induce inflammation (Hao et al., 2010). Cells incubated in EPA reduced expression of tumor necrosis factor alpha and IL-6 by 50% compared to cells incubated in media containing arachidonic acid (100 µmol/L) or media containing the combination of EPA and arachidonic acid (1:1 ratio, 100 µmol/L; $P \leq 0.01$; Hao et al., 2010). In a follow-up assay, cell cultures were stimulated with lipopolysaccharide (0.1 µg/mL) for 24 hours, and then EPA was added to the media (100 µmol/L), which resulted in > 150% increased production of anti-inflammatory cytokine IL-10 at 24 hours post-EPA addition ($P \leq 0.01$; Hao et al., 2010). Modulation of cytokine production through direct contact with EPA or DHA suggests a mode of action involving cell surface receptor function.

Anti-inflammatory IL-10 is produced by T cells and monocytes, and resolves cell mediated immune cell function by inhibiting $T_H1$ and Natural Killer cell IFNγ synthesis via counteracting synergistic IL-12, IL-1β, and co-stimulation of IL-18 (Tominaga et al., 2000; Skovbjerg et al., 2010). Along with resolving inflammation, IL-10 diminishes macrophage microbicidal activity and inhibits responsiveness to IFNγ; dysregulation of resolving inflammation is a factor development of autoimmune disorders (Duque and Descoteaux, 2014; Varzaneh et al., 2014). In the case of modern poultry production, IL-10 serves to resolve systemic inflammation that may occur from environmental, management, or pathogenic stress.

Infection via different bacterial species results in a specific characteristic cytokine production by immune cells. In an *in vitro* experiment, peripheral blood mononuclear cells collected from human donors were infected by a panel of 37 Gram-positive and -negative bacterial species representing 5 phyla and assessed for cytokine production (Skovbjerg et al., 2010). Infection with Gram-positive bacteria stimulated 1.5 times more IL-1β, 7 times more IFNγ, and 9 times more IL-12 than Gram-negative species ($P \leq 0.05$; Skovbjerg et al., 2010). Gram-negative bacterial infection induced an average of 1.5 times more IL-6 and 3.3 times more IL-10 than Gram-positive species ($P \leq 0.01$), demonstrating fundamental differences in innate immune cell cytokine production based on pathogen membrane components (Skovbjerg et al., 2010).

Proteins in serum are associated with regulation of host resilience to pathogen-associated molecular patterns, which are how antigen presenting cells and lymphocytes recognize foreign microbes within the body (Warren et al., 2010). Macrophages from human and mouse blood were activated by lipopolysaccharide *in vitro* (≤ 2000 ng/mL) for 18 hours

and resulted in human macrophages producing 35 times more IL-6 compared to mouse macrophages (Warren et al., 2010). The more resilient a species is to lipopolysaccharide exposure *in vivo*, the greater the macrophages will tolerate the toxin and reduce response and expression of IL-6 *in vitro*, suggesting that immune cell responses are controlled by circulating cytokines (Warren et al., 2010). Measuring cytokine expression or production is a useful tool for evaluating inflammatory status and identifying patterns in live animal models.

In addition to cytokines, acute phase proteins like serum amyloid A secreted by the liver of chickens and other vertebrates are a useful way to measure stress as a result of infection, trauma, or inflammatory insult as a non-specific systemic reaction by the innate immune system (O'Reilly and Eckersall, 2014). The primary function of serum amyloid A is to transport lipoprotein and modulate metabolism during an acute phase inflammatory response, which allows cholesterol to remain in damaged tissues for repair and actively remove lipid debris from bacteria and tissue (O'Reilly and Eckersall, 2014). IL-1β and IL-6 work in concert to stimulate hepatocellular production of serum amyloid A and increase circulating plasma concentrations by up to 1,000-fold (Uhlar and Whitehead, 1999). Prolonged chronic inflammation or infection may result in persistently elevated circulating serum amyloid A levels, which may result in amyloidosis where proteinaceous accumulations of amyloid A aggregate intercellularly among various tissues causing organ dysfunction or failure (Saif et al., 2008; O'Reilly and Eckersall, 2014). The anti-inflammatory effects of EPA and DHA supplementation my help prevent or reduce chronic states of inflammation in poultry.

Cellular storage of ALA and membrane incorporation of EPA and DHA are important for their effect on modulating inflammation. Peroxisome proliferator factor gamma (PPARγ)

in mammals and avian species is the master regulator of adipogensis (Sun et al., 2014). When PPARγ binds to polyunsaturated fatty acids such as EPA, the fatty acid functions as an activating ligand resulting in increased transcriptional activity for adipogenesis (Edwards and O'Flaherty, 2008). In a broiler study, genetically selected lean broilers expressed an average of 15% less PPARγ compared to heaver genetic lines in abdominal fat tissue and PPARγ genetic expression in abdominal fat tissue increased by an average of 35% from 2 to 7 weeks of age ($P \leq 0.01$; Sun et al., 2014). The results of a broiler study demonstrated that PPARγ expression increased with age and was a factor in producing a phenotypically heavier bird (Sun et al., 2014). Consuming ALA, EPA, or DHA results in increased PPARγ expression and the expression levels are linked to the onset of oocyte maturation and egg laying (Sato et al., 2004). Further examination of PPARγ expression during periods of inflammation would help to characterize how specific omega-3 fatty acids exert effects on adipogenesis and cellular metabolism in a variety of poultry models.

***Lipid Metabolites***

In addition to indirect and direct effects on cytokine modulation, EPA and DHA are further metabolized to generate anti-inflammatory lipid metabolites (Serhan, 2014). Post-absorptive modification by rate limiting elongases and desaturases results in competition among ALA and linoleic acid which generates a shift to less inflammatory eicosanoid (prostaglandins, leukotrienes, and lipoxins), resolvin, and protectin production (Figure 1.1; Voss et al., 1991; Bautista-Ortega et al., 2009). Many of the human health claims regarding the anti-inflammatory effects from ALA, EPA, or DHA consumption stems from competition and displacement of arachidonic acid from cell membranes and synthesis inhibition of pro-

inflammatory lipid metabolites (de Pablo et al., 2002), which is another mechanism of how EPA and DHA modulate the inflammatory response (Figure 1.1).

***Membrane Fluidity and Peroxidation***

As previously discussed, cell membrane fatty acid incorporation of dietary EPA and DHA into cell membrane phospholipids displaces arachidonic acid (Newman et al., 2002a). The EPA and DHA displacement also causes changes in cell membrane fluidity which impacts cell phagocytic activity and expression of surface proteins including major histocompatibility complexes and adhesions (de Pablo and de Cienfuegos, 2000). In an *in vitro* experiment, EPA (66 μmol/L) or DHA (61 μmol/L) was added to media with human monocytes and then stimulated with IFNγ and incubated for 48 hours (Hughes et al., 1996). Monocytes supplemented with EPA inhibited relative median expression intensity of human leukocyte antigens-DR (HLA-DR), HLA-DP, and intracellular adhesion molecule-1 (ICAM-1) by an average of 0.1-fold compared to control ($P \leq 0.05$; Hughes et al., 1996). Antigen presenting cells require HLA-DR and -DP in order to express major histocompatibility complex class II molecules for adhesion via ICAM-1 to T cells during cell-cell contact, which results in T cell activation and initiation of an immune response (Hughes et al., 1996).

In the same *in vitro* experiment, monocytes supplemented with DHA media suppressed expression of HLA-DR, HLA-DP, and ICAM-1 by an average of 0.07-fold compared to control, which would result in reduced initiation of immune responses ($P \leq 0.05$; Hughes et al., 1996). Supporting results were observed during an *in vivo* experiment with broilers fed diets supplemented with 0, 3, 5, and 6% fish oil from 19 to 47 days of age (Al-Khalifa et al., 2012). Broilers fed 6% fish oil diets responded with reduced monocyte

phagocytosis and lymphocyte proliferation compared to the 5% fish oil treatment ($P \leq 0.05$; Al-Khalifa et al., 2012). The results from the human monocyte study and broiler experiment demonstrated that EPA and DHA decrease stimulated monocyte reactivity and antigen presentation due to changes in membrane fluidity, suggesting that immune cell responses may be muted during periods of inflammation or pathogenic infection.

Omega-3 fatty acids ALA, EPA, and DHA are prone to peroxidation due to their polyunsaturated bonds. Cell membrane incorporation of EPA and DHA increases membrane lipid peroxidation and free radical formation, which suppresses antigen presentation and expression of cell surface molecules by lymphocytes (Gruner et al., 1986; de Pablo and de Cienfuegos, 2000). However, membrane incorporation of EPA and DHA also affects cellular production of reactive oxygen species used for oxidative bursts. Human monocytes were collected for a study from individuals supplemented with dietary fish oil containing EPA (3.6 g/day) and DHA (2.4 g/day) for 6 weeks and the harvested monocytes were activated using latex particles (0.05 mL) to measure superoxide production (Fisher et al., 1990). Monocytes from EPA and DHA supplemented individuals reduced superoxide production by half compared to baseline control values ($P \leq 0.01$; Fisher et al., 1990). Results from the study suggest that dietary EPA and DHA suppress oxidative bursts by activated monocytes, which would reduce cellular killing capacity when phagocytizing pathogens. In addition to reducing pro-inflammatory cytokine signaling, dietary EPA and DHA supplementation also reduce the activity of stimulated immune cells.

Based on the immune cell types altered by dietary EPA and DHA either directly by acting as second messengers intracellularly, indirectly through modulating receptor activation and cytokine expression, or via their lipid products, functional omega-3 fatty acids regulate

anti-inflammatory effects multimodally (de Pablo and de Cienfuegos, 2000). Several studies have demonstrated that ALA, DHA, and EPA can be readily transferred into meat and egg products creating value-added foods (Fraeye et al., 2012; Ribeiro et al., 2013). Dietary ALA, EPA, and DHA supplementation are known to improve human health and reduce incidence of inflammatory health risks, which has generated a market for value-added egg and meat products (Skulas-Ray et al., 2011; Tur et al., 2012). Feeding an omega-3 fatty acid supplemented poultry diet to generate value-added eggs and meat may be providing a benefit to the bird as well.

### 1.4.2 Influence on Bone Metabolism

Laying hens often suffer from osteoporosis, commonly referred to as soft bones or layer fatigue, at the end of laying cycles due to their high demand for calcium (Saif et al., 2008). It is common for broilers to develop lameness issues or bone deformities due to their prolific growth rate (Knowles et al., 2008; Grupioni et al., 2015). Bone turnover rate plays an important role in retention of bone mass and dietary ALA, EPA, and DHA may be able to improve bone health in these at-risk animals.

Bone turnover rate is a balance between bone mineral resorption and synthesis, which is regulated by the RANKL/RANK/OPG pathway (Boyle et al., 2003). Dysregulation of bone resorption and synthesis may lead to bone pathology and problems with shell quality (Saif et al., 2008). Receptor activator of NF-$\kappa$B ligand (RANKL) is secreted locally by osteoblasts in the bone marrow and systemically by lymph nodes and thymus when circulating calcium levels are low (Boyce and Xing, 2007; Boyce and Xing, 2008). The binding of RANKL to RANK cell surface receptors results in target cell activation and

mineral resorption (Boyle et al., 2003). Osteoclastic precursor cells differentiate into mature osteoclasts when activated by RANKL (Boyce and Xing, 2007; Boyce and Xing, 2008). With further maturation, osteoclasts become multinucleated and responsible for bone mineral resorption releasing calcium into the blood stream (Boyle et al., 2003; Boyce and Xing, 2007). During inflammatory states where IL-1 and IL-6 are expressed, RANKL is up regulated and bone is in a resorptive state (Boyle et al., 2003; Boyce and Xing, 2008). When RANKL secretion is up regulated, the net state of bone becomes catabolic and osteoclastic resorptive activity dominates, out pacing bone mineral deposition by osteoblastic cells (Figure 1.4a; Boyle et al., 2003; Boyce and Xing, 2008).

Osteoblastic cells and bone marrow are responsible for local secretion of osteoprotegerin when circulating calcium levels are high (OPG; Boyle et al., 2003; Boyce

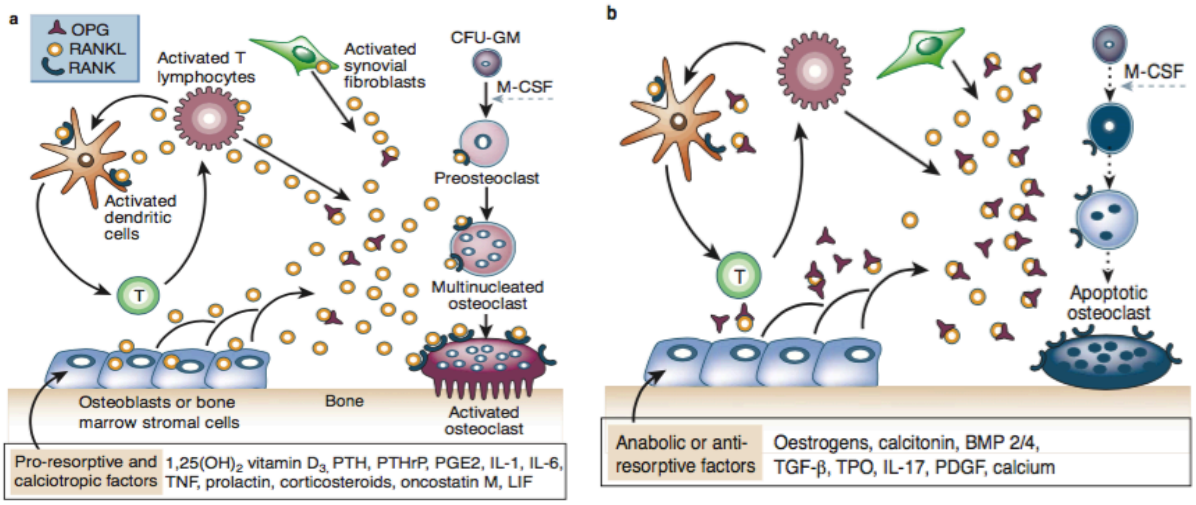


**Figure 1.4.** Adapted from Boyle et al. (2003). Bone remodeling and synthesis via RANKL/RANK/OPG pathway. A) Bone pro-resorptive state where receptor activator of Nuclear Factor Kappa B ligand (RANKL) is locally produced by osteoblasts and binds to RANK cell receptors activating dendritic cells, lymphocytes, fibroblasts, and bone resorptive osteoclasts. B) Bone anabolic state where osteoprotegerin (OPG) is predominantly produced by osteoblasts and binds to free RANKL resulting in inhibition of osteoclastic bone mineral resorption.

and Xing, 2007). Circulating OPG binds to free RANKL, making RANKL unavailable for RANK cell receptor binding (Boyle et al., 2003; Boyce and Xing, 2007). Reduced

concentrations of free RANKL in bone results in inhibition of osteoclastic activation, osteoclastogenesis, and survival of pre-existing osteoclasts (Boyle et al., 2003; Boyce and Xing, 2007). Therefore, when OPG is up regulated, decreasing the RANKL:OPG ratio, bone shifts into a net anabolic state of bone synthesis where osteoblasts utilize blood calcium for bone mineral deposition at a faster rate than osteoclastic resorption (Figure 1.4b; Boyle et al., 2003; Boyce and Xing, 2008).

Omega-3 fatty acids EPA and DHA are involved in the cellular mechanisms of bone remodeling. Similar to inflammatory modulation, EPA and DHA regulate bone turnover through intracellular signaling pathways, cytokine synthesis, growth factors, and lipid metabolites (Kajarabille et al., 2013). Eicosanoid metabolites derived from EPA and applied to *in vitro* cell cultures containing primary osteoclasts from mouse bone marrow resulted in inhibition of growth and bone resorption due to reduced cell differentiation (Sun et al., 2003; Herrera et al., 2008). Eicosanoid metabolites from EPA and DHA were also hypothesized to modulate osteoblast function, thereby increasing bone formation rates in rodent models (Watkins et al., 2003). Possible mechanisms of action have been identified using *in vitro* models demonstrating that EPA and DHA exert anti-osteoclastogenic activity by suppressing proliferation and differentiation of osteoclast precursor cells (Nakanishi and Tsukamoto, 2015; Kim et al., 2017). Using mouse bone marrow derived macrophages *in vitro*, DHA (0, 12.5, 25, and 50 μM) application to the culture suppressed proliferation in a dose-dependent manner (Kim et al., 2017). Addition of DHA to media attenuated cell osteoclastic differentiation by blocking RANKL cell activation and inhibited intracellular NF-$\kappa$B signaling cascades, which was demonstrated by suppression of NF-$\kappa$B transcriptional activity (Kim et al., 2017). Additionally, mature osteoclasts treated with DHA media resulted in

accelerated apoptosis due to expression of Bim, a protein that controls osteoclastic apoptosis (Kim et al., 2017). This agreed with results from Nakaishi and Tsukamoto (2015) who found that *in vitro* EPA and DHA treatment of mouse bone marrow cells inhibited osteoclastogenesis by suppressing gene expression of RANKL, cyclooxygenase 2, and IL-6, which regulate and activate NF-$\kappa$B. When comparing the potency of DHA to EPA, DHA application to murine monocyte cell lines reduced osteoclastic proliferation at concentrations > 100 μM and reduced intracellular NF-$\kappa$B activation by 20% compared to EPA treated cells ($P \leq 0.05$; Rahman et al., 2008). The modulating effects of EPA and DHA on bone metabolism appear to be associated with cytokine expression and regulation of the RANKL/RANK/OPG pathway.

Cytokines IL-1β and IL-6 potentiate bone resorption (Kruger et al., 2010). Circulating IL-1β up regulates prostaglandin $E_2$ ($PGE_2$) production and inhibits OPG secretion, which induces osteoclast formation as previously described through the RANKL/RANK/OPG pathways (Boyce and Xing, 2007; Kruger et al., 2010). Circulating IL-6 induces osteoclast differentiation by up-regulating $PGE_2$ production, which then increases RANKL synthesis shifting the RANKL:OPG ratio towards resorptive activity in bone (Boyce and Xing, 2007; Kruger et al., 2010). Both IL-1β and IL-6 up regulate expression of RANK cell surface receptors, enabling precursor osteoclastic cells to become activated via RANKL binding (Boyce and Xing, 2007; Kruger et al., 2010). It is possible that when EPA and DHA down regulate production of IL-1β, IL-6, and $PGE_2$, the net effect would inhibit osteoclast formation and support an anabolic state of bone mineral synthesis by osteoblasts (Kruger et al., 2010). It is clear that EPA and DHA promote a state of bone mineral synthesis *in vitro*.

Further investigation using *in vivo* models would help determine if practical applications exist for improving bone health in poultry.

A recent review of human studies reported that greater total polyunsaturated fatty acid consumption of both omega-3 and omega-6 fatty acids result in increased bone mineral density and reduced risk of fragility and fractures (Longo and Ward, 2016). The observed increase in bone mineral density may have been due to increased dietary PUFA consumption, which would aid in fat soluble vitamin D dependent calcium absorption (Longo and Ward, 2016). The individual effects of ALA, EPA, and DHA were not clear from epidemiological reviews and additional experimentation is needed to elucidate their specific roles and influence on bone metabolism (Longo and Ward, 2016). Although not fully defined, dietary ALA, EPA, and DHA modulate aspects of bone metabolism, and supplementation may improve bone health by increasing bone strength for poultry that are prone to lameness or osteoporosis.

Several studies have demonstrated that consuming functional ingredients containing dietary omega-3 fatty acids influence bone mineral metabolism. In a study using 8 week old ovariectomized mice that generated osteoporosis, it was found that feeding 5% fish oil diets to mice resulted in decreased osteoclastogenesis and increased bone mass retention compared to the unsupplemented control ($P \leq 0.01$; Sun et al., 2003). A survey of human clinical cases positively correlated fish consumption with bone mass retention in women diagnosed with osteoporosis (Moon et al., 2012) and additional studies indicated that dietary ALA, EPA, and DHA resulted in increased bone formation and peak bone mass in adolescent teenagers (Kajarabille et al., 2013). Research in the UK using free-range brown laying hens associated

reduced broken bones and deformation with dietary ALA supplementation, which is a relevant health and animal welfare concern (Tarlton et al., 2013; Toscano et al., 2015).

Dual-energy x-ray absorptiometry (DEXA) is used for diagnosis of osteoporosis in clinical practice and is the gold standard established by the World Health Organization (WHO Study Group, 1994). Scanning via DEXA quantifies bone mineral content and density within a defined area and has been used to assess poultry models since the mid-1990's (Mitchell et al., 1997; Longo and Ward, 2016). A murine study using DEXA analysis found that feeding 12% tuna oil diets for 8 weeks to 5 week old female rats resulted in increased tibial bone mineral content by 10%, increased tibial bone density by 26%, and reduced blood serum peroxidation by 25% compared to the corn oil-fed control ($P \leq 0.05$; Lukas et al., 2011). Results from the murine study suggest that dietary EPA and DHA directly contributed to the increases observed in bone mineral retention (Lukas et al., 2011). Although bone mineral density is measured to assess bone structure and composition, it has a weak association with clinical signs of lameness in broiler chickens, such as limping or abnormal gait (Talaty et al., 2010). In order to gain a comprehensive understanding of bone health in poultry, imaging and quantitative techniques along with observation for phenotypic lameness should be examined.

## 1.5 Lipopolysaccharide-Induced Systemic Inflammation

### 1.5.1 Lipopolysaccharide Administration

Lipopolysaccharides (LPS) are structural components of Gram-negative bacteria outer membrane and are recognized by mammalian and avian immune cells as pathogenic foreign molecules (Schwechheimer and Kuehn, 2015). A "sickness behavior" can be induced

in chickens with LPS injection intraperitoneally or intracerebroventricularly, which has been observed in 5 week old chickens using doses of 0.5 to 5.0 mg LPS/bird (Johnson et al., 1993). The behaviors described were clinical signs of an acute phase inflammatory response, which includes a febrile response, swelling, redness, elevated plasma corticosterone and increased pro-inflammatory cytokine synthesis of the affected area (Johnson et al., 1993; Leshchinsky and Klasing, 2001). Controlled LPS administration, although not identical to pathogenic infection, serves as a viable model for inducing systemic inflammation in poultry models due to the responses of depressed feed consumption, Toll-like receptor 4 stimulation, and increased splenic IL-1β, IFNγ, and IL-10 gene expression (Korver et al., 1998; Jiang et al., 2010; Munyaka et al., 2013). Further use of LPS administration in poultry experiments would be useful for evaluating protective anti-inflammatory effects of ALA, EPA, and DHA in an induced stress scenario.

### 1.5.2 Toll-like Receptor 4

Mammalian and avian species possess cells surface receptors known as toll-like receptor 4 (TLR4), which activate when LPS along with LPS binding protein, CD14, and MD-2 form a receptor ligand with a recognizable pathogen associated molecular pattern in the extracellular environment (Lu et al., 2008; Kannaki et al., 2010). Once TLR4 is activated via LPS stimulation, the receptor undergoes oligomerization and through a series of downstream amplifications mediate pro-inflammatory cytokine and interferon gene activation via NF-$\kappa$B transcription factor which translocates from the cytosol to the cell nucleus when activated (Figure 1.2; Lu et al., 2008). Chickens have TLR4 membrane receptors on a wide variety of immune and tissue cell types including heterophils,

monocytes, macrophages, spleen, tonsil, bursa, thymus, liver, brain, kidney, lung, muscle, and heart cells (Kannaki et al., 2010). The relative ubiquity of TLR4 membrane expression throughout various chicken cell types suggests they would be reliable candidates for inducing LPS inflammatory responses.

### 1.5.3 Lipopolysaccharide Poultry Models

Intraperitoneal and intravenous LPS administration are consistent methods for inducing inflammatory states in laying hens and broilers (Table 1.2), establishing inflammatory LPS challenges as a viable model for investigating inflammation in poultry (Cheng et al., 2004; Jiang et al., 2010). The tolerance level to LPS administration is greater for poultry varieties compared to other species, where poultry LPS dosages may be ≥ 10 times compared to swine models to stimulate systemic inflammation (Xie et al., 2000; Leshchinsky and Klasing, 2001). In a broiler experiment, 5.0 mg LPS/kg body weight was injected intravenously in broilers 21 days of age (Xie et al., 2000). The result of LPS administration was elevated plasma IL-6 and heterophil concentrations by 5 and 2 times, respectively, compared to saline injected control birds 12 and 24 hours post-injection ($P < 0.05$; Xie et al., 2000). Total plasma proteins increased by 15% in the LPS injected broilers 24 and 48 hours post-injection, demonstrating a functioning LPS inflammatory model at a 5.0 mg/kg dosage ($P < 0.05$; Xie et al., 2000).

For species comparison purposes, swine models require a much lower dosage of LPS than broilers to induce a similar response. Weaned swine were fed 5% fish oil diets from 21 to 42 days of age followed by an intraperitoneal injection of LPS at 100 μg/kg (Liu et al.,

2012; Liu et al., 2013). Feeding 5% dietary fish oil treatments to pigs, 4 hours post-LPS administration, resulted in reduction of TLR4 gene expression in jejunum by 43% and ileum by 36% compared to control pigs fed 5% corn oil diets ($P < 0.05$; Liu et al., 2012; Liu et al., 2013). Results from the swine experiment demonstrated a response to LPS administration at a lower dosage compared to chickens, which was attenuated by dietary fish oil supplementation. Investigation into dietary ALA, EPA, and DHA protective effects on poultry varieties during controlled LPS inflammation would help characterize poultry specific changes in inflammatory response due to dietary supplementation.

**Table 1.2.** Poultry models comparing dietary flaxseed oil and fish oil in response to inflammatory LPS challenge.

| Authors | Model | LPS | Omega-3 | Measure | Outcome |
|---|---|---|---|---|---|
| Korver et al. (1998) | Broiler 14 d old | 0.3 mg/bird, 3 IP doses once every 2 d | 1.0, 1.5, 2.0% fish oil | Relative liver weight; hepatocellular cytosolic metallothionein | LPS injection resulted in 10% ↑ in relative liver weight; 19% ↑ metallothionein; no dietary effect. |
| Korver and Klasing (1997) | Broiler 14 d old | 0.3 mg/bird, 3 IP doses once every 2 d | 0.5, 1.0, 2.0% flaxseed oil or fish oil | *In vitro* macrophage cytokines; hepatocellular cytosolic metallothionein | Fish oil enriched macrophages ↓ IL-1 compared to control; metallothionein ↓ as fish oil ↑ post-LPS injections; LPS injections ↑ metallothionein by 125%. |
| Leshchinsky and Klasing (2001) | Broiler and layer chicks 34 d old | 0 to 1000 mg LPS/well | None | *In vitro* splenic cytokines | IL-1β and IFNγ splenic gene expression ↓ in broilers compared to layers. |
| De Boever et al. (2008) | Broilers 21 d and 35 d old | 1.0 mg/kg, 2 IV doses 2 d or 7 d apart | None | Plasma cytokines | IL-6 greatest plasma concentration 3 h post-initial LPS injection; second LPS injection ↓ IL-6 peak compared to initial injection. |
| Sijben et al. (2003) | Layer chicks 37 to 38 d old | 1.0 mg/bird single IV dose | 5% flaxseed oil or fish oil | Splenic cytokines | IL-6, IL-8, IL-18, IFNγ ↑ 2 h post-LPS injection compared to saline injection; LPS injection in fish oil-fed birds ↑ IFNγ compared to LPS injection in flaxseed oil and control fed layers. |

Previous experiments have evaluated a range of LPS dosages from 0.1 to 5.0 mg/kg intraperitoneally in laying hen and broiler varieties demonstrating differences in biologic response (Leshchinsky and Klasing, 2001). It was found that LPS injection in laying hens, regardless of dosage, resulted in elevated body temperature 4 to 12 h post-injection compared to control ($P < 0.05$; Leshchinsky and Klasing, 2001). Only injection of 5.0 mg LPS/kg body weight in broilers produced an elevated body temperature compared to control ($P < 0.05$; Leshchinsky and Klasing, 2001). The difference in body temperature response to LPS administration suggests that broilers have a greater tolerance to LPS compared to hens, which was an influence of genetics on biological response.

Few poultry experiments using LPS and dietary omega-3 fatty acid supplementation have been performed that investigate protective anti-inflammatory effect or investigation into bone health (Table 1.2). In one experiment broilers were fed 2% fish oil supplemented diets from 0 to 14 days of age and then intraperitoneally injected with 0.3 mg LPS/bird at 10, 12, and 14 days of age (Korver and Klasing, 1997). Feeding the fish oil treatment to broilers resulted in reduced release of IL-1 by peritoneal macrophages by 20% compared to the control broilers fed corn oil diets ($P < 0.01$), which demonstrates inflammatory modulation by EPA and DHA treatment (Korver and Klasing, 1997). In a follow-up experiment, intraperitoneally injecting fish oil-fed broilers with 0.3 mg LPS/bird at 10, 12, and 14 d of age resulted in a 17% reduction in body weight and 11% reduction in feed intake from 10 to 14 d of age ($P < 0.05$; Korver et al., 1998). The LPS administration also increased relative liver weight by 10% and liver cytosolic metallothionein by 19% ($P < 0.05$), a protein that binds heavy metal ions and is associated with the acute phase response when synthesized (Korver et al., 1998). The results from repeated low dose LPS injections suggest that broilers

may become refractory to the antigen and > 2% fish oil may be needed to attenuate inflammation. Additional experimentation using greater LPS dosages or investigation using greater dietary EPA and DHA supplementation may help better characterize responses to LPS administration and EPA and DHA supplementation in broilers.

Data is limited regarding dietary omega-3 fatty acid supplementation (ALA, EPA, or DHA) and the responses of LPS exposure in pullets, laying hens in peak production, and end-of-cycle laying hens. A study using 4 day old pullets fed diets containing 5% fish oil or 5% flaxseed oil until 37 and 38 days of age (Sijben et al., 2003). On day 37 and 38, pullets were administered a single 1.0 mg/kg intravenous LPS dose, and splenic cytokine gene expression was measured 2 hours post-injection (Sijben et al., 2003). Splenic cytokine gene expression increased 6-fold for IL-6, 5-fold for IL-18, and 12-fold for IFNγ compared to saline infused control, while IFNγ increased in fish oil-fed chicks but not flaxseed oil and control-fed chicks ($P < 0.05$; Sijben et al., 2003). Results from the pullet experiment support that inflammatory modulation differences are influenced by EPA and DHA. Dietary EPA and DHA from fish oil may have practical applications in reducing inflammation in laying hens once dose-dependent responses are better defined.

Initial investigations of the potential health benefits from consuming dietary omega-3 fatty acids were focused on human epidemiological and observational case studies (Cotton et al., 2011; Mozaffarian and Wu., 2011; Tur et al., 2012). The potential health benefits have led consumers to incorporate value-added foods like omega-3 fatty acid enriched eggs and meat into their diets demonstrated by specialty eggs making up 26.4% of the egg market (Fraeye et al., 2012; Goyal et al., 2014; USDA, 2017b). Producers use omega-3 fatty acid supplemented poultry diets to create these value-added meat and eggs (Fraeye et al., 2012;

Ribeiro et al., 2013). Dietary omega-3 fatty acid supplementation used to create these value-added products may also be improving poultry health and welfare during commercial production (Tarlton et al., 2013; Toscano et al., 2015). Previous omega-3 fatty acid experiments used *in vitro* assays and cell cultures derived from murine models to investigate cell responses to ALA, EPA, or DHA media application (Rahman et al., 2008; Kruger et al., 2010; Lukas et al., 2011); however, there has not been *in vivo* investigation into the protective effects of different omega-3 fatty acids on inflammation and how they may alter bone health in aged laying hens and growing broilers under periods of stress. This dissertation seeks to determine the responses to dietary ALA or EPA and DHA supplementation of an aged laying hen model with a developed immune system and active skeletal turnover. In addition, this dissertation will pursue how immunologically naïve broilers with a growing skeletal frame respond to dietary ALA or EPA and DHA supplementation. Dietary ALA or EPA and DHA supplementation in poultry diets may potentially serve a dual purpose of producing value-added healthy food for consumers and improving the health and welfare of commercial poultry.

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# CHAPTER 2. COMPARATIVE OMEGA-3 FATTY ACID ENRICHMENT OF EGG YOLKS FROM FIRST-CYCLE LAYING HENS FED FLAXSEED OIL OR GROUND FLAXSEED

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## 2.1 Abstract

Value-added eggs can be generated by supplementing laying hen diets with functional ingredients, such as flaxseed, containing the omega-3 fatty acid, alpha-linolenic acid (ALA). Omega-3 fatty acids, specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have anti-inflammatory properties and are linked to reducing heart disease. Flaxseed is commonly used for ALA supplementation; however, the impact of dietary flaxseed source (extracted degummed oil vs. ground seed) on fatty acid transfer to egg yolk in laying hens is unknown. Therefore, dietary flaxseed oil and ground flaxseed were evaluated for ALA, EPA, and DHA deposition in egg yolks of Hy-Line W-36 laying hens over an 8 wk feeding period (25 to 33 wk old). Hens (n = 132) were randomly housed 3 birds/cage (4 replicates/treatment) for each of 11 treatments. Diets consisted of a control diet, 5 flaxseed oil diets (0.5, 1.0, 2.0, 3.0, or 5.0% flaxseed oil), and 5 ground flaxseed diets (calculated flaxseed oil concentration

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0.5, 1.0, 2.0, 3.0, 5.0% from ground flaxseed). Increasing dietary flaxseed oil and ground flaxseed concentrations led to increased egg yolk total omega-3 fatty acid (ALA, EPA, and DHA) concentration ($P \leq 0.01$). Egg yolk EPA and DHA concentration were not different due to flax source ($P = 0.21$). Supplementing ≥ 3% dietary flaxseed oil resulted in > 2 times the egg yolk total omega-3 fatty acid deposition compared to the equivalent ground flaxseed inclusion ($P \leq 0.01$). Hens receiving either flaxseed oil or ground flaxseed had reduced BW change as dietary concentrations increased ($P = 0.02$). Feed efficiency (egg mass ÷ feed intake) increased as flaxseed oil supplementation increased, whereas feeding ground flaxseed decreased feed efficiency ($P \leq 0.01$). Determination of flaxseed oil nitrogen corrected apparent metabolizable energy resulted in 7,488 kcal/kg on an as-fed basis. Dietary flaxseed oil, with greater egg yolk total omega-3 fatty acid deposition and increased feed efficiency, proved to be an efficient alternative to ground flaxseed for producing value-added eggs, warranting further investigation of dietary ALA, EPA, and DHA to determine potential health and welfare benefits of supplementing poultry diets.

Key words: ALA, EPA, DHA, value-added, energy

## 2.2 Introduction

Health conscious consumers want to eat food that supports healthy lifestyles. Certain omega-3 fatty acids contained in functional ingredients and value-added foods are known to possess anti-inflammatory properties that are linked to reducing health risks, such as heart disease and osteoporosis (Kruger et al., 2010; Tur et al., 2012; Goyal et al., 2014). This has led to consumer demand for value-added omega-3 fatty acid enriched egg products, which now captures 10% of the marketing of retail shell eggs and egg products (USDA, 2016).

Dietary alpha-linolenic acid (ALA; 18:3 omega-3 fatty acid), found in flaxseeds, can be supplemented in poultry diets to generate value-added omega-3 fatty acid enriched eggs (Tarlton et al., 2013; Toscano et al., 2015). It is important to understand how ALA ingredients impact dietary omega-3 fatty acid transfer to the egg yolk and practical applications as a poultry feed ingredient so that producers will have a reference for making these value-added eggs.

Hens readily absorb and transfer omega-3 fatty acids from dietary sources for deposition into the yolk (Cherian and Sim, 1991). On average, it takes 2 wk for a laying hen to adjust to an omega-3 fatty acid supplemented diet and reach a transfer plateau of dietary omega-3 fatty acid incorporation into developing ovarian follicles (Cherian and Sim, 1991; Nain et al., 2012). The beneficial anti-inflammatory properties for reducing health risks have been attributed to longer chain omega-3 fatty acids eicosapentaenoic (EPA; 20:5 omega-3 fatty acid) and docosahexaenoic acids (DHA; 22:6 omega-3 fatty acid; Cottin et al., 2011; Tur et al., 2012). Laying hens have the ability, although not efficient (< 6%), to elongate and desaturate ALA, an essential fatty acid and predominant omega-3 fatty acid in flaxseed, to the functional omega-3 fatty acids EPA and DHA (Burdge and Calder, 2006; Zivkovic et al., 2011; Gregory et al., 2013). Egg yolk fatty acid content is finite due to the 10% total fat contained within an egg and reaches a plateau of saturation, which is directly influenced by the total ALA, EPA, and DHA omega-3 fatty acid composition within the diet (Cherian and Sim, 1991; Nain et al., 2012).

The fatty acid composition of a feed ingredient has a direct effect on fatty acid profile and deposition in poultry meat and eggs (Jia and Slominski, 2010; Nain et al., 2012). Similar to other monogastric animals, poultry species have a limited endogenous enzymatic ability to

modify the structure of dietary fatty acids compared to ruminant species (Haug et al., 2014). Poultry do not host the microbial populations responsible for the expression of elongases and desaturases like those contained within the rumen modifying dietary lipids (Haug et al., 2014). Long chain fatty acids, such as ALA, are added to triglycerides for long-term energy storage during post-absorptive metabolism and contain the relatively unaltered fatty acids in adipocyte lipid droplets (Cherian and Sim, 1991). Longer chain fatty acids, including EPA and DHA, are almost exclusively deposited for storage in the form of phospholipids, particularly phosphatidylethanolamine in egg yolk (Jiang et al., 1991). Producers are able to create value-added omega-3 fatty acid enriched eggs by using the laying hen's physiological ability to deposit dietary ALA, EPA, and DHA directly into egg yolk.

Ground flaxseed, also known as linseed, is an ALA source and is used by poultry producers in the US for enriching commercial table eggs and meat products (Samman et al., 2009; Petrovic et al., 2012; Lopes et al., 2013). Flaxseed contains 7 times the amount of ALA compared to soybean and corn oil with 3 times less omega-6 linoleic acid (LA; 18:2 omega-6 fatty acid) content, properties which contribute to its use as an ALA supplement in poultry formulation (NRC, 1994). Whole flaxseed contains 30 to 40% fat with 50% of the fat composition consisting of ALA and 15% LA (Kratzer and Vohra, 1996). Flaxseed products effectively deliver ALA to poultry meat and eggs due to high concentration and bioavailability (Jia et al., 2008; Fraeye et al., 2012). However, the transfer rates of flaxseed ingredients have not been determined. Eggs from unsupplemented laying hens may contain 93 mg ALA and 173 mg total omega-3 fatty acid (ALA, EPA, and DHA)/50 g egg (Jia et al., 2008; Samman et al., 2009). Supplementation of 15% dietary flaxseed can increase ALA and

total omega-3 fatty acid content to 358 mg and 468 mg/50 g egg, respectively (Jia et al., 2008; Samman et al., 2009).

There appears to be a tolerable limit to how much flaxseed can be included in a ration, due to anti-nutrients or palatability, without negatively affecting bird performance and feed efficiency. Digestion and absorption may be impaired due to various anti-nutrients found in flaxseed such as cyanogenic glycosides and phytic acid, or due to increased viscosity of ingesta caused by mucilage (Alzueta et al., 2003; Goyal et al., 2014). Laying hens fed diets with 20% ground flaxseed, compared to 0 and 10% treatments, resulted in inadequate weight gain, reduced egg production, and increased feed intake, which significantly reduced feed efficiency (Leeson et al., 2000). Anti-nutrients or reduced nitrogen corrected apparent metabolizable energy ($AME_n$) due to flaxseed in the diet were suspected as the cause for the adjustment in laying hen feed intake (Leeson et al., 2000). Few energy values have been published for whole, ground, or oil flaxseed sources since the mid 1990's (NRC, 1994; Lee et al., 1995). Determining a current $AME_n$ value of flaxseed oil would be beneficial for future diet formulation and provide an update in oil characteristics from current flax crops.

Yet to be investigated is how the source of flaxseed ingredient may affect the efficiency of ALA transfer from the laying hen diet into the developing egg yolk. Milled or ground flaxseed still contains the components of the seed's cellular matrix that may trap some of the lipid fraction as seen with other seed types (Cassady et al., 2009). Trapped lipid may be a factor in reducing digestibility or availability of dietary ALA in ground flaxseed (Cassady et al., 2009). A human study comparing whole, ground, and oil flaxseed sources found that supplementing 6 g of ALA for 4 wk resulted in 77% greater plasma ALA levels in

test subjects fed flaxseed oil compared to test subjects fed ground flaxseed, while feeding whole flaxseed did not appear to increase plasma ALA levels (Austria et al., 2008). Test subjects from each treatment group experienced gastrointestinal discomfort, especially from those that consumed whole flaxseeds (Austria et al., 2008). The discomfort led to dietary non-compliance in the study, stressing the importance of how increased flaxseed or oil consumption may change the intestinal microenvironment (Austria et al., 2008).

In order to further investigate how ALA sources affect the fatty acid transfer rate from laying hen diet to the egg yolk, extracted degummed flaxseed oil and conventional ground flaxseed ingredients were fed at increasing inclusions in experimental ALA supplemented laying hen diets. Dietary $AME_n$ of flaxseed oil was determined to fill the gap in knowledge for future research and industry application. It was hypothesized that an extracted degummed flaxseed oil ingredient would have an improved rate of ALA deposition to egg yolk compared to a ground flaxseed ingredient when supplemented in a laying hen ration during peak production, with no adverse effects on $AME_n$ and performance.

## 2.3 Materials and Methods

### 2.3.1 Animals and Housing

The Institutional Animal Care and Use Committee (IACUC) at Iowa State University (Ames, IA) approved the protocol and the experiment was conducted in accordance with university policies and the Ag Guide (2010). Single-Comb White Leghorn laying hens (n = 132, Hy-Line W-36, age = 25 wk old) were obtained from a commercial source (Bancroft, IA) as they were approaching peak production and reproductive efficiency. Laying hens were randomly placed into 3 tier conventional cages (Poultry Layer Cage, Safeguard, New

Holland, PA), resulting in 3 hens/cage at a density of 696 $cm^2$/bird. Each single cage of 3 hens represented an experimental unit (EU). Treatments were assigned in a completely randomized design, allowing for 11 dietary treatments with 4 replicates/treatment. Hens were allowed *ad libitum* access to feed and water during the 8 wk experiment from 25 to 33 wk of age. Lighting started at 13.5L:10.5D with 30 minutes of increasing light/wk until 15.5L:8.5D was achieved 4 wk into the experimental period. House temperature was maintained between 18 to 27° C for the 8 wk experimental period.

### 2.3.2 Flaxseed Source Supplemented Diets

Experimental diets were formulated to meet or exceed the NRC (1994) requirements of commercial layer hens. Flaxseed oil diets were formulated by adding the degummed flaxseed oil at the expense of soy oil in the diet (Table 2.1). Formulations consisted of a control diet, 5 flaxseed oil diets, and 5 ground flaxseed diets (calculated flaxseed oil concentrations for treatment diets were 0.5, 1.0, 2.0, 3.0, and 5.0%). A twelfth treatment diet contained 5.0% added flaxseed oil with an antioxidant stabilizer (n = 12 additional hens, internal data not reported here). The ground flaxseed ingredient was created by grinding whole flaxseed through a 4.0 mm screen (Thomas-Wiley Laboratory Mill Model 4, Arthur H. Thomas Company, Philadelphia, PA) with corn as a carrier. To obtain the calculated oil concentrations in the ground flaxseed diets, the ground flaxseed was included at 1.5, 3.0, 6.0, 9.0, and 15.0% in the diet (Table 2.2).

### 2.3.3 Performance

Laying hens were monitored twice daily for the duration of the 8 wk experiment in accordance with IACUC policy. Eggs were collected daily for hen-housed egg production (HHEP; no mortality occurred, therefore hen-day egg production was not reported) calculation and average daily feed intake (ADFI) was determined by measuring weekly disappearance of feed: HHEP % = (eggs laid ÷ hens housed ÷ d) × 100; ADFI = start feed weight in kg – end feed weight in kg. Body weight (BW) was recorded at the start, 4 wk, and 8 wk. Egg weight (EW), average daily egg mass (ADEM), and feed efficiency were measured weekly from 0 to 8 wk: ADEM = average EW in g × (HHEP % ÷ 100). Feed efficiency (FE) was reported as kg eggs per kg FI: FE = EM in kg ÷ FI in kg.

### 2.3.4 Egg Yolk Analysis

A pooled sample of 5 egg yolks from each EU was used to measure egg solids and yolk fatty acid profile at 4, 6, and 8 wk of the experiment. Fatty acid analysis of egg yolk, as previously described by Sun et al. (2013) and Nam et al. (2001) using gas chromatography (HP 6890, Hewlett Packard Co., Palo Alto, CA), was performed starting at 4 wk to allow an adjustment period for maximal transfer of fatty acids to egg yolk.

Egg yolk total omega-3 fatty acid (ALA, EPA, and DHA) deposition in mg was calculated using the egg yolk omega-3 fatty acid content (%) obtained from the previously described egg yolk fatty acid analysis and applied to a 50 g egg with 10% fat content (AEB, 2017). The extrapolated omega-3 fatty acid deposition values were calculated using the following formula: Egg yolk total omega-3 fatty acid deposition in mg = average egg yolk ALA, EPA, and DHA content in % ÷ 100 × 5000 mg fat.

### 2.3.5 $AME_n$ Experiment

After the initial 8 wk omega-3 fatty acid supplementation experiment, all birds (now 33 wk of age) were utilized in a 2 wk study to determine the $AME_n$ content of the flaxseed oil, as this information is currently lacking in the literature. The laying hens (n = 144, including the unreported $12^{th}$ diet group) were removed from their respective cages and separated, randomly rearranged using the same 3 tier cages as previously described so that each cage contained 3 hens that were new cage-mates. This ensured that the previous flaxseed dietary supplementation would not adversely affect the $AME_n$ experiment. Treatments were assigned in a completely randomized design allowing for 4 dietary treatments with 12 replicates/treatment for the $AME_n$ experiment. Each EU consisted of 3 hens/cage with identical bird density as previously mentioned in the flaxseed supplementation experiment. Hens were managed as previously described with *ad libitum* access to feed and water for the 2 wk $AME_n$ experiment from 33 to 35 wk of age. Performance data during this 2 wk $AME_n$ experiment was not reported, because the hens were adjusting to the new experimental diets.

A basal diet with titanium dioxide (0.30%) and increasing levels of flaxseed oil (0.0, 3.0, 6.0, and 9.0% added to the basal diet) were used to generate 4 $AME_n$ treatment diets. Experimental diets were formulated to meet or exceed the NRC (1994) requirements of commercial laying hens (Table 2.3). These diets were fed for a 2 wk adjustment period, which served as a transition period for the previous flaxseed egg yolk fatty acid deposition experiment, in order to collect excreta on d 14 for $AME_n$ determination and regression analysis.

Feed samples were ground through a 0.5 mm screen and subsequently dried for 24 h at 100°C for DM determination. Pooled excreta samples were dried at 75°C in a convection oven for 3 d and subsequently ground through a 1.0 mm screen as previously described by Ehr et al. (2015)**.** The ether extract of the feed samples was determined by AOAC (2010) Official Method 920.39, traditional Soxhlet extraction using diethyl ether at the University of Missouri Agricultural Experiment Station Chemistry Laboratories (AESCL, Columbia, MO). For the feed and excreta samples, N concentration was determined by thermal combustion (TruMac N Analyzer, LECO Corp., St. Joseph, MI); gross energy (GE) was determined using an adiabatic oxygen bomb calorimeter (Parr Instrument Co., Moline, IL); and Ti concentration was determined using the method outlined by Leone (1973). All samples of excreta and feed were analyzed in duplicate. The diet $AME_n$ determination was calculated using the following formula from Leeson and Summers (2001) modified for Ti: $AME_n = GE_{Diet} - (GE_{Excreta} \times Ti_{Diet} \div Ti_{Excreta}) - 8.22 \times (N_{Diet} - N_{Excreta} \times Ti_{Diet} \div Ti_{Excreta})$.

### 2.3.6 Statistical Analysis

Data were analyzed by repeated measures ANOVA using SAS (SAS 9.4, 2012, SAS Institute Inc., Cary, NC) with diet, week, and diet × week interaction included in the model. The 11 dietary treatments were assigned to the EU (3 hen/cages) following a completely randomized design, with 4 replicates/treatment. Orthogonal contrasts were used to test the response variables of the control against the 10 flax source supplemented diets and the 5 flaxseed oil vs. 5 ground flaxseed supplemented diets. There was only one control group for the experiment, therefore responses variables were tested for linear, quadratic, and cubic orthogonal contrasts of dietary oil % (0.5, 1.0, 2.0, 3.0, 5.0%) and dietary oil % × flax source

(flaxseed oil or ground flaxseed) interaction for the 5 flaxseed oil and 5 ground flaxseed supplemented treatments. For the purposes of this experiment, linear and quadratic functions were used to explain responses in the biological model. Linear fitment indicated progressive response to dietary inclusion and quadratic fitment represented an upper limit for response in relation to dietary inclusion. Cubic contrasts were included for the reader to make further inferences if desired. In all cases $P \leq 0.05$ was accepted as significant.

## 2.4 Results

### 2.4.1 Supplementation Period Performance

During the dietary flax source supplementation period, no differences were observed for FI, EM, or egg solids for any of the orthogonal contrasts tested ($P \geq 0.07$). Quadratic contrast of oil % × flax source resulted in increasing then plateauing HHEP as inclusion rate of flaxseed oil increased ($P = 0.01$). As ground flaxseed inclusion rate increased, HHEP declined then plateaued ($P = 0.01$). Linear contrast of oil % × flax source resulted in increasing FE as dietary flaxseed oil was increased in the flaxseed oil treatments ($P = 0.01$). Increasing ground flaxseed dietary inclusion resulted in decreasing FE ($P = 0.01$). Linear contrast of dietary oil % resulted in decreasing EW as increasing inclusion of either flax source (flaxseed oil or ground flaxseed equivalent) was added to the diet ($P = 0.05$). Gains in BW decreased as oil % increased in flaxseed oil or ground flaxseed equivalent diets linearly ($P = 0.02$). Change in BW decreased for ground flaxseed fed hens 24 times more compared to flaxseed oil fed hens as dietary inclusion of each respective flax source increased ($P = 0.02$; Table 2.4).

### 2.4.2 Egg Yolk Omega-3 Fatty Acid Deposition

Egg yolk total omega-3 fatty acid (ALA, EPA, and DHA) concentration increased linearly as oil % increased for flaxseed oil and ground flaxseed supplemented dietary treatments ($P < 0.01$). Increasing inclusion of flaxseed oil supplementation resulted in an egg yolk total omega-3 fatty acid deposition rate (slope value) that was approximately 2 times greater compared to equivalent inclusion of ground flaxseed dietary treatments ($P < 0.01$). Specifically, the flaxseed oil treatments resulted in a linear equation where the egg yolk lipid fraction omega-3 fatty acid (%) = 1.604 × flaxseed oil concentration (% of diet) + 2.171 ($R^2 = 0.880$; $P < 0.01$) while ground flaxseed treatments resulted in a linear equation where the egg yolk lipid fraction omega-3 fatty acid (%) = 0.783 × flaxseed oil concentration (% of diet) + 2.310 ($R^2 = 0.808$; $P < 0.01$; Figure 2.1). No differences were observed for oil % inclusion and flax source on egg yolk total omega-3 fatty acid concentration or egg yolk EPA and DHA concentration using quadratic and cubic analysis ($P \geq 0.30$). Egg yolk EPA and DHA concentration was not affected by flax source ($P = 0.26$). However, as oil % increased in flaxseed oil and mill flaxseed dietary treatments, egg yolk EPA and DHA concentration increased linearly ($P < 0.01$; Figure 2.2).

The average total omega-3 fatty acid concentration values from each level of flaxseed oil and ground flaxseed treatments were used to extrapolate the total omega-3 fatty acid deposition (mg) for a 50 g egg containing 10% fat. The linear equation for flaxseed oil supplementation was y (mg/50 g egg) = 80.184 × x (% ingredient in diet) + 108.5 ($R^2 = 0.999$) and ground flaxseed supplementation was y (mg/50 g egg) = 13.055 × x (% ingredient in diet) + 115.5 (Figure 2.3; $R^2 = 0.990$), also as shown in Figure 2.3.

### 2.4.3 Oil $AME_n$ Determination

The linear regression equation of $AME_n$ vs. dietary flaxseed oil concentration was y (kcal/kg as fed) = 74.88 × x (flaxseed oil concentration % of diet) + 2,750 ($P < 0.01$; $R^2 = 0.941$). The slope of the regression line equated to the $AME_n$ value of the flaxseed oil ingredient, which was 7,488 kcal/kg on an as-fed basis (Figure 2.4). The mean analyzed $AME_n$ values for the experimental diets were 2,762, 2,948, 3,218, and 3,421 kcal/kg as the flaxseed oil concentration increased from 0.0, 3.0, 6.0, and 9.0 % for each diet, respectively.

## 2.5 Discussion

This experiment was designed to evaluate the impact of different dietary omega-3 fatty acid sources (extracted degummed oil vs. ground seed) on egg yolk deposition and to determine the $AME_n$ of flaxseed oil. No significant differences were observed in ADFI for hens fed diets containing ground flaxseed. If any were to be observed, the 5.0% ground flaxseed treatment would have been expected to suppress ADFI due to anti-nutrients present in or palatability of the ground seeds (Table 2.4). However, the rate of BW gain of hens fed ground flaxseed declined 24 times more than hens fed flaxseed oil as dietary inclusions increased. The difference in rate of BW change may have been due to anti-nutrients present in the ground flaxseed causing impaired digestion or absorption of the dietary nutrients (Gonzalez-Esquerra and Leeson, 2000; Leeson et al., 2000).

Cyanogenic glycosides including linustatin, neolinustatin, and linmarin present in the ground flaxseed may have caused a reduction in BW change due to loss of effective intestinal epithelial cell absorptive function (Oomah et al., 1992; Feng et al., 2003; Kajla et al., 2015). In addition, phytic acid present in ground flaxseed may result in protein-mineral-phytic acid

complexes that are not bioavailable, reducing BW gain by exacerbating impaired nutrient absorption (Erdman, 1979; Feng et al., 2003; Goyal et al., 2014). Trypsin inhibitors present in flaxseed may have played a minor role in decreasing nutrient bioavailability, but the quantity found in flaxseed is insignificant compared to levels found in soybean (Bhatty, 1993; Feng et al., 2003). It has been well documented that mucilage, a water-soluble polysaccharide found in flaxseed, increases chicken intestinal content viscosity (Rodriguez et al., 2001). The increased viscosity inhibits nutrient digestion and absorption of the intestinal ingesta (Alzueta et al., 2003). Similar declines in performance were observed in FE for hens fed ground flaxseed diets as the inclusion increased. Data from this current work suggests that feeding hens ground flaxseed exerted physiologic effects impairing nutrient storage and anabolic activity compared to equivalent inclusions of supplemental flaxseed oil.

When analyzing the total omega-3 fatty acid (ALA, EPA, and DHA) transfer from the diet into the egg yolk, dietary inclusions of flaxseed oil incorporated total omega-3 fatty acids into yolks at 2.0 times the rate of ground flaxseed (Figure 2.1). This difference in rates was likely because the structural components of the cell wall entrapped the lipid fraction, as seen in other types of seeds such as almonds (Ellis et al., 2004; Mandalari et al., 2008). The presence of anti-nutrients likely contributed to the decreased transfer of ALA due to reduced bioavailability or absorption of the dietary lipids. Extracted degummed flaxseed oil treatments resulted in delivering dietary ALA to egg yolk without seed components interfering with intestinal utilization. In an experiment with up to 7% dietary flaxseed oil inclusion, total omega-3 fatty acid deposition in the muscle tissue of broiler chickens was increased in a curvilinear manner (Kartikasari et al., 2012). The lack of quadratic fit or plateau in the work reported here suggests that the maximum saturation limit of total omega-

3 fatty acid deposition to the egg yolk was not reached and would explain the linear fit of omega-3 fatty acid egg yolk content as dietary oil % increased in the flax source treatment groups. EPA and DHA egg yolk inclusion was not different based on flax source supplemented treatments suggesting that laying hens deposited modified portions of ALA to egg yolk at a finite but constant rate.

Flaxseed oil has 2 distinct advantages over ground flaxseed when comparing egg yolk total omega-3 fatty acid deposition into a 50 g egg. The first advantage is that flaxseed oil has a more efficient ALA transfer rate compared to ground flaxseed (Figure 2.1 and 2.2). Flaxseed oil, as an ingredient, only requires 1.8% dietary inclusion to achieve a target 250 mg total omega-3 fatty acid content for a 50 g egg (Figure 2.3). It would require 10.3% ground flaxseed to achieve the same target 250 mg total omega-3 fatty acid content per 50 g egg (Figure 2.3). The second advantage lies in the economics when applying wholesale flaxseed oil ($1.41/kg) and flaxseed crop ($0.45/kg) prices to the diets (USDA, 2015). Using 1.8% dietary flaxseed oil would cost $20.52 flaxseed oil/metric ton of finished feed compared to using 10.3% ground flaxseed, which would cost $46.35 ground flaxseed/metric ton of finished feed. Flaxseed oil takes up less volume in a finished feed and delivers targeted mg of total omega-3 fatty acids into a 50 g egg for less than half the cost than ground flaxseed. The advantages of flaxseed oil suggest that it is a superior dietary supplement for generating value-added omega-3 fatty acid enriched eggs than ground flaxseed.

The $AME_n$ of the flaxseed oil ingredient was determined after the 8 wk omega-3 fatty acid flaxseed source supplementation to egg yolk experiment, as few data are published regarding the metabolizable energy of flaxseed oil. The energy value found was 7,488 kcal/kg of flaxseed oil as fed, which was a less than the $AME_n$ value of 8,100 ± 540 kcal/kg

reported by Lee et al. (1995). Flaxseed oil may be used as an energy source when formulating diets, but with less energy compared to the corn and soy oil values listed in the NRC (1994). Previous investigations of flaxseed $AME_n$ reported different age-sensitive tolerances to dietary flaxseed by poultry. In an experiment feeding 10% flaxseed supplemented diets to broiler chicks, significantly lower tolerance was observed, manifesting as diarrhea, compared to mature single comb white leghorn roosters fed flaxseed diets (Gonzalez-Esquerra and Leeson, 2000).

Processing also affects $AME_n$ of flaxseed supplemented diets. Pelleting (4,578 kcal/kg) or crumbling (4,277 kcal/kg) significantly increases $AME_n$ fed to roosters compared to mash (3,659 kcal/kg; Gonzalez-Esquerra and Leeson, 2000). Extrusion processing of an ALA feed supplement containing 60% flaxseed improved $AME_n$ by 18% (Bean and Leeson, 2002). The additional pressure and heat of processing may release trapped oil from the matrix of the cell or the additional heat may destroy some of the toxic anti-nutrients in the feed (Calet, 1965; Gonzalez-Esquerra and Leeson, 2000). Therefore, pelleting and extrusion may be a method to offset the energy deficit of flaxseed oil compared to soy oil.

Flaxseed ingredients are an effective laying hen feed supplement for generating value-added omega-3 fatty acid enriched eggs. The results reported here demonstrate that degummed flaxseed oil is a well-tolerated, energy dense feed ingredient with a more efficient ability to transfer ALA, but not EPA and DHA, to egg yolk as compared to ground flaxseed. Producers can benefit from using flaxseed oil as an alternative to ground flaxseed because it takes less feed volume for supplementation and it is more cost effective (about half of the cost). Producers can use the regression equations from this experiment to develop custom omega-3 fatty acid supplemented diets to adapt to changes in consumer egg preferences.

Future investigation should include characterization of performance and more importantly the health benefits of poultry consuming ALA, EPA, and DHA supplemented diets.

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## TABLES

**Table 2.1.** Calculated and analyzed values for 11 control and experimental laying hen diets used to evaluate the transfer rate of dietary omega-3 fatty acids into the egg yolk from 25 to 33 wk of age.

| | Control | Flaxseed oil[1] | | | | | Ground flaxseed (% oil concentration) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Ingredients (%)** | **diet** | **0.5** | **1.0** | **2.0** | **3.0** | **5.0** | **0.5** | **1.0** | **2.0** | **3.0** | **5.0** |
| Corn | 42.86 | 42.86 | 42.86 | 42.86 | 42.86 | 42.86 | 41.57 | 40.27 | 37.68 | 35.08 | 29.89 |
| Soybean meal 48% CP[2] | 34.20 | 34.20 | 34.20 | 34.20 | 34.20 | 34.20 | 33.99 | 33.78 | 33.37 | 32.95 | 32.12 |
| DDGS[3] | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 |
| Meat and bone meal | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 |
| Flaxseed (ground) | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1.50 | 3.00 | 6.00 | 9.00 | 15.00 |
| Flaxseed oil | 0.00 | 0.50 | 1.00 | 2.00 | 3.00 | 5.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| Soy oil | 5.86 | 5.36 | 4.86 | 3.86 | 2.86 | 0.86 | 5.87 | 5.89 | 5.92 | 5.95 | 6.01 |
| Sodium chloride | 0.41 | 0.41 | 0.41 | 0.41 | 0.41 | 0.41 | 0.40 | 0.40 | 0.40 | 0.40 | 0.39 |
| DL-methionine | 0.28 | 0.28 | 0.28 | 0.28 | 0.28 | 0.28 | 0.28 | 0.28 | 0.28 | 0.28 | 0.29 |
| L-threonine | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 | 0.04 | 0.05 | 0.05 | 0.06 |
| Calcium carbonate[4] | 9.80 | 9.80 | 9.80 | 9.80 | 9.80 | 9.80 | 9.80 | 9.80 | 9.80 | 9.80 | 9.78 |
| Dicalcium phosphate | 1.94 | 1.94 | 1.94 | 1.94 | 1.94 | 1.94 | 1.93 | 1.93 | 1.92 | 1.91 | 1.89 |
| Choline chloride | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 |
| V and M premix[5] | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 |
| **Calculated values** | | | | | | | | | | | |
| ME (kcal/kg)[6] | 2900 | 2900 | 2900 | 2900 | 2900 | 2900 | 2900 | 2900 | 2900 | 2900 | 2900 |
| Omega-3 fatty acids (mg)[7] | 473 | 710 | 946 | 1419 | 1892 | 2837 | 754 | 1036 | 1598 | 2161 | 3286 |
| Omega-6 fatty acids (mg)[8] | 4095 | 3911 | 3726 | 3356 | 2986 | 2247 | 4154 | 4217 | 4339 | 4461 | 4705 |
| Omega-6:3 ratio | 8.7 | 5.5 | 3.9 | 2.4 | 1.6 | 0.8 | 5.5 | 4.1 | 2.7 | 2.1 | 1.4 |
| **Analyzed values (%)** | | | | | | | | | | | |
| Crude protein | 21.51 | 22.48 | 21.84 | 22.29 | 20.49 | 22.25 | 21.01 | 21.33 | 22.16 | 22.26 | 22.40 |
| Crude fat | 6.67 | 7.08 | 6.90 | 7.01 | 6.49 | 7.39 | 7.79 | 8.53 | 9.27 | 10.90 | 13.36 |
| Crude fiber | 3.43 | 3.41 | 3.22 | 3.05 | 2.74 | 3.35 | 3.30 | 3.18 | 3.49 | 3.41 | 4.33 |
| Moisture | 9.51 | 9.05 | 9.68 | 8.88 | 9.64 | 6.06 | 9.50 | 8.70 | 9.10 | 8.81 | 8.03 |
| Ash | 13.68 | 14.10 | 13.47 | 13.58 | 11.71 | 12.01 | 12.35 | 11.60 | 12.10 | 11.90 | 12.59 |

**Table 2.1 continued.**

[1]Extracted degummed flaxseed oil was added at the expense of 5.86% soy oil to generate individual diets that contained 0.5, 1.0, 2.0, 3.0, and 5.0% flaxseed oil, along with the 5.0% flaxseed oil with antioxidant diet.

[2]CP = crude protein.

[3]DDGS = distillers dried grains with solubles.

[4]Calcium carbonate added was a 50/50 mixture of small (≤ 2 mm) and large particle (> 2 mm).

[5]Vitamin and mineral premix provided per kg of diet: selenium 200 µg; vitamin A 6,600 IU; vitamin $D_3$ 2,200 IU; vitamin E 14.3 IU; menadione 880 µg; vitamin $B_{12}$ 9.4 µg; biotin 33 µg; choline 358 mg; folic acid 1.1 mg; niacin 33 mg; pantothenic acid 8.8 mg; pyridoxine 880 µg; riboflavin 4.4 mg; thiamine 1.1 mg; iron 226 mg; magnesium 100 mg; manganese 220 mg; zinc 220 mg; copper 22 mg; iodine 675 µg.

[6]The ME value of flaxseed oil and soy oil ingredients were assigned a value of 8,800 kcal/kg for the diet calculations.

[7]Calculated dietary omega-3 fatty acid content (alpha-linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid) mg per 100 g of completed feed.

[8]Calculated dietary omega-6 fatty acid content (linoleic acid and arachidonic acid) mg per 100 g of completed feed.

**Table 2.2.** Analyzed crude fat, fatty acid, and total omega-3 fatty acid concentrations in extracted degummed flaxseed oil and ground flaxseed ingredients.

| Fatty acid[1] (C:double bond) | Flaxseed oil % | Ground flaxseed % |
|---|---|---|
| Myristic acid (14:0) | 0.06 | 0.08 |
| Palmitic acid (16:0) | 5.72 | 6.06 |
| Palmitoleic acid (16:1) | 0.13 | 0.70 |
| Margaric acid (17:0) | 0.05 | 0.00 |
| Stearic acid (18:0) | 3.72 | 4.05 |
| Oleic acid (18:1) | 18.49 | 18.66 |
| Vaccenic acid (18:1) | 0.69 | 0.00 |
| Linoleic acid (18:2) | 15.19 | 15.24 |
| Alpha-linolenic acid (18:3) | 54.21 | 55.21 |
| Arachidic acid (20:0) | 0.58 | 0.00 |
| Arachidonic acid (20:4) | 0.09 | 0.00 |
| Eicosapentaenoic acid (20:5) | 0.16 | 0.00 |
| Docosapentaenoic acid (22:5) | 0.38 | 0.00 |
| Docosahexaenoic acid (22:6) | 0.46 | 0.00 |
| Crude fat (%)[2] | > 99 | 34 |
| Total omega-3 fatty acid (%)[3] | 54.83 | 55.21 |

[1]The fatty acid values represent the composition (%) of the crude fat in each ingredient.
[2]Crude fat (%) was the analyzed fat content of each feed ingredient.
[3]Total omega-3 fatty acid (%) was the sum of alpha-linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid.

**Table 2.3.** Calculated compositions and analyzed values of 4 laying hen diets used for the apparent metabolizable energy, nitrogen corrected ($AME_n$) assay fed from 33 to 35 wk of age.

| | Basal | Flaxseed oil concentration (%) | | |
|---|---|---|---|---|
| **Ingredients** | **0.0** | **3.0** | **6.0** | **9.0** |
| Corn | 60.18 | 58.37 | 56.57 | 54.76 |
| Meat and bone meal | 2.00 | 1.94 | 1.88 | 1.82 |
| Soybean meal 48 | 25.01 | 24.26 | 23.51 | 22.76 |
| Soy oil | 1.33 | 1.29 | 1.25 | 1.21 |
| Flaxseed oil | 0.00 | 3.00 | 6.00 | 9.00 |
| Sodium chloride | 0.42 | 0.40 | 0.39 | 0.38 |
| DL-methionine | 0.15 | 0.15 | 0.14 | 0.14 |
| Calcium carbonate[1] | 9.24 | 8.96 | 8.68 | 8.40 |
| Dicalcium phosphate | 0.87 | 0.84 | 0.82 | 0.79 |
| Titanium dioxide | 0.30 | 0.29 | 0.28 | 0.27 |
| Phytase | 0.00075 | 0.00073 | 0.00071 | 0.00068 |
| V and M premix[2] | 0.50 | 0.49 | 0.47 | 0.46 |
| **Calculated values (%)** | | | | |
| ME (kcal/kg) | 2800 | - | - | - |
| Calcium | 4.00 | 3.88 | 3.76 | 3.64 |
| Available phosphorus | 0.35 | 0.34 | 0.33 | 0.32 |
| **Analyzed values (%)** | | | | |
| Dietary $AME_n$ as-fed (kcal/kg) | 2762 | 2948 | 3218 | 3421 |
| Crude protein | 18.02 | 17.72 | 17.13 | 16.76 |
| Crude fat | 2.95 | 4.84 | 7.89 | 10.80 |
| Crude fiber | 3.18 | 2.30 | 2.35 | 2.19 |
| Moisture | 10.14 | 9.99 | 9.68 | 9.61 |
| Ash | 14.04 | 14.29 | 11.21 | 10.63 |

[1]Calcium carbonate added was a 50/50 mixture of small (≤ 2 mm) and large particle (> 2 mm).

[2]Vitamin and mineral premix provided per kg of diet: selenium 200 μg; vitamin A 6,600 IU; vitamin $D_3$ 2,200 IU; vitamin E 14.3 IU; menadione 880 μg; vitamin $B_{12}$ 9.4 μg; biotin 33 μg; choline 358 mg; folic acid 1.1 mg; niacin 33 mg; pantothenic acid 8.8 mg; pyridoxine 880 μg; riboflavin 4.4 mg; thiamine 1.1 mg; iron 226 mg; magnesium 100 mg; manganese 220 mg; zinc 220 mg; copper 22 mg; iodine 675 μg.

**Table 2.4.** Laying hen performance[1] from 25 to 33 wk of age as affected by increasing dietary inclusion of flaxseed oil (FSO) or ground flaxseed (GFS).[2]

| | ADFI (g/bird) | | HHEP (%) | | Feed efficiency (kg eggs/kg feed) | | Egg weight (g/egg) | | ADEM (g/bird) | | Egg solids (%) | | BW change (g) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Oil (%)** | **FSO** | **GFS** | **FSO** | **GFS** | **FSO** | **GFS** | **FSO** | **GFS** | **FSO** | **GFS** | **FSO** | **GFS** | **FSO** | **GFS** |
| **Control 0.0** | 93.5 | | 95.8 | | 0.600 | | 57.6 | | 55.3 | | 23.6 | | 75.1 | |
| **0.5** | 92.3 | 92.9 | 91.4 | 98.3 | 0.583 | 0.640 | 58.7 | 59.4 | 53.8 | 58.4 | 23.3 | 23.5 | 54.8 | 79.2 |
| **1.0** | 94.4 | 93.1 | 96.9 | 96.6 | 0.597 | 0.603 | 58.0 | 57.9 | 56.2 | 56.0 | 23.5 | 23.4 | 78.6 | 74.8 |
| **2.0** | 93.8 | 92.7 | 98.3 | 97.7 | 0.586 | 0.606 | 55.8 | 57.3 | 54.9 | 56.1 | 23.7 | 23.6 | 80.5 | 38.1 |
| **3.0** | 94.5 | 93.1 | 96.4 | 95.1 | 0.593 | 0.589 | 57.7 | 57.5 | 55.8 | 54.7 | 23.4 | 23.5 | 77.2 | 58.8 |
| **5.0** | 90.8 | 93.8 | 96.2 | 96.9 | 0.602 | 0.591 | 56.4 | 57.2 | 54.3 | 55.5 | 23.0 | 23.5 | 61.1 | 24.2 |
| **SEM** | 1.59 | | 1.23 | | 0.0115 | | 0.98 | | 1.05 | | 0.22 | | 11.71 | |
| **Contrast *P*-values** | | | | | | | | | | | | | | |
| Control vs. Flax | 0.82 | | 0.67 | | 0.93 | | 1.00 | | 0.78 | | 0.64 | | 0.32 | |
| FSO vs. GFS | 0.97 | | 0.18 | | 0.07 | | 0.39 | | 0.10 | | 0.53 | | 0.04 | |
| **Linear** | | | | | | | | | | | | | | |
| Oil % | 0.69 | | 0.58 | | 0.24 | | 0.05 | | 0.17 | | 0.49 | | 0.02 | |
| Oil % × Flax | 0.33 | | 0.09 | | 0.01 | | 0.94 | | 0.30 | | 0.26 | | 0.02 | |
| **Quadratic** | | | | | | | | | | | | | | |
| Oil % | 0.37 | | 0.24 | | 0.17 | | 0.23 | | 0.77 | | 0.19 | | 0.37 | |
| Oil % × Flax | 0.20 | | 0.01 | | 0.29 | | 0.92 | | 0.07 | | 0.28 | | 0.14 | |
| **Cubic** | | | | | | | | | | | | | | |
| Oil % | 0.94 | | 0.03 | | 0.78 | | 0.13 | | 0.97 | | 0.43 | | 0.73 | |
| Oil % × Flax | 0.94 | | 0.15 | | 0.46 | | 0.64 | | 0.61 | | 0.67 | | 0.12 | |

[1]Performance parameters measured were average daily feed intake (ADFI), hen-housed egg production (HHEP), feed efficiency, egg weight, average daily egg mass (ADEM), egg solids 4, 6, and 8 wk average, and body weight (BW) change from 25 to 33 wk of age.
[2]Hens were fed a control diet or one of the following omega-3 fatty acid supplemented diets: degummed flaxseed oil (FSO) treatments containing 0.5, 1.0, 2.0, 3.0, or 5.0% FSO content, or ground flaxseed (GFS) treatments containing 0.5, 1.0, 2.0, 3.0, or 5.0% FSO concentration (GFS included at 1.5, 3.0, 6.0, 9.0, or 15.0% of the diet, respectively).

## FIGURES

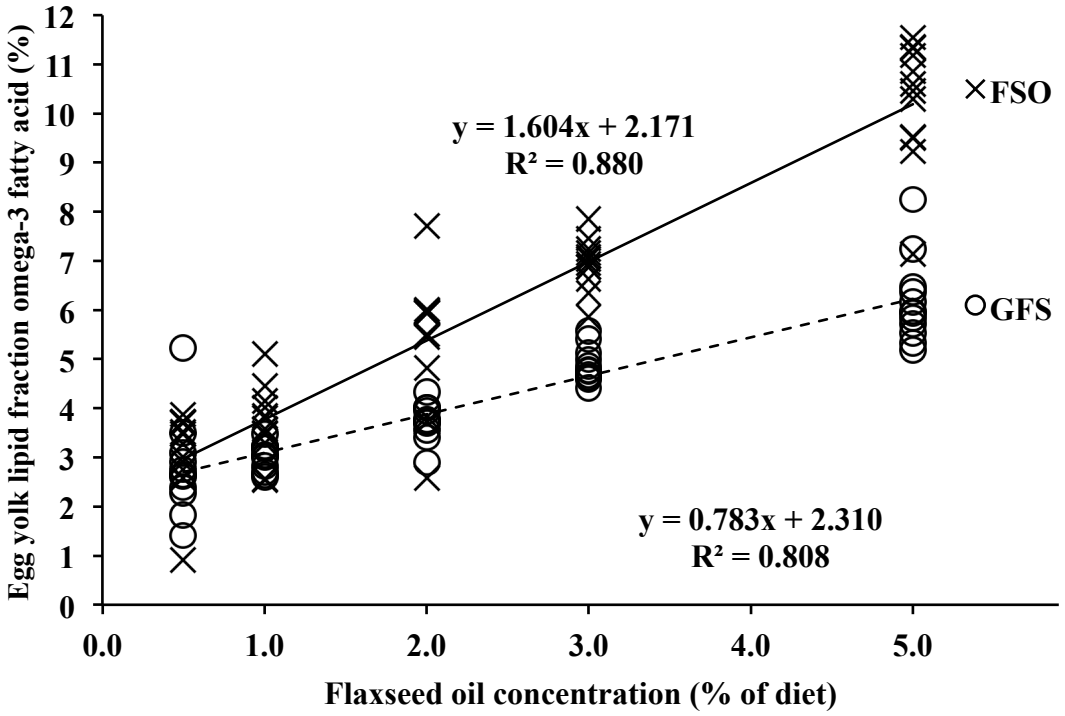


**Figure 2.1.** Egg yolk total omega-3 fatty acid content (ALA, EPA, and DHA) from hens (25 to 33 wk of age) fed one of the following experimental diets: flaxseed oil (FSO) treatments containing 0.5, 1.0, 2.0, 3.0, or 5.0% FSO content, or ground flaxseed (GFS) treatments containing 0.5, 1.0, 2.0, 3.0, or 5.0% FSO concentration (GFS included at 1.5, 3.0, 6.0, 9.0, or 15.0% of the diet respectively). Increasing dietary inclusions of FSO or GFS resulted in linear increases of total omega-3 fatty acid deposition into egg yolk ($P \leq 0.01$). FSO slope SE = ± 0.0779; intercept SE = ± 0.2184. GFS slope SE = ± 0.0501; intercept SE = ± 0.1403.

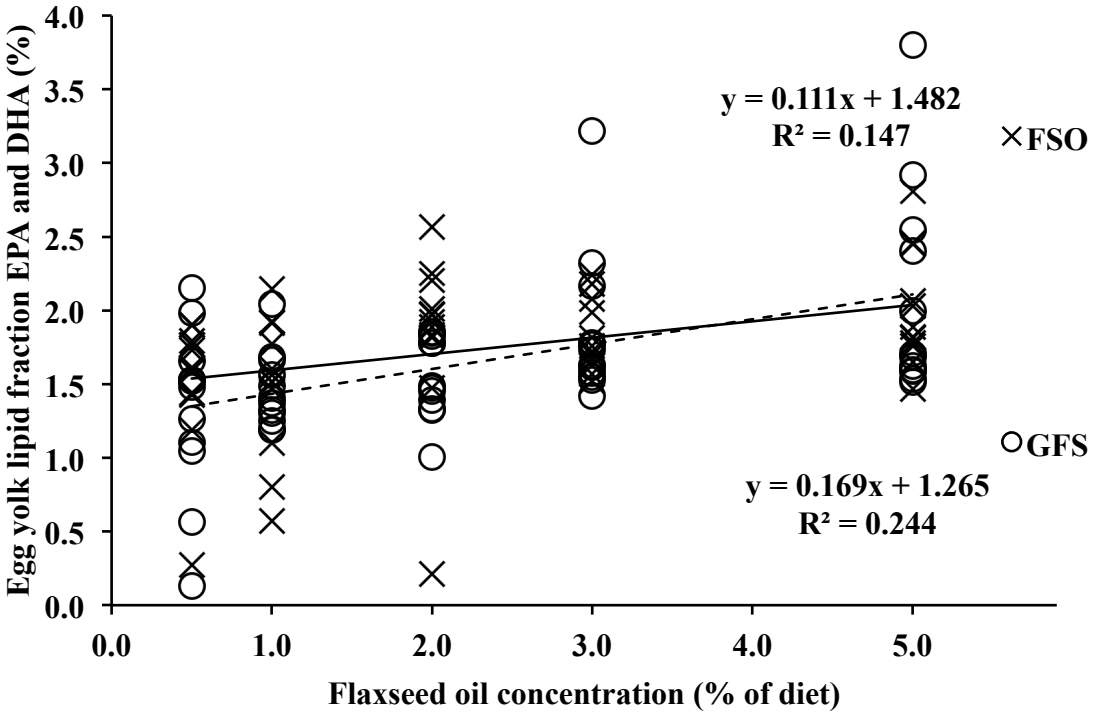


**Figure 2.2.** Egg yolk EPA and DHA content from hens (25 to 33 wk of age) fed one of the following experimental diets: flaxseed oil (FSO) treatments containing 0.5, 1.0, 2.0, 3.0, or 5.0% FSO content, or ground flaxseed (GFS) treatments containing 0.5, 1.0, 2.0, 3.0, or 5.0% FSO concentration (GFS included at 1.5, 3.0, 6.0, 9.0, or 15.0% of the diet respectively). Increasing dietary inclusions of FSO or GFS resulted in increased EPA and DHA deposition into egg yolk ($P \leq 0.01$); however, there was no difference in EPA and DHA egg yolk deposition based on flaxseed ingredient ($P = 0.21$). FSO slope SE = ± 0.0351; intercept SE = ± 0.0984. GFS slope SE = ± 0.0389; intercept SE = ± 0.1091.

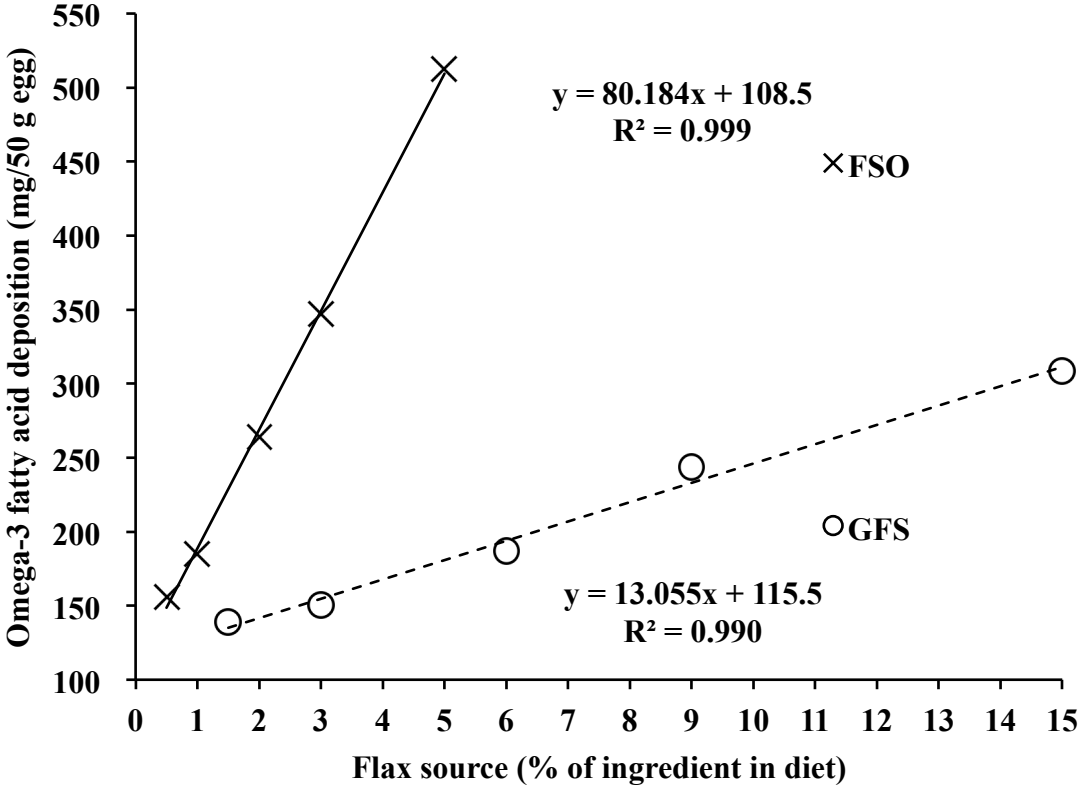


**Figure 2.3.** Calculated egg yolk total omega-3 fatty acid (ALA, EPA, and DHA) deposition (mg) per 50 g egg containing 10% total fat (AEB, 2017). Extrapolated omega-3 fatty acid deposition values were calculated using egg yolk total omega-3 fatty acid content (%) from hens (25 to 33 wk of age) fed the following experimental diets: flaxseed oil (FSO) treatments containing 0.5, 1.0, 2.0, 3.0, or 5.0% FSO content, or ground flaxseed (GFS) treatments containing 0.5, 1.0, 2.0, 3.0, or 5.0% FSO concentration (GFS included at 1.5, 3.0, 6.0, 9.0, or 15.0% of the diet respectively). FSO slope SE = ± 3.8950; intercept SE = ± 10.92. GFS slope SE = ± 0.8350; intercept SE = ± 7.02.

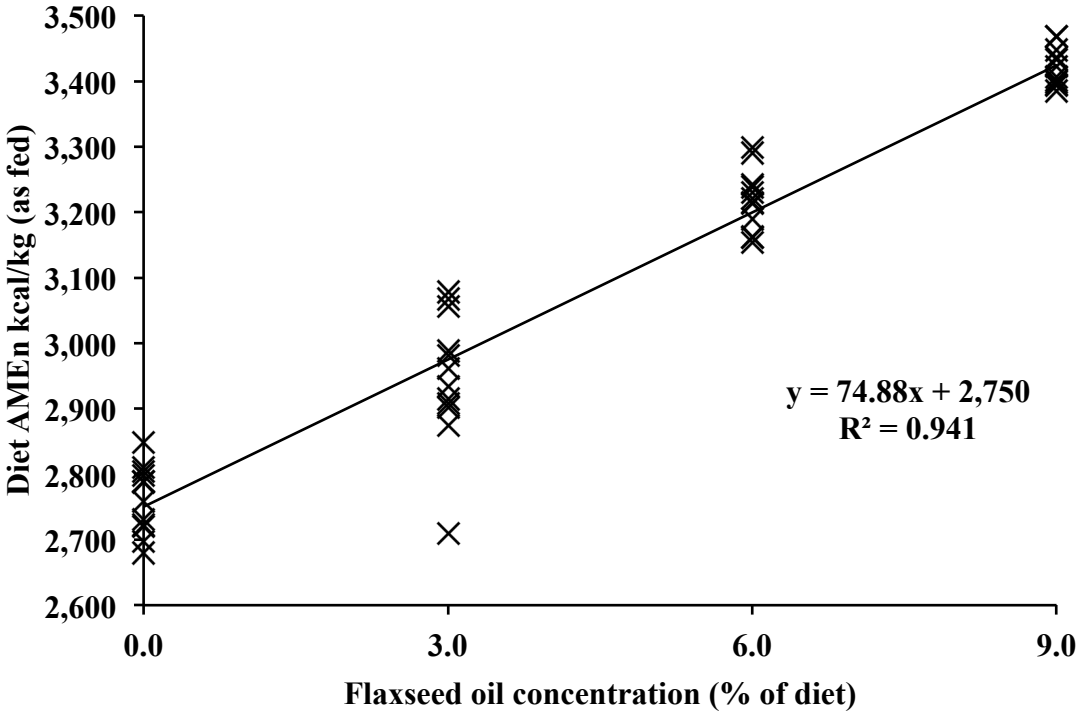


**Figure 2.4.** Dietary $AME_n$ values on an as-fed basis for hens fed diets containing 0.0, 3.0, 6.0, and 9.0% extracted degummed flaxseed oil (FSO) added to a basal diet for 2 wk (33 to 35 wk of age). Increasing dietary inclusion of FSO resulted in a linear increase in $AME_n$ ($P \leq 0.01$). Slope SE = ± 2.771; intercept SE = ± 15.6.

# CHAPTER 3. PRODUCTION AND HEALTH RESPONSES OF AGED LAYING HENS TO DIETARY FLAXSEED OIL OR FISH OIL SUPPLEMENTATION

A manuscript to be submitted to *Poultry Science*

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## 3.1 Abstract

Supplementation of omega-3 fatty acids in laying hen diets generates value-added eggs for consumers, and may also improve laying hen performance and health. Alpha-linolenic (ALA), eicosapentaenoic (EPA), and docosahexaenoic acids (DHA) are omega-3 fatty acids linked to exerting positive bone health and anti-inflammatory effects. Two sources of dietary omega-3 fatty acids, flaxseed and fish oils, were evaluated over a 12-wk period in end-of-second-cycle Hy-Line W-36 laying hens (n = 234; 122 to 134 wk of age). Experimental diets consisted of a 4% flaxseed oil diet (2,200 mg ALA/100 g feed), a 4% fish oil diet (1,200 mg EPA + DHA/100 g feed), and a 4% soy oil diet as control (280 mg ALA/100 g feed). Hens were randomly housed 6 birds/cage (13 replicates/treatment) for each of the 3 treatments. Production performance, bone strength, bone conformation, and bone mineral content were evaluated during the 12-wk trial period. Immediately following the supplementation period, 1 hen/experimental unit (EU) received saline and another hen/EU

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received a single lipopolysaccharide (LPS; 1 mg/kg) injection to induce acute systemic inflammation. Liver was harvested 12 h post-LPS challenge for qRT-PCR cytokine analysis. Dietary treatments were independently contrasted to the control using PROC MIXED in SAS with significance considered at $P \leq 0.05$. During the supplementation period, hens fed flaxseed oil consumed 1.2 ± 0.19 g/hen/d more feed than the control, while hens fed fish oil consumed 0.8 ± 0.19 g/hen/d less feed and laid 2.39 g ± 0.556 lighter eggs than the control ($P \leq 0.01$). No differences were observed in bone strength, conformation, or ash comparing either dietary treatment to the control ($P \geq 0.40$). Hens fed the control diet and injected with LPS resulted in increased IL-1β, IL-6, IL-18, and PPARγ expression by > 4-fold compared to saline-injected control hens ($P \leq 0.05$). Feeding flaxseed oil or fish oil to hens resulted in an intermediate response in inflammatory cytokine gene expression compared to control ($P \geq 0.06$), demonstrating anti-inflammatory protection. The data from this experiment demonstrated that flaxseed oil or fish oil supplementation did not improve performance and did not have an effect on bone health in aged laying hens. Although both oils provided anti-inflammatory protection, it is unclear if that effect is beneficial when generating value-added eggs in commercial applications.

Key words: ALA, EPA, DHA, inflammation, lipopolysaccharide

## 3.2 Introduction

Feeding laying hens diets supplemented with omega-3 fatty acids can yield value-added enriched eggs, as demonstrated in Chapter 2 of this dissertation (Fraeye et al., 2012). The essential omega-3 fatty acid alpha-linolenic acid (ALA; C18:3), found in flaxseed oil, is the precursor for endogenously synthesized eicosapentaenoic acid (EPA; C20:5) and

docosahexaenoic acid (DHA; C22:6; Burdge and Calder, 2006). Dietary omega-3 fatty acids found in fish oil, specifically EPA and DHA, have been reported to modulate bone remodeling activity and exhibit anti-inflammatory properties in murine and *in vitro* experiments (Sun et al., 2003; Moon et al., 2012; Tur et al., 2012). Laying hens are at risk for and often suffer from osteoporosis near or at the end of production cycles, a condition commonly referred to as soft bones or layer fatigue (Saif et al., 2008). Dietary ALA supplementation has been linked to reducing the incidence of fractures in free-range laying hens (Tarlton et al., 2013). Laying hens are also under physical and environmental stress during commercial egg production, which contributes to inflammation (Lay et al., 2011; Zhao et al., 2016). Dietary ALA or EPA and DHA supplementation for these vulnerable hens may help mitigate bone and inflammation related health concerns, serving an overlooked second benefit to generating value-added enriched eggs for consumers.

Flax plant species naturally produce the essential omega-3 fatty acid ALA, which is stored in their seeds (Goyal et al., 2014). Flaxseeds contain 40% total fat and have been used as a dietary ALA source in laying-hen diets (Fraeye et al., 2012). The abundance of ALA in flaxseed ranges from 39 to 60% of the fatty acid profile, dependent upon genetic variety (Goyal et al., 2014). Omega-3 fatty acid-containing ingredients from marine sources, such as fish and microalgae, naturally contain EPA and DHA in varying quantities, which can be concentrated and blended into different concentrations during oil extraction (Gunstone, 2006; Mozaffarian and Wu, 2011; Fraeye et al., 2012). Although plant-derived ALA can be converted into EPA and DHA through endogenous elongation, desaturation, and beta-oxidative reactions, birds have a limited ability to do so (Harris et al., 2008; Gregory et al., 2011; Gregory et al., 2013). Depending on avian species, only 5% of dietary ALA is

endogenously converted to EPA and < 1% to DHA (Burdge and Calder, 2006; Zivkovic et al., 2011). Because not all omega-3 fatty acids (ALA, EPA, and DHA) have the same biological functions, consuming different proportions of the fatty acids may result in different effects on bone remodeling and attenuating inflammation.

Previous studies investigating end-of-cycle laying hens (≥ 72 wk of age) housed in cage systems concluded that up to 29% of broken bones were attributed to removal from cages and placement onto processing lines (Gregory and Wilkins, 1989; Knowles and Wilkins, 1998). Regardless of housing system, the incidence of broken bones, especially the keel (sternum), remains quite high for laying hens, 28 to 89% in aviary systems and 22 to 44% in furnished caged systems (Wilkins et al., 2011; Nasr et al., 2012). Tarlton et al. (2013) reported that a 10% dietary inclusion of an ALA feed additive (50% flaxseed and 50% carrier grain) reduced incidence of fracture and bone deformity in free-range laying hens compared to hens fed unsupplemented conventional diets. Further investigation is needed to evaluate the health benefits of ALA or EPA and DHA supplementation in end-of-cycle laying hens with weakened bones, susceptible to fractures, using the predominant laying hen strain and housing system found in the US (Hy-Line W-36 in a conventional cage system).

Aged laying hens housed in commercial environments are exposed to bacterial and physical stressors, such as commensal *E. coli* and feeder competition (Lay et al., 2011; Zhao et al., 2016). Overstimulation of the immune system and chronic inflammation by pathogenic infections reduce laying hen performance, which includes egg production and quality (Saif et al., 2008; Bai et al., 2014). Dietary EPA and DHA were found to play functional anti-inflammatory roles in pre-clinical human studies, where the test subjects receiving the dietary supplementation had reduced hepatic synthesis of acute phase proteins and pro-inflammatory

cytokine IL-6 (Cottin et al., 2011; Skulas-Ray, 2015). Therefore, dietary supplementation of omega-3 fatty acids (ALA or EPA and DHA) may mitigate inflammatory stress encountered by laying hens in commercial environments by reducing the intensity of an inflammatory acute phase response (Korver and Klasing, 1997; Bai et al., 2014).

End-of-cycle laying hens make an ideal model for investigating the effect of dietary supplements on bone health and mitigation of inflammatory stress because of their rapid bone turnover and physiological stress (Cook, 2000; Rodenburg et al., 2008; Wilkins et al., 2011). Commercial environments are dynamic, potentially posing physiological stress in a multifactorial fashion with housing, management, handling, infectious disease, and nutrition impacting hen health and performance (Silversides et al., 2006, 2012; Van Hoorebeke et al., 2010). An inducible stress model needs to be applied to aged laying hens in order to investigate the protective effects that supplementation of dietary ALA or EPA and DHA may offer.

Previous experiments have demonstrated that lipopolysaccharide (LPS) administration to laying hens and broilers resulted in controlled pro-inflammatory acute phase response and systemic inflammation (Korver et al., 1998; Leshchinsky and Klasing, 2001; Munyaka et al., 2013). Lipopolysaccharides are structural outer membrane components of Gram-negative bacteria and recognized as pathogenic molecules (Schwechheimer and Kuehn, 2015). Although not identical to pathogenic challenges, LPS administration is a well-described model for induced systemic inflammation in poultry (Korver et al., 1998; Jiang et al., 2010; Munyaka et al., 2013). Lipopolysaccharide exposure in laying hens and broilers resulted in depressed feed consumption, Toll-like receptor 4 stimulation, and increased

splenic interleukin-1 beta (IL-1β), interferon gamma (IFNγ), and IL-10 cytokine gene expression (Korver et al., 1998; Jiang et al., 2010; Munyaka et al., 2013).

The objective of this experiment was to investigate the effect of dietary omega-3 fatty acid supplementation (flaxseed oil as ALA source; fish oil as EPA and DHA source) on end-of-second-cycle laying hens with regards to a) performance and bone health of the hens during and at the end of a supplementation period, and b) inflammatory response (cytokine gene expression) of the hens to a post-supplementation acute LPS challenge. The hypothesis of this experiment was that bioactive EPA and DHA supplementation would improve the bone health of aged laying hens and provide anti-inflammatory protection during a period of stress without negatively impacting performance, which may be beneficial for the producer and commercial hens.

## 3.3 Materials and Methods

### 3.3.1 Animals and Housing

The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee. Laying hens were cared for in accordance with the FASS Guide for the Care and Use of Agricultural Animals in Research and Teaching (2010). Two hundred thirty-four Hy-Line W-36 laying hens (122 wk of age; BW 1.628 ± 0.0078 kg) were obtained from a commercial producer (Litchfield, MN) and transported to the Poultry Research and Teaching Unit at Iowa State University, where they were housed in single-tier conventional cages (W 30.5 × D 43.2 × H 45.2 cm) with 360° nipple waterlines at a stocking density of 438 $cm^2$/hen. The lighting program was 15.5L:8.5D (light from 5:00 am to 8:30 pm) with 30 lux light intensity, as recommended by the Performance Standards Manual (Hy-

Line, 2012). Manure was collected in shallow pits below the cages for the duration of the experiment. All hens were observed twice daily and house temperature ranged from 20 to 27°C.

### 3.3.2 Omega-3 Fatty Acid Supplementation Period

Hens were fed corn-soybean meal-wheat middlings-based experimental diets as follows: 1) basal diet + 4% soy oil (280 mg ALA/100 g feed; control); 2) basal diet + 4% flaxseed oil (2,200 mg ALA supplemented/100 g feed; flaxseed oil); or 3) basal diet + 4% fish oil (720 mg EPA and 480 mg DHA omega-3 fatty acids supplemented/100 g feed; fish oil). All diets were mixed every 2 wk and met or exceeded the NRC (1994) nutritional requirements for laying hens (Table 3.1). Experimental units (EU) consisted of 2 adjacent cages housing 3 hens/cage (438 $cm^2$/hen stocking density) that shared a single feed trough (6 hens/EU). Experimental diets were assigned in a randomized complete block design to 13 EU/diet. The 4% flaxseed oil and 4% fish oil dietary inclusions resulted in unequal total omega-3 fatty acid concentrations in the treatment diets (Table 3.2). Therefore, the flaxseed oil and fish oil treatments were independently compared to the soy oil control in this experiment. Each hen was offered a range of 95 to 100 g/d feed in an attempt to control and equalize feed consumption across the dietary regimens. Previous work has demonstrated that it may take laying hens up to 6 wk to reach an omega-3 fatty acid transfer rate plateau (Ehr et al., 2017). Therefore, the dietary supplementation period lasted 12 consecutive wk (122 to 134 wk of age) to allow for omega-3 fatty acid incorporation into laying hen tissues and egg yolk.

### 3.3.3 Feed Sample Measurements

Feed samples were collected for every batch generated and stored at -20°C. Samples were ground through a 0.5 mm screen (Thomas-Wiley Laboratory Mill Model 4, Arthur H. Thomas Company, Philadelphia, PA) and dried at 100°C (Precision, Jouan, Inc., Winchester, VA) for 18 h to determine dry matter (DM) content. Ground feed samples for each regimen were pooled by 4-wk period. Fat content was measured by Soxhlet extraction using diethyl ether at the University of Missouri Agricultural Experiment Station Chemistry Laboratories (Method 920.39; AOAC, 2010; AESCL, Columbia, MO). Nitrogen concentration was determined by thermocombustion (TruMac N Analyzer, LECO Corp., St. Joseph, MI) and GE was determined using an adiabatic oxygen bomb calorimeter (Parr Instrument Co., Moline, IL).

### 3.3.4 Production Performance Measurements

Feed disappearance, which included wasted feed from the trough, was measured weekly for feed intake and feed efficiency (FE) calculations: Average daily feed intake (ADFI) = start feed weight in kg – end feed weight in kg after 7 d ÷ 7 d ÷ hens in cage; FE = egg mass in kg ÷ FI in kg. Eggs were collected and recorded daily and used for calculation of hen-day egg production (HDEP, %), i.e., HDEP% = (eggs laid ÷ living hens ÷ d) × 100. Eggs from 2 consecutive d of production were weighed weekly and used together with HDEP in calculating average daily egg mass (g/hen), i.e., average daily egg mass (ADEM) = average egg weight in g × (HDEP% ÷ 100). Hens were individually weighed with a digital scale (Defender 3000, Ohaus Corp., Parsippany, NJ) at the start of the experiment for a baseline body weight (BW), and then at 4, 8 and 12 wk during the dietary supplementation period.

Eggs from each EU were individually measured over 2 consecutive d for egg quality parameters at the start of the experiment, 4, 8, and 12 wk into the dietary supplementation period using a Digital Egg Tester (DET6000, Nabel Co., Ltd., Kyoto-shi, Japan). The recorded egg quality parameters included egg weight, albumen height, Haugh unit, shell breaking strength, and shell thickness. The Haugh unit was calculated as follows from Silversides and Villeneuve (1994): Haugh unit = 100 × Log[albumen height in mm – (1.7 × egg weight in $g^{0.37}$ + 7.6)].

The albumen and yolk of 4 eggs from 8 replicates of each dietary treatment were pooled and homogenized (Immersion Hand Blender 59735, Hamilton Beach/Proctor-Silex, Inc., Southern Pines, NC) for egg solids DM analysis. Five g of each homogenized sample was weighed onto a dry, pre-weighed aluminum dish in duplicate and placed in a 100°C drying oven. Samples were removed from the oven after 18 h and placed into a desiccator, allowed to cool to 21°C, and then weighed. Egg solids content was calculated as follows: egg solids content = (dried egg sample in g ÷ wet egg sample in g) × 100.

### 3.3.5 Fatty Acid Analysis

A pooled sample of 13 egg yolks, 1 egg from each EU, was collected for fatty acid analysis at 4, 8, and 12 wk during the dietary supplementation period. The analysis started at 4 wk to allow an adjustment period for maximal transfer of omega-3 fatty acids to egg yolk (Cherian and Sim, 1991). The fatty acid analysis of the egg yolks and ground feed samples was performed as described by Nam et al. (2001) and Sun et al. (2013) using gas chromatography (HP 6890, Hewlett Packard Co., Palo Alto, CA).

### 3.3.6 Bone Measurements

Laying hens were evaluated for keel (sternum) bone conformation at the start of the experiment and then at 4, 8, and 12 wk of the dietary supplementation period. Each hen was supported by the thighs and dorsal surface of the back and held in a vertical position to allow the entire length of the keel to be palpated using the index finger and thumb. Every hen was examined and assigned a keel bone conformation palpation score on a 3-point scale (Table 3.3) adapted from Wilkins et al. (2004) and Tarlton et al. (2013).

At the conclusion of the 12-wk dietary supplementation period, one hen randomly selected from each of 8 separate EU was euthanized via $CO_2$ asphyxiation following the AVMA (2013) euthanasia guidelines. The use of 8 replicates per dietary regimen, rather than 13, was due to financial constraints. The left tibia was collected for bone ash analysis and the right tibia was used for breaking strength analysis. The left tibia soft tissue was manually removed after being autoclaved for 45 min at 121°C at 18 psi. Cleaned tibiae were placed in a drying oven at 100°C for 18 h before fat extraction in a Soxhlet fat extractor for 48 h using hexane as a solvent. Fat extracted bones were placed back into a drying oven for 18 h at 100°C to obtain their dry weight. The dried fat-extracted bone were ashed in a muffle furnace (Model 550-126, Fisher Scientific, Pittsburg, PA) at 580°C for 18 h. All dried and ashed samples were stored in a desiccator after immediate removal from ovens and furnaces and were allowed to cool to room temperature (21°C) prior to recording dry weight. Bone ash was calculated as follows: bone ash% = (bone ash weight ÷ dried fat-extracted bone weight) × 100.

To determine bone breaking strength, soft tissue was manually removed from the right tibia without autoclaving to preserve the structural integrity of the bone. Individual

tibiae were broken at the midpoint using methods adapted from Fleming et al. (1998) to measure bone breaking strength using Texture Exponent Software and TA.XTPlus Texture Analyzer fit with a TA-92A Self Supporting 3-Point Bend Rig (Texture Technologies Corp., Hamilton, MA).

### 3.3.7 Post-supplementation Inflammatory Challenge

At the end of the 12 wk dietary supplementation period, one randomly selected hen from each EU was injected intraperitoneally with 1 mg/kg LPS in sterile saline vehicle (L2630, *E. coli* 0111:B4 LPS, Sigma-Aldrich Co. LLC., St. Louis, MO) and a second randomly selected hen per EU was injected with the equivalent volume of sterile saline. Hens were given *ad libitum* access to experimental diets (control, flaxseed oil, or fish oil) and water throughout the inflammatory challenge. Twelve hours post-injection of LPS or saline, hens were euthanized via $CO_2$ asphyxiation and liver was harvested, flash frozen in liquid nitrogen, and stored at -80°C for further analysis.

### 3.3.8 Quantitative Real-Time PCR

RNA extraction and DNase cleanup followed manufacturer directions using flash frozen liver samples (RNeasy Mini Kit, Qiagen, Valencia, CA). Samples of RNA were analyzed in triplicate by quantitative real-time PCR (qRT-PCR; DNA Engine Opticon 2 System, Bio-Rad Lab. Inc., Hercules, CA) using 1-step SYBR Green master mix (QuantiTect SYBR Green RT-PCR, Qiagen, Valencia, CA) adapted from methods described by Kaiser et al. (2006). Relative RNA expression levels were measured for IL-1β, IL-6, IL-18, IFNγ, serum amyloid A (SAA), IL-10, and peroxisome proliferator-activated receptor gamma

(PPARγ) using ribosomal 28s subunit as a reference gene. Forward and reverse primers were generated (Integrated DNA Technologies, Coralville, IA) from sequences listed in Table 3.4 with referenced source. Adjusted cycle threshold (Adj. Ct) value and normalized gene expression level (fold change) were calculated using the following adapted formulas (Eldaghayes et al., 2006; Kaiser et al., 2012): Adj. Ct = 40 − [mean $Ct_{\text{test gene}}$ + ($median_{28s}$ − mean $Ct_{28s}$)] × ($slope_{\text{test gene}} \div slope_{28s}$); fold change = $2^{[-(Ct_{\text{test gene treatment}} - Ct_{\text{28s treatment}}) - (Ct_{\text{test gene control}} - Ct_{\text{28s control}})]}$.

### 3.3.9 Statistical Analysis

Data from the 12-wk supplementation period were analyzed by repeated measures ANOVA using the MIXED procedure in SAS (SAS 9.4, 2012, SAS Institute Inc., Cary, NC). Experimental diets were assigned in a randomized complete block design with each treatment consisting of 13 replicates with 6 hens/EU. Experimental diet (diet), time (wk), and the interaction (diet*wk) were used as fixed effects in the model with the random effect of cage nested within experimental diet. In all cases, flaxseed oil and fish oil treatments contained different total omega-3 fatty acid inclusions and were therefore not directly comparable.

Significant effects within the model included the soy oil control, flaxseed oil, and fish oil experimental diets, hence orthogonal contrasts were used to separate and test the individual dietary effect of flaxseed oil or fish oil treatment compared to the soy oil control diet. These data included HDEP, ADFI, egg weight, ADEM, FE, BW, egg quality parameters, and bone measurements with an experimental focus on the main effect of individual dietary treatment compared to control. Significant differences within the model due to time (wk) were presented for completeness.

The data from the LPS inflammatory challenge were analyzed using the MIXED procedure in SAS. Experimental diet (diet) and inflammatory challenge (challenge) and their interaction (diet*challenge) were used as fixed effects in the model with cage nested within experimental diet included as a random effect. Significant effects within the model included soy oil control, flaxseed oil, and fish oil experimental diets, therefore orthogonal contrasts were used to separate and test the individual dietary effect of flaxseed oil or fish oil treatment compared to the soy oil control. Orthogonal contrasts were used to test the effect of challenge (saline injection vs. LPS injection) and interactions (diet*challenge) separated by using flaxseed oil or fish oil treatment independently compared to the soy oil control. In all cases, significance was considered at $P \leq 0.05$.

## 3.4 Results

### 3.4.1 Omega-3 Fatty Acid Supplementation Impact on Production Performance

All inferences were based on orthogonal contrasts. Interactions (diet*wk) within the overall model were not considered significantly different because flaxseed oil and fish oil dietary treatments were independently contrasted to the soy oil control.

No differences were observed between hens receiving the flaxseed oil diet compared to the control hens in HDEP, FE, egg weight, ADEM, BW ($P \geq 0.56$; Table 3.5) or egg quality parameters ($P \geq 0.42$; Table 3.6) throughout the supplementation period. However, ADFI was 1.2 ± 0.19 g/hen greater for hens fed the flaxseed oil diet than for the control hens ($P \leq 0.01$).

Hens receiving the fish oil diet consumed 0.8 ± 0.19 g/hen/d less feed and laid 2.39 ± 0.556 g lighter eggs compared to the control hens ($P \leq 0.01$). No differences were detected

between hens fed the fish oil diet and the control hens in HDEP, FE, ADEM, or BW ($P \geq 0.17$; Table 3.5); and the same was true with egg quality measurements ($P \geq 0.13$; Table 3.6).

Time (wk) affected the production performance of HDEP, FI, FE, ADEM, BW, egg weight, albumen height, Haugh unit, and shell thickness for hens fed experimental diets ($P \leq 0.01$). All hens steadily declined in HDEP until wk 7 and then increased wk 10 through wk 12 of the dietary supplementation period, which was a result of hens progressing towards the end of production and the start of natural molt. The difference in HDEP over time affected ADEM and FE for all hens ($P \leq 0.01$). All hens consumed more feed as the supplementation period progressed ($P \leq 0.01$; Figure 3.1) and lost an average of 60 ± 10.5 g body weight until 8 wk, which normalized to baseline weight by 12 wk of dietary supplementation ($P \leq 0.01$; Figure 3.2A).

Time (wk) affected the albumen height and Haugh unit for all hens, which remained the same for 4 to 8 wk then increased wk 12 by 0.4 ± 0.08 mm and 2.3 ± 0.57 units, respectively ($P \leq 0.01$; Figure 3.2B). Egg weight increased for all hens compared to the starting value at 0 wk ($P \leq 0.01$; Figure 3.2C). Shell thickness increased for all hens from wk 4 to wk 8 by 0.05 ± 0.003 mm average and the thicker shell was maintained until the end of the 12 wk supplementation period ($P \leq 0.01$; Figure 3.2D).

### 3.4.2 Omega-3 Fatty Acid Transfer to Egg Yolk

Egg yolk fatty acid analysis confirmed that ALA, EPA, and DHA transfer occurred due to flaxseed oil and fish oil dietary supplementation, which followed their respective dietary fatty acid profiles. Egg yolks from hens fed the flaxseed oil diet contained the greatest ALA content (6.91%), resulting in 7 times lower omega-6:omega-3 fatty acid ratio compared

to yolks from the control hens. Egg yolks from hens fed the fish oil diet contained a greater EPA and DHA content (0.57% and 2.74%, respectively), resulting in 4 times lower omega-6:omega-3 fatty acid ratio compared to yolks from the soy oil-diet control hens (Table 3.7).

### 3.4.3 Omega-3 Fatty Acid Supplementation Impact on Bone Measurements

No dietary effects of the flaxseed oil supplementation were observed on keel bone conformation score, tibia bone ash, or bone breaking strength compared to the soy oil control diet ($P \geq 0.72$; Table 3.8). The same held true with the fish oil supplementation on bone conformation score ($P = 0.79$), tibia bone ash and bone breaking strength ($P \geq 0.40$; Table 3.8).

Differences in keel bone conformation score were detected over time (wk) for all hens, where the average score increased by 0.29 ± 0.035 from 4 to 8 wk and then decreased by 0.23 ± 0.035 from 8 to 12 wk back to baseline level ($P \leq 0.01$; Figure 3.2A).

### 3.4.4 Responses to Post-supplementation Inflammatory Challenge

To obtain baseline reference values and confirm immune system stimulation, the control hens were administered LPS or saline injections. Lipopolysaccharide administration given to the control hens caused increased relative gene expression of inflammatory cytokines IL-β, IL-6, and IL-18 by 4.4, 26.2, and 7.2-fold, respectively, compared to the control hens injected with saline ($P \leq 0.05$). Lipopolysaccharide injection to the control hens caused a 5.4-fold increase of PPARγ, the gene responsible for stimulating lipid uptake and adipogenesis, compared to the control hens injected with saline ($P = 0.02$; Figure 3.3A).

Hens fed the flaxseed oil supplemented diets and administered LPS or saline injection were contrasted to the respective reference hens fed the control diet. No differences due to dietary effect were observed for mRNA inflammatory cytokine gene expression, acute phase protein SAA, anti-inflammatory cytokine IL-10, or PPARγ for hens fed the flaxseed oil diet or soy oil control diet ($P \geq 0.79$). Lipopolysaccharide injection given to hens fed the flaxseed oil and control diets caused an increase in gene expression of inflammatory cytokines IL-6 and IL-18 by 10.0 and 4.4-fold, respectively, compared to hens fed flaxseed oil and control hens injected with saline ($P \leq 0.01$). Gene expression for PPARγ was 3.1-fold more in hens fed flaxseed oil and control hens injected with LPS compared to the hens fed flaxseed oil and control hens injected with saline ($P \leq 0.01$; Figure 3.3B).

Both LPS and saline injections given to hens fed the flaxseed oil diet resulted in a trending reduction in gene expression of inflammatory cytokines IL-1β, IL-6, IL-18 and PPARγ when compared to the LPS-injected control hens ($P \geq 0.08$). In addition, both LPS and saline injections given to hens fed the flaxseed oil diet responded with a trending increase in gene expression of inflammatory cytokines IL-1β, IL-6, IL-18 and PPARγ compared to the saline-injected control hens ($P \geq 0.08$). Overall, feeding flaxseed oil diets to hens demonstrated a trending intermediate response in gene expression of inflammatory cytokines at levels between those of the reference control hens injected with LPS or saline ($P \geq 0.08$; Figure 3.3A).

Additional laying hens were injected with LPS or saline to evaluate the protective anti-inflammatory effects of dietary fish oil supplementation. Hens fed the fish oil diet and injected with LPS or saline were contrasted to the reference control hens. Feeding the fish oil dietary treatment or the soy oil control diet to hens did not cause differences in mRNA gene

expression of inflammatory cytokines, acute phase protein SAA, anti-inflammatory cytokine IL-10, or PPARγ ($P \geq 0.43$). Lipopolysaccharide injection to hens fed the fish oil and control diets caused increased gene expression for inflammatory cytokines IL-6 and IL-18 by 7.5 and 4.3-fold, respectively, compared to saline injected hens fed the fish oil and control diets ($P \leq 0.01$). Lipopolysaccharide administration to hens fed the fish oil and control diets resulted in a 2.9-fold increase in gene expression of PPARγ compared to hens fed fish oil and control diets injected with saline ($P \leq 0.01$; Figure 3.3B).

Administration of LPS and saline injections to hens fed the fish oil diet resulted in a trending reduction in gene expression of inflammatory cytokines IL-1β, IL-6, IL-18 and PPARγ when compared to hens fed the soy oil control diet injected with LPS ($P \geq 0.06$). In addition, LPS and saline injection given to hens fed the fish oil diet resulted in a trending increase in gene expression of inflammatory cytokines IL-1β, IL-6, IL-18 and PPARγ compared to hens fed the soy oil control diet injected with saline ($P \geq 0.06$). Overall, feeding fish oil dietary treatments to hens resulted in a trending intermediate response in gene expression of inflammatory cytokines at levels between those of the reference control hens injected with LPS and saline ($P \geq 0.06$; Figure 3.3A).

## 3.5 Discussion

### 3.5.1 Production Performance during the Supplementation Period

Prior to the dietary supplementation period (122 to 134 wk of age), the hens performed to the expected breed standards. They were molted from 65 to 70 wk of age, then continued into second-cycle production. Upon experimental initiation at 122 wk of age, the hens had declined in HDEP to < 65 ± 1.8%, which was in agreement with previous work

reporting declining performance in hens 85 to 102 wk old (Khalaji et al., 2014). Furthermore, molting the hens in the current experiment caused a negative impact on eggshell proportion and increased egg deformities, which was in agreement with data reported by Arpášová et al. (2010) in 70 to 110 wk old molted hens.

The extended length of second-cycle production may have contributed to the declining flock performance observed in this experiment. Pooled average flock HDEP declined linearly from 122 to 130 wk of age and stabilized at approximately 50 ± 1.8% HDEP, as did FE, which eventually stabilized at 0.330 ± 0.0112 kg egg mass/kg feed (Figure 3.1). The flock started their second-cycle of egg production at 70 wk of age and had been in continuous production for 64 wk by the end of the 12 wk experiment. Physiological exhaustion may have caused the hens to decrease or go out of production and enter a natural molt to recover from such a long laying cycle. This may explain why BW and egg quality was lowest at 4 and 8 wk in this experiment. The 4 to 8 wk period (126 to 130 wk of age) of low performance may have provided time for recuperation, which allowed the flock to rebound by 12 wk (134 wk of age) and recover BW and egg quality losses, specifically Haugh unit, albumen height, and shell thickness (Figure 3.2A, 3.2B, and 3.2D). The flock performance data suggest that laying hens can persist in second-cycle egg production for a 54 wk timeframe until spontaneously going out of production or molting.

The pooled average flock BW was lowest (1.559 ± 0.0105 kg) at 8 wk in the experiment, which may have contributed to the elevated 8 wk flock bone conformation score of 1.95 ± 0.035. Leaner hens have less muscle and fat to impede fracture and lesion detection during keel bone examination and palpation (Wilkins et al., 2004; Casey-Trott et al., 2015). In addition, the tissue surrounding bone acts as protective padding, therefore reduced muscle

and fat on a skeletal frame may have allowed for more fractures or bone lesions to occur. The bone conformation scores returned to baseline levels at 12 wk in the experiment, which coincided with BW values returning back to baseline levels.

Feeding dietary flaxseed oil or fish oil supplementation to aged laying hens did not improve performance, egg quality, or bone health compared to hens fed the soy oil control diet. The lack of performance improvements was in agreement with the report by Toscano et al. (2012, 2015) who found that high inclusions of dietary omega-3 fatty acid supplementation (1,978 mg ALA, 346 mg EPA, and 303 mg DHA/100 g feed) fed to brown laying hens in aviary housing reduced performance with minimal increases in biomechanical bone strength. These findings contrast with Tarlton et al. (2013) reporting that dietary ALA supplementation (≥ 2,500 mg ALA/100 g feed) fed to free-range brown laying hens decreased keel fractures by 60% and increased keel bone breaking strength by 5 N compared to hens fed unsupplemented conventional diets, due to the dietary omega-6:omega-3 fatty acid ratio (Toscano et al., 2015). The conflicting results from the previously described studies suggest that further experimentation is needed.

The laying hens fed fish oil supplemented diets in this experiment consumed 0.8 ± 0.19 g feed/hen/d less compared to the control hens, which may have contributed to the 2.39 ± 0.556 g reduced egg weight. In a separate experiment, 36 wk old laying hens were fed 6% microencapsulated fish oil supplemented diets over a 3 wk supplementation period (Lawlor et al., 2010). Feed intake was not suppressed over the course of the 3 wk experiment, suggesting that microencapsulated oil might be better tolerated by laying hens than a free fish oil (Lawlor et al., 2010). Free fish oil supplementation included at ≥ 4% may be unpalatable

to laying hens, and microencapsulation of oil may be able to mask the unpleasant odors or flavors (Lawlor et al., 2010).

Environmental housing, management, and genetics are contributing factors in keel bone fractures and should be considered when evaluating the dietary effects of ALA or EPA and DHA supplementation on modulating bone and overall bone health (Silversides et al., 2012; Campbell et al., 2016; Stratmann et al., 2016). This experiment has shown that ALA or EPA and DHA supplementation did not decrease egg production or bone health in aged laying hens. In order to improve laying hen bone health in dynamic housing systems, the combination of genetic selection and dietary omega-3 fatty acid supplementation may be required to generate stronger bone biomechanical properties rather than supplementation alone (Fleming et al., 2004; Stratmann et al., 2016).

### 3.5.2 Responses to Post-supplementation Inflammatory Challenge

After the 12-wk omega-3 fatty acid (ALA or EPA and DHA) supplementation period, mRNA gene expression in the liver was altered in end-of-second-cycle laying hens due to the LPS challenge. Clinical signs of inflammation, such as fever and swelling, that manifest due to LPS exposure are modulated by activated immune cells producing cytokines, resulting in a pro-inflammatory state (MacKay and Lester, 1992; Nakamura et al., 1998; Xie et al., 2000). The LPS injection in hens resulted in increased gene expression of inflammatory cytokines IL-6 and IL-18 compared to saline injected hens regardless of experimental diet (flaxseed oil, fish oil, and soy oil control), demonstrating the influence of 1.0 mg/kg LPS dosage in 134 wk old hens. Pro-inflammatory cytokine production was expected from the LPS challenge due to previous experiments demonstrating acute phase response in 34 d old broiler and layer lines

(Leshchinsky and Klasing, 2001; Warren et al., 2010). The LPS injection in hens also resulted in increased expression of PPARγ, which is the master regulator of adipogenesis (Sun et al., 2014). When PPARγ is up-regulated, cells are signaled to store fatty acids (Sun et al., 2014), including ALA, EPA, and DHA that possess anti-inflammatory properties. The data between the two dietary treatments cannot be directly compared, as the same 4% dietary oil inclusion yielded unequal total omega-3 fatty acid supplementation, with the fish oil providing 45% less total omega-3 fatty acids than the flaxseed oil. Although unequal in total omega-3 fatty acid supplementation, hens fed flaxseed oil (ALA source) and fish oil (EPA and DHA source) diets generated similar responses to the inflammatory challenge.

An interaction occurred where hens in the flaxseed oil and fish oil dietary treatments (LPS and saline-injected) produced intermediate mRNA expression levels of inflammatory cytokines IL-1β, IL-6, IL-18 and PPARγ when compared to hens in the soy oil control group injected with LPS or saline. The control hens injected with LPS generated ≥ 4.4-fold increases in inflammatory cytokine expression compared to their saline-injected control counterparts. The lower peak inflammatory cytokine expression of hens fed ALA and EPA and DHA supplemented diets compared to the LPS-injected control hens, while not significant, indicated that the supplementation diminished cellular response to inflammatory stimulation. Dietary ALA or EPA and DHA supplementation was able to mute the acute phase inflammatory response without shutting down the immune system in aged laying hens. These results imply that even though inflammation is suppressed the immune system may still be able to clear out pathogens and prevent infections. Further investigation would be necessary to determine the effect of dietary omega-3 fatty acid supplementation on pathogen clearance during active infection of laying hens.

Inflammation may potentially result in discomfort and loss of performance in production animals, but inflammatory signaling serves an important function in the event of pathogenic infection. Interferon γ gene expression is up regulated through IL-1β, IL-18, and IL-12, synergistically activating T cell subsets (Tominaga et al., 2000). Several mechanisms have been proposed for the inflammatory actions of EPA and DHA, including competition with omega-6 fatty acids for rate limiting enzymes (Voss et al., 1991), synthesis of less inflammatory prostaglandin variants (Gonzalez et al., 2011), and modulation of macrophage pro-inflammatory cytokine synthesis (Hao et al., 2010). The intermediate IL-1β and IL-18 gene expression in this experiment suggests that circulating IFNγ would be greater in the ALA or EPA and DHA supplemented hens compared to the control hens injected with saline.

Circulating levels of IFNγ at primed concentrations could potentially reduce pathogenic infection by increasing detection and elimination of invading bacteria and viruses before cellular infection and replication occurs (Irons and Fritsche, 2005; Spits et al., 2016). However, the reduced peak expression of IFNγ in hens supplemented with ALA or EPA and DHA compared to hens fed the soy oil control and injected with LPS suggests that macrophage activation and microbial clearance via the $T_H1$ immune response may be reduced (Irons and Fritsche, 2005; Spits et al., 2016). Suppressing inflammatory signaling in extreme conditions, such as sepsis, could theoretically result in prolonged infection of Gram-positive bacteria or viruses normally eliminated by IL-10 and IFNγ (de Pablo et al., 2002; Irons and Fritsche, 2005; Skovbjerg et al., 2010). Dietary ALA or EPA and DHA supplementation appears to mute the immune response, but may still be effective at pathogenic elimination despite blunting peak inflammatory gene expression levels.

As previously mentioned, immunologically developed end-of-second cycle laying hens are able to endogenously metabolize ≤ 5% of dietary ALA to EPA and DHA (Burdge and Calder, 2006; Zivkovic et al., 2011). The flaxseed oil treatment may have provided enough ALA substrate for adequate endogenous EPA and DHA synthesis over the 12 wk experiment to attenuate inflammation in a similar fashion as the fish oil treatment. Mechanistically, the current gene expression data may support that EPA and DHA cell membrane incorporation interferes with toll-like receptor 4 (TLR4) signaling, the cell surface receptor that dimerizes and signals when bound to LPS (Rockett et al., 2011). Membrane lipid raft clusters that contain TLR4 protein components become disorganized spatially and in size due to the presence of EPA and DHA in the lipid bilayer (Rockett et al., 2011). Membrane fluidity also becomes impaired due to EPA and DHA incorporation, further preventing protein recruitment into lipid raft region for TLR4 activation (Hao et al., 2010). Intracellular EPA and DHA may also directly inhibit the TLR4 pathway by acting as second messengers that inhibit pro-inflammatory gene transcription and translation (Hao et al., 2010). In this current experiment, it may be speculated that feeding flaxseed oil and fish oil supplemented diets inhibited the TLR4 pathway resulting in the intermediate cytokine responses during LPS inflammatory stimulation.

Collecting liver samples from hens 12 h post-LPS challenge allowed adequate time for physiological changes in gene expression to occur. These data parallel with the phenotypic changes observed by Cheng et al. (2004) who found that relative liver weights increased 15% in 6 wk old pullets that exhibited clinical lethargy 12 h post-LPS injection. Additional time point sampling before and after LPS administration would provide insight

into whether the dietary treatments accelerated, resolved, or prolonged inflammatory gene expression over time in response to LPS stimulation.

This is the first time hens of this age and stage of production have been utilized for investigation into omega-3 fatty acid anti-inflammatory protection and immune responses. Previous work in mouse models reported anti-inflammatory effects on $CD8^+$ T cells and adipocytes using 3.5% dietary flaxseed oil (Monk et al., 2016). The dietary oil inclusion level in this experiment was commercially relevant at 4%, which is the upper limit of what feed mills can handle when manufacturing an omega-3 fatty acid enriched diet. If producers in the industry are using oil supplementation at levels to generate value-added eggs, then they should be made aware of the effects that flaxseed oil and fish oil have on their laying hens' immune system. As previously discussed, reducing inflammation may not always be in an animals best interest. Immune suppression may improve laying hen production performance in a clean research environment where disease challenge and feed competition are not factors. However, in commercial environments that contain bacteria and competition, anti-inflammatory immune suppression may not be ideal for eliminating certain pathogens, such as viruses. Further investigation is needed to verify the role EPA and DHA play *in vivo* during pathogenic infections.

This experiment disproved the hypothesis that fish oil supplementation, providing EPA and DHA, would improve aged laying hen bone health. Omega-3 fatty acid supplementation in laying hen diets can generate value-added enriched eggs, and provide some level of anti-inflammatory protection but this protection may not be a practical benefit for producers faced with disease challenges. It is up to producers to consider the pros and cons related to health of using feed additives or supplements in animal diets.

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## TABLES

**Table 3.1.** Calculated and analyzed values for experimental soy oil (control), flaxseed oil, or fish oil diets[1] fed to Hy-Line W-36 laying hens for a 12-wk supplementation period used to evaluate dietary alpha-linolenic acid (ALA) or eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

| | Experimental diet formulations (%) | | |
|---|---|---|---|
| **Ingredient** | **Soy oil control** | **Flaxseed oil** | **Fish oil** |
| Corn | 49.53 | 49.53 | 49.53 |
| Soybean meal 48% crude protein | 21.57 | 21.57 | 21.57 |
| DDGS[2] | 5.00 | 5.00 | 5.00 |
| Wheat middlings | 5.00 | 5.00 | 5.00 |
| Meat and bone meal | 4.00 | 4.00 | 4.00 |
| Soy oil | 4.00 | 0.00 | 0.00 |
| Flaxseed oil | 0.00 | 4.00 | 0.00 |
| Fish oil | 0.00 | 0.00 | 4.00 |
| Calcium carbonate[3] | 9.76 | 9.76 | 9.76 |
| Dicalcium phosphate | 0.07 | 0.07 | 0.07 |
| Salt | 0.33 | 0.33 | 0.33 |
| Methionine hydroxy analogue | 0.15 | 0.15 | 0.15 |
| Choline chloride | 0.10 | 0.10 | 0.10 |
| V and M premix[4] | 0.50 | 0.50 | 0.50 |
| **Calculated values** | | | |
| Calcium | 4.23 | 4.23 | 4.23 |
| Available Phosphorus | 0.33 | 0.33 | 0.33 |
| **Analyzed values** | | | |
| Crude protein | 18.59 | 17.65 | 18.40 |
| Crude fat | 4.15 | 4.83 | 4.94 |
| Moisture | 10.56 | 10.06 | 10.30 |
| Ash | 13.85 | 14.49 | 14.47 |

[1]Experimental diets used a basal diet combined with 4% soy oil (control), 4% flaxseed oil (2,200 mg ALA/100 g diet), or 4% fish oil (720 mg EPA + 480 mg DHA/100 g diet) fed from 122 to 134 wk of age (12-wk supplementation period). Control and dietary treatments contained 78 laying hens with 13 replicate experimental units. Calculated ME for the soy oil control diet was 2,889 kcal/kg.

[2]DDGS = distillers dried grains with solubles.

[3]Calcium carbonate added was a 50/50 mixture of small (< 2 mm) and large (≥ 2 mm) particle.

[4]Vitamin and mineral premix provided per kg of diet: selenium 200 μg; vitamin A 6,600 IU; vitamin $D_3$ 2,200 IU; vitamin E 14.3 IU; menadione 880 μg; vitamin $B_{12}$ 9.4 μg; biotin 33 μg; choline 358 mg; folic acid 1.1 mg; niacin 33 mg; pantothenic acid 8.8 mg; pyridoxine 880 μg; riboflavin 4.4 mg; thiamine 1.1 mg; iron 226 mg; magnesium 100 mg; manganese 220 mg; zinc 220 mg; copper 22 mg; iodine 675 μg.

**Table 3.2.** Analyzed fatty acid profile of experimental soy oil (control), flaxseed oil, or fish oil diets[1] fed to Hy-Line W-36 laying hens for a 12-wk supplementation period used to evaluate dietary alpha-linolenic acid (ALA) or eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

| | Diet fatty acid composition (%) | | |
|---|---|---|---|
| **Fatty acid[2] (C:double bond)** | **Control** | **Flaxseed oil** | **Fish oil** |
| Myristic acid (14:0) | 0.24 | 0.29 | 4.78 |
| Palmitic acid (16:0) | 15.43 | 10.69 | 18.57 |
| Palmitoleic acid (16:1) | 0.37 | 0.45 | 5.26 |
| Margaric acid (17:0) | 0.00 | 0.00 | 0.34 |
| Stearic acid (18:0) | 4.15 | 3.79 | 4.23 |
| Oleic acid (18:1) | 20.87 | 20.99 | 17.85 |
| Linoleic acid (18:2) | 52.82 | 30.16 | 25.97 |
| Alpha-linolenic acid (18:3) | 5.44 | 32.95 | 2.17 |
| Arachidic acid (20:0) | 0.32 | 0.30 | 0.33 |
| Arachidonic acid (20:4) | 0.00 | 0.00 | 0.66 |
| Eicosapentaenoic acid (20:5) | 0.00 | 0.37 | 11.62 |
| Behenic acid (22:0) | 0.35 | 0.00 | 0.00 |
| Docosapentaenoic acid (22:5) | 0.00 | 0.00 | 1.39 |
| Docosahexaenoic acid (22:6) | 0.00 | 0.00 | 6.84 |
| **Calculated values** | | | |
| Total saturated fat | 20.49 | 15.09 | 28.25 |
| Total unsaturated fat | 79.51 | 84.91 | 71.75 |
| Total omega-3 fatty acid[3] | 5.44 | 33.32 | 20.63 |
| Total omega-6 fatty acid[4] | 52.82 | 30.16 | 26.63 |
| Omega-6:omega-3 ratio | 9.70 | 0.91 | 1.29 |

[1]Experimental diets were made every 2 wk and a pooled sample using feed from each new batch was used for fatty acid analysis. Experimental diets used a basal diet combined with 4% soy oil (control), 4% flaxseed oil (2,200 mg ALA/100 g diet), or 4% fish oil (720 mg EPA + 480 mg DHA/100 g diet) fed from 122 to 134 wk of age. Control and dietary treatments contained 78 laying hens with 13 replicate experimental units.

[2]Analyzed dietary crude fat was 4.15, 4.83, and 4.94% for the soy oil control, flaxseed oil, and fish oil diets respectively. The fatty acid values represent the percent composition within the crude fat for each experimental diet.

[3]Total omega-3 fatty acid was the sum of alpha-linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid.

[4]Total omega-6 fatty acid was the sum of linoleic acid and arachidonic acid.

**Table 3.3.** Keel (sternum) bone conformation palpation scale for laying hens fed experimental soy oil (control), flaxseed oil, or fish oil diets[1] for a 12-wk supplementation period used to evaluate dietary alpha-linolenic acid (ALA) or eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

| Score | Definition |
|---|---|
| **0** | Normal keel (sternum) with no fractures |
| **1** | Presence of keel damage from a minor fracture |
| **2** | Presence of keel damage from a moderate fracture and calcification |
| **3** | Presence of damage from a severe fracture, sigmoid calcification, or multiple fracture sites on keel |

[1]Experimental diets were applied for a 12-wk dietary supplementation period (122 to 134 wk of age). Laying hens were fed experimental diets containing 4% soy oil (control), 4% flaxseed oil (2,200 mg ALA/100 g diet), or 4% fish oil (720 mg EPA + 480 mg DHA/100 g diet).

**Table 3.4.** Forward (F) and reverse (R) primer sequences[1] for quantitative real-time PCR cytokine mRNA gene expression.

| Cytokine[2] | Primer Sequence | Amplicon size | Accession number or referenced source |
|---|---|---|---|
| **IL-1β** | F 5'-GCTCTACATGTCGTGTGTGATGAG-3'<br>R 5'-TGTCGATGTCCCGCATGA-3' | 100 | AJ245728 |
| **IL-6** | F 5'-GCTCGCCGGCTTCGA-3'<br>R 5'-GGTAGGTCTGAAAGGCGAACAG-3' | 125 | AJ250838 |
| **IL-18** | F 5'-AGGTGAAATCTGGCAGTGGAAT-3'<br>R 5'-ACCTGGACGCTGAATGCAA-3' | 25 | AJ276026 |
| **IFNγ** | F 5'-GTGAAGAAGGTGAAAGATATCATGGA-3'<br>R 5'-GCTTTGCGCTGGATTCTCA-3' | 720 | Y07922 |
| **SAA** | F 5'-TTCTGTGGGCTAGGTTCCTG-3'<br>R 5'-GCAAGTCAGCAACAACCAGA-3' | 111 | Q9PSM7 |
| **IL-10** | F 5'-CATGCTGCTGGGCCTGAA-3'<br>R 5'-CGTCTCCTTGATCTGCTTGATG-3' | 161 | AJ621614 |
| **PPARγ** | F 5'-GGGCGATCTTGACAGGAA-3'<br>R 5'-GCCTCCACAGAGCGAAAC-3' | 175 | Sun et al., 2014 |
| **28s** | F 5'-GGCGAAGCCAGAGGAAACT-3'<br>R 5'-GACGACCGATTTGCACGTC-3' | 62 | X59733 |

[1]Primers were designed using the corresponding accession number or listed reference.
[2]Cytokine gene expression analyzed for interleukin-1 β (IL-1β), interleukin-6 (IL-6), interleukin-18 (IL-18), interferon γ (IFNγ), serum amyloid A (SAA), interleukin-10 (IL-10), peroxisome proliferator-activated receptor gamma (PPARγ), and ribosomal 28s subunit reference gene (28s).

**Table 3.5.** Performance of Hy-Line W-36 laying hens fed experimental soy oil (control), flaxseed oil, or fish oil diets for a 12-wk supplementation period used to evaluate dietary alpha-linolenic acid (ALA) or eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

| Experimental diet[1] | Hen-day egg production (%) | Average daily feed intake (g/hen) | Feed efficiency (kg egg/kg feed) | Egg weight (g/egg) | Average daily egg mass (g/hen) | Body weight[2] (kg) |
|---|---|---|---|---|---|---|
| Soy oil control | 51.8 | 97.1 | 0.363 | 69.70 | 35.2 | 1.604 |
| Flaxseed oil | 51.3 | 98.3 | 0.350 | 69.37 | 34.3 | 1.607 |
| Fish oil | 53.5 | 96.3 | 0.363 | 67.31 | 35.0 | 1.572 |
| **SEM** | 2.40 | 0.19 | 0.0162 | 0.556 | 1.57 | 0.0164 |
| **Contrast *P*-value[3]** | | | | | | |
| Flax vs. Con | 0.76 | ≤ 0.01 | 0.56 | 0.67 | 0.69 | 0.87 |
| Fish vs. Con | 0.68 | ≤ 0.01 | 1.00 | ≤ 0.01 | 0.92 | 0.17 |

[1]Control and dietary treatments contained 78 laying hens with 13 replicate experimental units. Experimental diets used a basal diet combined with 4% soy oil (control), 4% flaxseed oil (2,200 mg ALA/100 g diet), or 4% fish oil (720 mg EPA + 480 mg DHA/100 g diet) from 122 to 134 wk of age.

[2]Body weight was measured at the start and every 4 wk (0, 4, 8, 12-wk) during the 12-wk dietary supplementation period (122 to 134 wk old). Starting body weight (0-wk) was used to attain a baseline.

[3]Orthogonal contrasts of flaxseed oil dietary treatment (Flax) vs. soy oil control (Con) and fish oil dietary treatment (Fish) vs. Con.

**Table 3.6.** Egg quality of Hy-Line W-36 laying hens fed experimental soy oil (control), flaxseed oil, or fish oil diets for a 12-wk supplementation period used to evaluate dietary alpha-linolenic acid (ALA) or eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

| Experimental diet[1] | Egg weight (g) | Albumen height (mm) | Haugh unit | Shell breaking strength (N) | Shell thickness (mm) | Egg solids (%) |
|---|---|---|---|---|---|---|
| Soy oil control | 68.9 | 7.1 | 81.5 | 26.1 | 0.32 | 24.3 |
| Flaxseed oil | 68.3 | 7.0 | 81.0 | 25.9 | 0.32 | 24.3 |
| Fish oil | 67.2 | 7.0 | 81.5 | 24.2 | 0.31 | 24.2 |
| **SEM** | 0.53 | 0.10 | 0.72 | 0.86 | 0.003 | 0.25 |
| **Contrast *P*-value[2]** | | | | | | |
| Flax vs. Con | 0.42 | 0.52 | 0.64 | 0.85 | 0.97 | 0.96 |
| Fish vs. Con | 0.03 | 0.67 | 0.96 | 0.13 | 0.43 | 0.71 |

[1]Control and dietary treatments contained 78 laying hens with 13 replicate experimental units. Experimental diets used a basal diet combined with 4% soy oil (control), 4% flaxseed oil (2,200 mg ALA/100 g diet), or 4% fish oil (720 mg EPA + 480 mg DHA/100 g diet) from 122 to 134 wk of age (12-wk supplementation period).

[2]Orthogonal contrasts of flaxseed oil dietary treatment (Flax) vs. soy oil control (Con) and fish oil dietary treatment (Fish) vs. Con.

**Table 3.7.** Analyzed fatty acid profile of dietary omega-3 fatty acid transfer to egg yolk[1] from Hy-Line W-36 laying hens fed experimental soy oil (control), flaxseed oil, or fish oil diets[2] for a 12-wk supplementation period used to evaluate dietary alpha-linolenic acid (ALA) or eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

| | Egg yolk fatty acid composition (%) | | |
|---|---|---|---|
| **Fatty acid[3] (C:double bond)** | **Control** | **Flaxseed oil** | **Fish oil** |
| Myristic acid (14:0) | 0.22 | 0.20 | 0.93 |
| Palmitic acid (16:0) | 26.86 | 23.98 | 30.35 |
| Palmitoleic acid (16:1) | 1.84 | 2.06 | 3.64 |
| Margaric acid (17:0) | 0.00 | 0.00 | 0.00 |
| Heptadecanoic (17:1) | 0.19 | 0.07 | 0.23 |
| Stearic acid (18:0) | 9.43 | 9.08 | 9.52 |
| Oleic acid (18:1) | 35.29 | 38.25 | 35.57 |
| Linoleic acid (18:2) | 22.37 | 16.98 | 14.41 |
| Alpha-linolenic acid (18:3) | 0.90 | 6.91 | 0.53 |
| Arachidic acid (20:0) | 0.00 | 0.00 | 0.00 |
| Arachidonic acid (20:4) | 2.24 | 1.19 | 0.78 |
| Eicosapentaenoic acid (20:5) | 0.00 | 0.00 | 0.57 |
| Behenic acid (22:0) | 0.00 | 0.00 | 0.26 |
| Docosapentaenoic acid (22:5) | 0.00 | 0.00 | 0.46 |
| Docosahexaenoic acid (22:6) | 0.65 | 1.28 | 2.74 |
| **Calculated values** | | | |
| Saturated fat | 36.51 | 33.26 | 41.06 |
| Unsaturated fat | 63.49 | 66.74 | 58.94 |
| Total omega-3 fatty acid[4] | 1.55 | 8.19 | 3.84 |
| Total omega-6 fatty acid[5] | 24.61 | 18.17 | 15.19 |
| Omega-6:omega-3 ratio | 15.86 | 2.22 | 3.96 |

[1]Egg yolks were sampled at 0, 4, 8, and 12-wk during the 12-wk supplementation period (122 to 134 wk of age). Eggs sampled at the start (0-wk) were used as a baseline for the supplementation period. One egg yolk per experimental unit was pooled by treatment diet for fatty acid analysis.

[2]Experimental diets used a basal diet combined with 4% soy oil (control), 4% flaxseed oil (2,200 mg ALA/100 g diet), or 4% fish oil (720 mg EPA + 480 mg DHA/100 g diet) from 122 to 134 wk of age. Control and dietary treatments contained 78 laying hens with 13 replicate experimental units.

[3]Analyzed egg yolk crude fat was 31.01, 30.96, and 31.32% for the soy oil control, flaxseed oil, and fish oil-fed hens respectively. The egg yolk fatty acid values represent the percent composition within the egg yolk crude fat for each experimental diet.

[4]Total omega-3 fatty acid was the sum of alpha-linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid.

[5]Total omega-6 fatty acid was the sum of linoleic acid and arachidonic acid.

**Table 3.8.** Bone evaluation of Hy-Line W-36 laying hens fed experimental soy oil (control), flaxseed oil, or fish oil diets for a 12-wk supplementation period used to evaluate dietary alpha-linolenic acid (ALA) or eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

| Experimental diet[1] | Bone conformation score (0 to 3)[2] | Tibia bone ash (%) | Bone breaking strength (N) |
|---|---|---|---|
| Soy oil control | 1.8 | 65.3 | 171.4 |
| Flaxseed oil | 1.8 | 64.8 | 169.1 |
| Fish oil | 1.8 | 64.4 | 161.7 |
| **SEM** | 0.04 | 0.86 | 7.96 |
| **Contrast *P*-value**[3] | | | |
| Flax vs. Con | 0.86 | 0.72 | 0.84 |
| Fish vs. Con | 0.79 | 0.50 | 0.40 |

[1]Control and dietary treatments contained 78 laying hens with 13 replicate experimental units. Experimental diets used a basal diet combined with 4% soy oil (control), 4% flaxseed oil (2,200 mg ALA/100 g diet), or 4% fish oil (720 mg EPA + 480 mg DHA/100 g diet) from 122 to 134 wk of age (12-wk supplementation period).

[2]Bone conformation palpation score was measured at the start and every 4 wk (0, 4, 8, 12-wk) during the 12-wk dietary supplementation period. Starting conformation score (0-wk) was used to attain a baseline value. Keel bone conformation palpation was scored on a 3 point scale where 0 = normal keel, 1 = keel with presence of damage from minor fracture, 2 = keel with presence of damage from moderate fracture, and 3 = presence of damage from multiple or severe keel fractures.

[3]Orthogonal contrasts of flaxseed oil dietary treatment (Flax) vs. soy oil control (Con) and fish oil dietary treatment (Fish) vs. Con.

## FIGURES

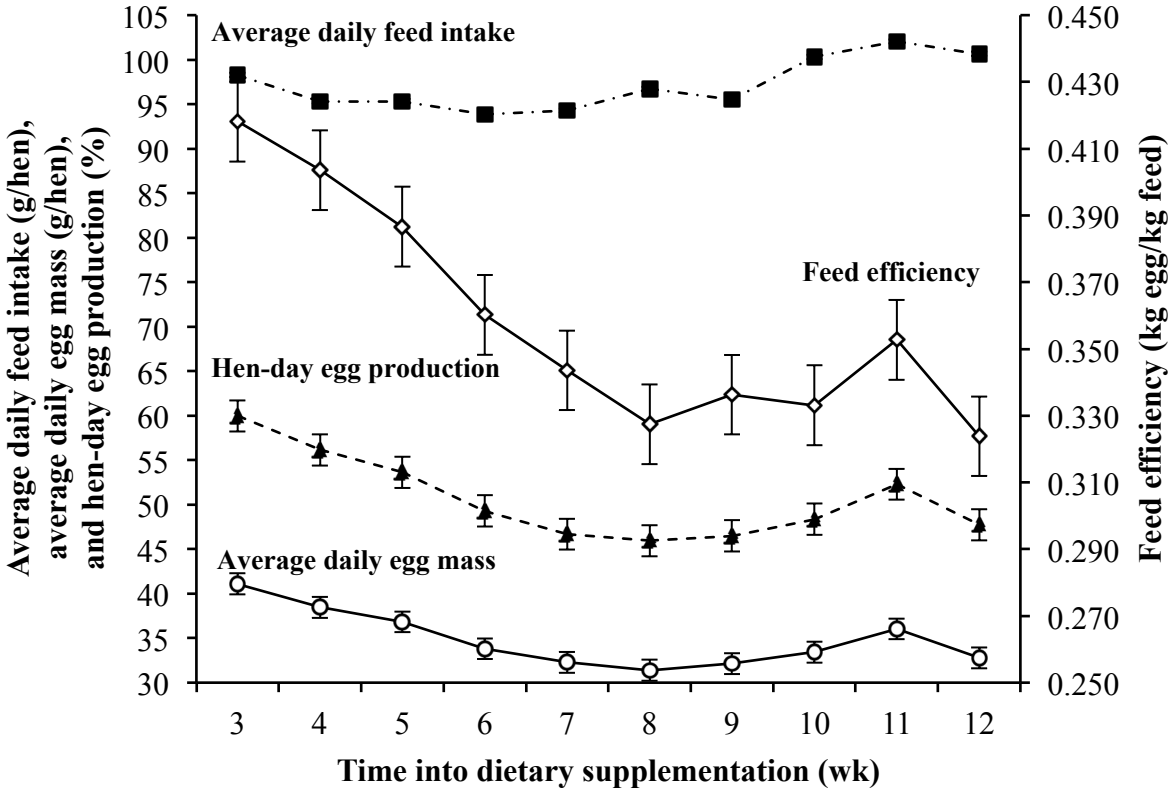


**Figure 3.1.** Pooled average flock performance for laying hens fed experimental soy oil control, flaxseed oil, and fish oil diets over a 12-wk experimental supplementation period (122 to 134 wk of age). Experimental diets used a basal diet combined with 4% soy oil (control), 4% flaxseed oil (2,200 mg ALA/100 g diet), or 4% fish oil (720 mg EPA + 480 mg DHA/100 g diet). Control and dietary treatments contained 78 laying hens with 13 replicate experimental units. The hens were given the first 2 wk of the experiment to adjust to their rations, data not included. Data shown as LS Means ± SEM for the effect of time (wk) on pooled values of average daily feed intake (■; g/hen), feed efficiency (◇; kg egg/kg feed), hen-day egg production (▲; %), and average daily egg mass (○; g/egg; $P \leq 0.01$).

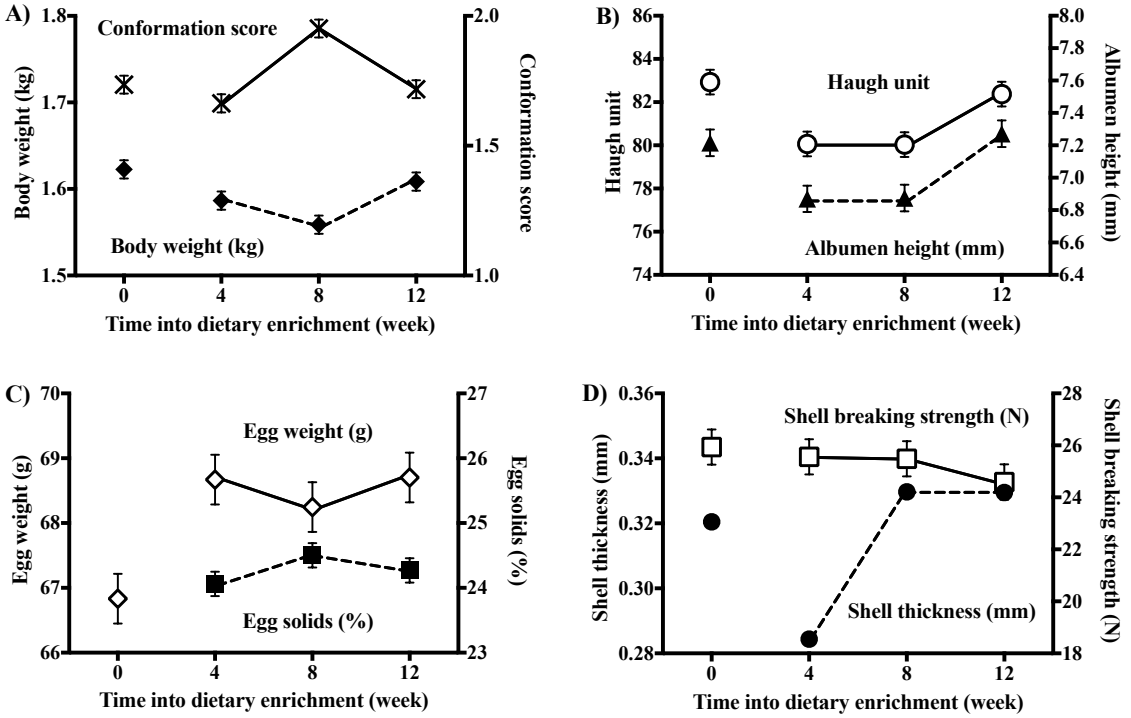


**Figure 3.2.** Pooled average flock bone conformation score, BW, and egg quality for laying hens fed experimental soy oil control, flaxseed oil, and fish oil diets over a 12-wk experimental supplementation period (122 to 134 wk of age). Experimental diets used a basal diet combined with 4% soy oil (control), 4% flaxseed oil (2,200 mg ALA/100 g diet), or 4% fish oil (720 mg EPA + 480 mg DHA/100 g diet). Control and dietary treatments contained 78 laying hens with 13 replicate experimental units. Data shown as LS Means ± SEM for the effect of time (wk) on pooled values of A) bone conformation score (×; $P \leq 0.01$) and BW (◆; $P \leq 0.01$), B) Haugh unit (○; $P \leq 0.01$) and albumen height (▲; $P \leq 0.01$), C) egg weight (◇; $P \leq 0.01$) and egg solids (■; $P = 0.12$), and D) shell breaking strength (□; $P = 0.30$) and shell thickness (●; $P \leq 0.01$).

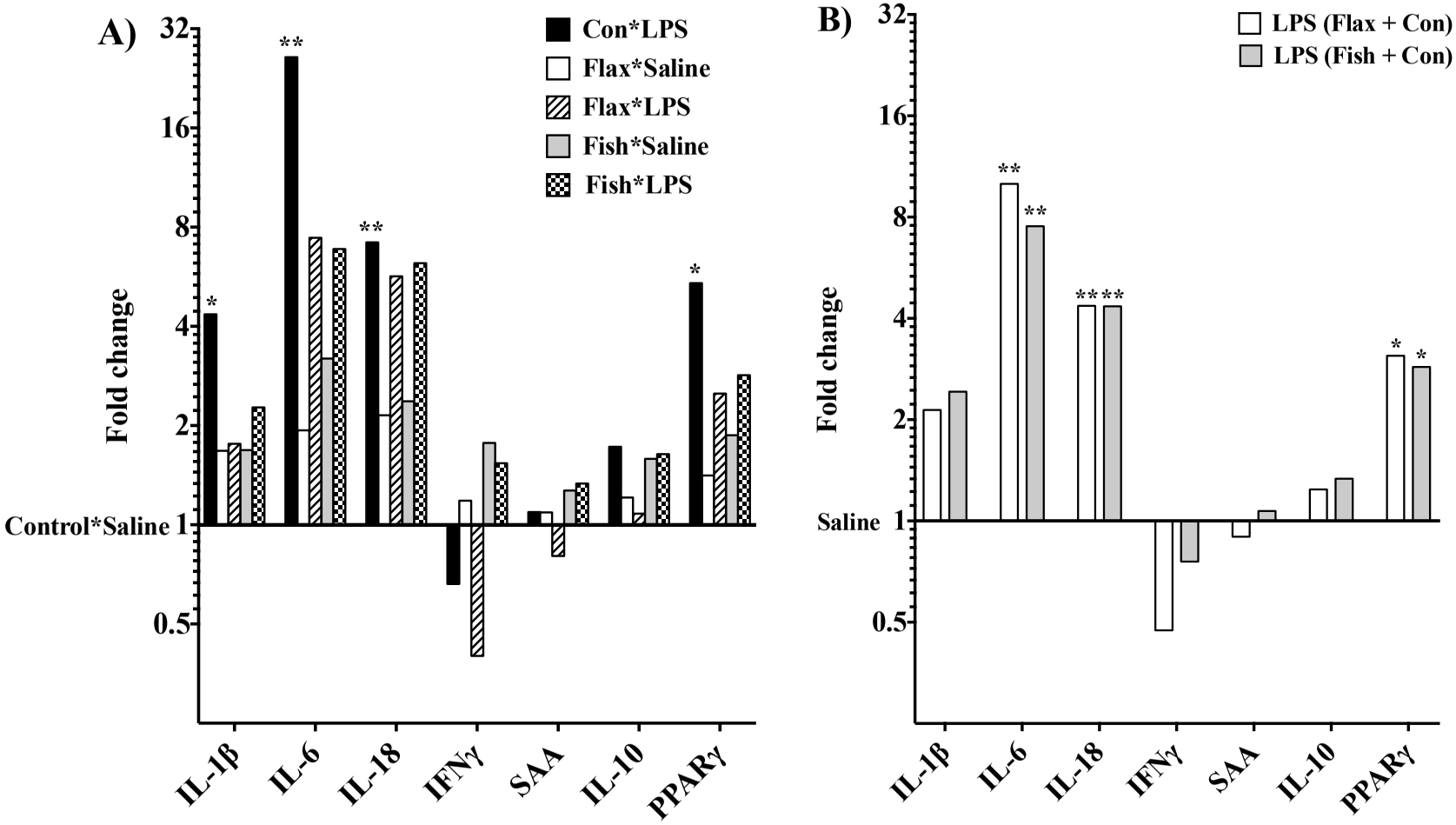


**Figure 3.3.** Liver relative mRNA gene expression using qRT-PCR for the A) interaction of inflammatory challenge (LPS vs. Saline) in laying hens fed experimental soy oil control (Con), flaxseed oil (Flax), or fish oil (Fish) diets over a 12-wk experimental supplementation period (122 to 134 wk of age) followed by a 12 h LPS challenge and B) the main effect of the inflammatory challenge (LPS vs. Saline) separated by flaxseed oil or fish oil dietary treatment with soy oil control. Experimental diets used a basal

**Figure 3.3 continued.**
diet combined with 4% soy oil (control), 4% flaxseed oil (2,200 mg ALA/100 g diet), or 4% fish oil (720 mg EPA + 480 mg DHA/100 g diet). Each bar represents A) the average normalized fold change of 5 samples for each factorial combination of diet*challenge and B) the average normalized fold change of 10 samples for each level of inflammatory challenge (LPS vs. Saline). $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$ in reference to relative baseline set at 1-fold change for A) hens fed the soy oil control diet and injected with saline (Control*Saline) and B) hens injected with saline (Saline) separated by flaxseed oil or fish oil dietary treatments with the soy oil control.

# CHAPTER 4. DIETARY FLAXSEED OIL OR FISH OIL SUPPLEMENTATION ON BONE HEALTH AND PERFORMANCE IN GROWING BROILERS

A manuscript to be submitted to *Poultry Science*

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## 4.1 Abstract

Broiler diets supplemented with omega-3 fatty acids can generate value-added meat for consumers and may provide a second benefit by improving broiler health. Rapid muscle and skeletal growth rates that have allowed broilers to become efficient production animals have also caused an increase in lameness and leg issues. Dietary alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are known to modulate bone mineral resorption *in vitro*. In order to investigate the effects of dietary flaxseed oil (ALA source) or fish oil (EPA and DHA source) supplementation on broiler bone health and performance, 6 d old Ross 308 broilers (n = 240) were fed experimental diets containing 4.1% omega-3 fatty acids or a palm oil control diet over a 28 d dietary supplementation period (6 to 34 d old, final BW of 2.412 ± 0.318 kg). Lameness, bone conformation, bone mineral content (BMC), and bone mineral density (BMD) were used to quantify bone health. Lameness score was assessed every 7 d based on locomotion; keel, legs, and wings were

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palpated on each bird for bone conformation score using a 3-point scale. After the dietary supplementation period, 1 bird/experimental unit (EU; 4 broilers = EU) was imaged with dual-energy x-ray absorptiometry (DEXA) for BMC, BMD, lean mass, and fat mass accretion, and a second bird/EU for breast yield. Performance and DEXA data were analyzed using PROC MIXED in SAS; lameness and bone conformation were analyzed using PROC GLIMMIX with Poisson distribution. Significant means were separated using Tukey's Test ($P \leq 0.05$). Feeding fish oil decreased BMD by 0.015 ± 0.0041 g/cm$^2$ compared to broilers fed flaxseed oil diets ($P \leq 0.05$), the control was intermediate. Broilers fed fish oil resulted in lower BW by 0.234 and 0.264 ± 0.0318 kg compared to feeding control and flaxseed oil diets, respectively ($P \leq 0.01$). Feeding the fish oil diet to broilers decreased breast yield by 1.301 ± 0.3553% compared to the control ($P \leq 0.05$). Feeding fish oil reduced performance even though feed intake was not different among treatments ($P = 0.43$). The combined results from bone analysis and performance suggest that the fish oil may not have been as stable as flaxseed oil, affecting fatty acid utilization. In order to avoid performance losses when generating value-added meat, flaxseed oil may be a more consistent ingredient for producers to use when supplementing broiler diets. However, the results from this experiment did not demonstrate a secondary benefit of improving broiler bone health or performance when supplemented with flaxseed oil or fish oil.

Key words: ALA, EPA, DHA, performance, DEXA

## 4.2 Introduction

Poultry diets supplemented with omega-3 fatty acids can create value-added foods, such as omega-3 fatty acid enriched broiler meat, for consumers of the health and functional

food markets (Ribeiro et al., 2013; Zhong et al., 2014). Genetic selection for efficient performance has created broilers with high metabolic demands that rapidly grow muscle and bone (Julian, 1998; Cook, 2000; Julian, 2005). Rapid growth rates and production stress in commercial flocks has lead to leg deformities and lameness becoming a common occurrence (Bradshaw et al., 2002; Saif et al., 2008; Grupioni et al., 2015). Omega-3 fatty acids, specifically bioactive eicosapentaenoic acid (EPA; C20:5) and docosahexaenoic acid (DHA: C22:6), are known for possessing anti-inflammatory properties and the ability to modulate bone remodeling activity *in vitro* (Sun et al., 2003; Herrera et al., 2008; Lukas et al., 2011). Alpha-linolenic acid (ALA; C18:3), the omega-3 fatty acid precursor of EPA and DHA, has been linked to reducing the occurrence of keel bone fractures in free-range laying hens by ≤ 60% (Tarlton et al., 2013; Toscano et al., 2015). Dietary omega-3 fatty acid supplementation used to generate value-added meat for health conscious consumers may also be able to provide health and welfare benefits for growing broilers.

Dietary ALA supplementation using flaxseed oil appears to influence bone mineral density in broilers (Zhong et al., 2014). A previous study fed a diet containing 3% flaxseed oil and 2% palm oil to broilers from 0 to 42 d of age reported increased tibial bone mineral density by 0.1 g/cm$^2$, average daily weight gain by 6.1 g, and average daily feed intake by 7.4 g compared to broilers fed a 5% lard diet ($P < 0.05$; Zhong et al., 2014). The observed increase in bone mineral density may have resulted from suppressed resorptive osteoclastic activity, which would allow osteoblasts to deposit mineral faster than it can be resorbed (Nakanishi and Tsukamoto, 2015; Kim et al., 2017). Previous experiments reported that EPA and DHA addition into bone marrow cell cultures resulted in apoptosis of mature osteoclasts and attenuated proliferation and differentiation of osteoclastic precursor cells, with DHA

having a stronger attenuating effect on osteoclasts than EPA (Rahman et al., 2008; Kim et al., 2017). However, poultry can only metabolize ≤ 5% dietary ALA to EPA and DHA due to over saturation of endogenous elongases and desaturases (Burdge and Calder, 2006; Zivkovic et al., 2011). Therefore, dietary fish oil supplementation, providing EPA and DHA, fed to broilers may result in greater bone mineral density compared to broilers fed dietary ALA supplemented diets, when provided at equivalent total omega-3 fatty acid concentrations.

Dietary ALA from flaxseed oil or EPA and DHA from fish oil have to be tested at levels that will induce a biological response when fed to broilers. Previous broiler experiments have ranged in maximum dietary oil inclusion from 4 to 10% for their high inclusion omega-3 fatty acid treatments (Ferrini et al., 2008; Navidshad et al., 2012). Other studies testing omega-3 fatty acid meal ingredients used a maximum of 15% ground flaxseed in supplemented broiler diets to evaluate growth performance (Jia et al., 2010; Ribeiro et al., 2013). Because a slight difference in broiler bone mineral density was detected using 3% flaxseed oil supplementation (Zhong et al., 2014), a higher dietary inclusion was used in the current experiment in order to induce a therapeutic response in broiler bone activity.

In order to investigate and compare the effects of dietary ALA from flaxseed oil or EPA and DHA from fish oil on broiler performance and bone health, equal concentrations of total omega-3 fatty acids at potentially therapeutic levels were supplemented in broiler diets for this experiment. The hypothesis was that dietary omega-3 fatty acid supplementation would improve bone characteristics without compromising performance compared to control, thereby improving animal health and welfare. Furthermore, it was hypothesized that dietary EPA and DHA supplementation would outperform dietary ALA supplementation.

## 4.3 Material and Methods

### 4.3.1 Animals and Housing

The protocol for this study was approved by the Iowa State University Institutional Animal Care and Use Committee. Broilers were cared for in accordance with the FASS Guide for the Care and Use of Agricultural Animals in Research and Teaching (2010). Two hundred and forty male Ross 308 hatching-day broiler chicks were obtained from a commercial hatchery (Bancroft, IA) and transported to the Poultry Research and Teaching Unit at Iowa State University. The poultry house was enclosed with cross ventilation and divided in half, resulting in 2 rooms containing 30 pens in each half of the house. Chicks were housed in floor pens (121.9 cm length × 121.9 cm width; 3,600 $cm^2$/bird at placement) with a supplemental 125-watt clear incandescent heat bulb in each pen. Each pen contained 10 cm of pine shavings placed on the concrete floor. Rooms were heated to 29°C at time of placement using a vented gas-fired heater (Modine High Efficiency, PDP300AE010SBAN, Modine Manufacturing Co., Racine, WI) and room temperature was reduced by 2.5°C every 7 d until 21°C was attained (by experimental d 15). The light program was 23L:1D for the first 7 d of life (experimental d -6 to 1) and then adjusted to 20L:4D for the duration of the experiment. Light intensity was 30 to 40 lux at bird level, as recommended by the Ross Broiler Management Manual (2009). Feed was provided *ad libitum* in one circular hanging feeder (30.5 cm diameter × 29.2 cm height) per pen (Model HF30, Brower Equipment, Houghton, IA). The feeders were placed at bedding level at the start of the experiment, and then were raised as the birds grew (7 cm at 10 d of age, 10 cm at 17 d of age, 13 cm at 21 d of age, and 15 cm from 28 to 34 d of age). Water was provided via Ziggity 360° nipple waterlines with 5 nipples per pen (Ziggity System Inc., Middlebury, IN) set to 7 cm of

column pressure and suspended 12 cm above bedding level from 0 to 14 d of age. Column pressure was increased to 10 cm from 14 to 28 d of age and then increased to 15 cm column pressure for the remainder of the experiment. Waterlines were raised to 22 cm above bedding level 14 to 21 d of age, 25 cm from 21 to 28 d of age, and 29 cm for the remainder of the experiment.

### 4.3.2 Treatments

Broilers were given a 5 d environmental acclimation period (0 to 5 d of age) prior to starting the experiment. At 6 d of age (baseline; experimental d 0) each bird was wing banded (Tab End Wing Band, Style 898, National Band & Tag Co., Newport, KY into the chicks' right wing web), weighed on a digital gram scale (Defender 3000, Ohaus Corp., Parsippany, NJ), sorted by 5 g increments, distributed equally by body weight (BW) to pens, and randomly assigned an experimental diet. All birds were physically examined for clinical signs of illness, stress, and were categorized as normal with a bone conformation palpation score of 0 before being enrolled into the experiment (Table 4.1). Broilers were fed diets for 28 consecutive d, from 6 to 34 d of age, which served as the dietary supplementation period allowing for omega-3 fatty acid incorporation into the broiler tissues.

The experiment was a randomized complete block design where pen was the experimental unit (EU) consisting of 4 broilers/pen with 20 EU/diet. Each half of the poultry house was a block with equal assignment of experimental diets. In order to evaluate broiler performance and bone remodeling responses to omega-3 fatty acid supplementation, 4.1% dietary ALA or EPA and DHA was supplemented into the treatment diets, which was a 50% increase in omega-3 fatty acid concentration from previous work (Lopes et al., 2013; Tarlton

et al., 2013; Zhong et al., 2014). Palm oil was used as the control fat source because it was composed of 50% saturated fatty acids, 42% monounsaturated fatty acids, and ≤ 8% polyunsaturated fatty acids with little to no omega-3 or omega-6 fatty acids. Addition of palm oil to experimental diets had little impact on omega-6:omega-3 fatty acid ratios, due to its fatty acid profile. Three experimental diets were compared using a corn-soybean meal-wheat middlings basal diet (Table 4.2) formulated to meet or exceed NRC (1994) requirements: 1) palm oil control (no supplemental omega-3 fatty acids; BW 0.157 kg ± 0.0010), 2) flaxseed oil (4.1% ALA; BW 0.157 ± 0.0010), and 3) fish oil (2.5% EPA and 1.6% DHA; BW 0.158 ± 0.0010) diet. A starter diet was formulated for 0 to 21 d of the experiment and a grower formulation was used from 22 to 28 d. All feeders were manually agitated daily to keep feed flow uniform across experimental pens.

### 4.3.3 Fatty Acid Analysis

Feed samples from each experimental diet were collected for every batch generated and stored at -20°C. Samples were ground through a 0.5 mm screen (Thomas-Wiley Laboratory Mill Model 4, Arthur H. Thomas Company, Philadelphia, PA) and were analyzed for fatty acid profiles using gas chromatography (HP 6890, Hewlett Packard Co., Palo Alto, CA). The fatty acid analysis was performed using methods previously described by Nam et al. (2001) and Sun et al. (2013).

### 4.3.4 Bone Health

Visually assessed lameness, bone conformation, mineral content, and mineral density were measured to determine the bone health of broilers fed experimental diets. All

broilers were visually assigned a lameness score in their home pens every 7 d. A single handler selected 1 bird to move using a plastic paddle (50 cm length handle; 13 cm length × 10 cm width paddle surface) that was positioned behind the bird. After the bird had attempted or completed a 1-m walk, a second researcher assigned a lameness score using a 3-point lameness scale (Table 4.1). This method was repeated until all birds in each pen had been assigned a lameness score. Broilers were evaluated for bone conformation at the start of the experiment (6 d of age) and then every 7 d. Keel (sternum), leg, and wing bones were gently palpated and a bone conformation score was assigned on a 3-point scale (Table 4.1).

At the beginning of the experiment (6 d of age), 8 broilers were euthanized by $CO_2$ asphyxiation following guidelines from the AVMA Panel on Euthanasia (2013) for subsequent imaging using dual-energy x-ray absorptiometry (DEXA; Discovery A, Hologic Inc., Marlborough, MA) following methods previously described by Mitchell et al. (1997). Baseline imaging provided starting bone mineral content (BMC; g) and bone mineral density (BMD; g/cm$^2$). BMC was measured to evaluate total mineral content within bone and BMD was measured to evaluate the distribution of mineral in an aerial plane. At the end of the experiment, 1 broiler/EU (240 = 80 broilers/diet) was euthanized and imaged to obtain BMC and BMD values after the 28 d dietary supplementation period.

### 4.3.5 Production Performance

All broilers were observed twice daily in accordance with university policy. Broilers were weighed individually on a digital gram scale at the start of the experiment (6 d of age) and then every 7 d for 28 consecutive d of dietary supplementation. Feed disappearance was measured every 7 d for feed intake (FI), feed conversion ratio (FCR), and average daily gain

(ADG) was calculated as follows: FI = start feed weight kg – end feed weight kg; FCR = FI ÷ (end BW kg – start BW kg); and ADG = (end BW kg – start BW kg) ÷ d.

Baseline DEXA imaging provided starting total body mass (kg), fat mass (kg), and lean tissue + BMC (kg). After the 28 d dietary supplementation period, 1 bird/EU (240 birds = 80/diet) was euthanized and imaged to obtain the 28 d performance values. The breast muscle (Pectoralis major and minor) were excised and weighed to obtain breast yield calculated as follows: Breast yield (%) = breast muscle weight ÷ total BW × 100.

### 4.3.6 Statistical Analysis

Data were analyzed using the UNIVARIATE procedure in SAS 9.4 (SAS Institute Inc., Cary, NC) to test for normality by plotting the predicted residuals and the quantile-quantile values. The experiment was a randomized complete block design consisting of 3 experimental diets (palm oil control, flaxseed oil, and fish oil) and 20 EU replicates for each diet (n = 240 broilers). Each half of the poultry house was a block with experimental diets equally assigned to each block. Performance measurements (BW, ADG, FI, FCR, and breast yield) and DEXA data (fat accretion, lean accretion, BMC, and BMD) were analyzed separately using the MIXED procedure in SAS. The statistical model included experimental diet (palm oil control, flaxseed oil, and fish oil) as fixed effects. The random effect of pen nested within experimental diet was used in the model.

Lameness and bone conformation data were non-normally distributed and therefore were analyzed using the GLIMMIX procedure in SAS with Poisson distribution. The statistical model included experimental diet (control, flaxseed oil, and fish oil), time (7 d intervals for 28 consecutive d), and the interaction of diet*time as fixed effects. Three

random effects, pen nested within diet, pen nested within time and bird nested within pen and diet were included in the model. The I-Link option was used to transform the data back to its original scale and the PDIFF option was used to obtain the LS-means. Tukey's Honestly Significant Difference (HSD) Test was used to separate means and identify significant differences. In all cases, a *P*-value $\leq 0.05$ was considered to be significant.

## 4.4 Results

### 4.4.1 Fatty Acid Analysis

The control diet, containing 14% palm oil, contained double the concentration of palmitic acid compared to the flaxseed oil and fish oil diets. The flaxseed oil diet contained 12 times the ALA concentration compared to the palm oil control and fish oil diets. The fish oil diet contained 19.55% EPA and 10.87% DHA. Neither of the longer chain omega-3 fatty acids EPA or DHA were detected in the palm oil control diet. The use of palm oil allowed the omega-6:omega-3 fatty acid ratios to be maintained at 9.46, 0.64, and 0.39 in the palm oil control, flaxseed oil, and fish oil diets, respectively (Table 4.3).

### 4.4.2 Bone Health

Bone health was investigated by visually and physically assessing each broiler using 3-point scales (Table 4.1), where a score of 0 was considered normal and a score of 3 considered severe (non-ambulatory or broken bone). There was no significant interaction of diet*time or changes over time observed for lameness score or bone conformation in broilers fed experimental diets ($P \geq 0.96$). Feeding the palm oil control, flaxseed oil, or fish oil supplemented diets to broilers from 6 to 34 d of age did not result in observable differences

in lameness score ($P$ = 0.83) or bone conformation score and broilers remained normal ($P$ = 1.00; Table 4.4). Feeding the experimental diets to broilers did not result in differences when DEXA scanned for BMC, which averaged 28.57 ± 0.810 g across the 3 diets ($P$ = 0.21). However, feeding the flaxseed oil diet to broilers resulted in increased BMD by 0.015 ± 0.0041 g/cm$^2$ compared to broilers fed the fish oil diet ($P$ =0.04; Table 4.4).

### 4.4.3 Production Performance

Starting BW was not different ($P$ = 0.42) with broiler chicks weighing an average of 0.157 ± 0.0010 kg. After the 28 d dietary supplementation period, the 34 d old broilers fed the palm oil control and flaxseed oil diets increased in BW gain by 0.234 and 0.264 ± 0.0318 kg, respectively, compared to broilers fed the fish oil diet ($P \leq 0.01$). No differences were observed in feed intake among broilers fed the experimental diets from 6 to 34 d of age with an average consumption of 4.250 ± 0.0413 kg/bird ($P$ = 0.43). Over the 28 d experiment, feeding the palm oil control and flaxseed oil diets to broilers resulted in a 10% improvement in FCR compared to the fish oil-fed broilers ($P \leq 0.01$). Fat accretion was not different among broilers fed experimental diets, averaging 0.140 ± 0.0160 kg/bird ($P$ = 0.87). Feeding the flaxseed oil diet to broilers resulted in a trending increase in lean tissue accretion, where 0.183 ± 0.0563 kg more lean tissue was gained compared to broilers fed the fish oil diet ($P$ = 0.07). Feeding the fish oil diet to broilers resulted in decreased breast yield by 1.301 ± 0.3553% compared to broilers fed the palm oil control diet ($P$ = 0.05; Table 4.5).

## 4.5 Discussion

The hypothesis that broilers fed the fish oil diet, supplying EPA and DHA, would outperform broilers supplemented with ALA was not supported by the current data. Feeding the fish oil diet to broilers resulted in lower BMD than feeding the flaxseed oil diets that provided ALA. Previous experiments have demonstrated that EPA and DHA addition to bone marrow cell cultures *in vitro* inhibited osteoclastic bone mineral resorption and allowed for increased osteoblastic bone mineral deposition (Watkins et al., 2000; Watkins et al., 2003; Lukas et al., 2011). However, in the current experiment dietary flaxseed oil or fish oil supplemented diets fed to broilers from 6 to 34 d of age did not improve bone characteristics measured *in vivo*. A positive correlation between BW to BMD has been reported, with lean mass more associated with BMD than fat mass (Ho-Pham et al., 2014). The broilers in the current experiment fed the palm oil control and flaxseed oil diets were heavier in BW compared to broilers fed the fish oil diet, which may have contributed to the differences reported in BMD.

*In vitro* and murine studies have demonstrated that EPA and DHA are bioactive omega-3 fatty acids that attenuate activity, differentiation, and proliferation of osteoclastic precursor cells (Rahman et al., 2008; Lukas et al., 2011; Kim et al., 2017). However, Zhong et al. (2014) reported that broiler tibia weight, length, and BMD were not influenced by flaxseed oil supplementation alone, but in combination with palm oil as a saturated fat stabilizing the polyunsaturated fatty acids. The 3 diets in the current experiment contained some level of palm oil in their composition to maintain an equal fat concentration of 14% across all 3 diets. The palm oil control diet contained 14% palm oil, the flaxseed oil diet (4.1% ALA) contained 6.53% palm oil and 7.48% flaxseed oil, and the fish oil diet (4.1%

EPA and DHA) contained 0.33% palm oil and 13.68% fish oil. The increased BMD observed in broilers fed the flaxseed oil diet may be attributed to alteration of lipid stability with the addition of saturated fats supplied by 6.53% palm oil compared to the fish oil diet, which only contained 0.33% palm oil. A study by Kang et al. (2001) reported that 3.5% dietary palm oil supplementation mixed with ground flaxseed, fish meal, and fish oil fed to laying hens resulted in reduced thiobarbituric acid reactive substances in egg, muscle, and liver by ≥ 10% compared to hens fed a 0% palm oil diet ($P < 0.05$). These findings suggest that dietary saturated fat content and oxidative stability are critical factors along with the omega-6:omega-3 fatty acid ratio in attenuating osteoclastic bone resorption *in vivo*.

In the current experiment, significant differences were observed in performance. Feeding the fish oil diet to broilers resulted in reduced BW gain, breast muscle yield, and increased FCR even though FI was not different among broilers fed the 3 experimental diets. Part of these performance differences may be attributed to nutrient partitioning influenced by fatty acid type and deposition into tissue, where it has been reported that muscle preferentially metabolizes polyunsaturated fatty acids (Newman et al., 2002a, 2002b; Ferrini et al., 2008). A study that fed 8% fish oil, 8% sunflower oil, or 8% tallow diets to broilers from 21 to 56 d of age reported that feeding 8% fish oil or 8% sunflower oil diets, high in polyunsaturated fatty acids, to broilers resulted in a 50% reduction of abdominal fat pad weight compared to broilers fed the 8% tallow diet ($P < 0.01$; Newman et al., 2002b). Breast muscle yield and total fat content within the breast muscle tissue was not different among the treatments of that experiment (Newman et al., 2002b). The performance data from the current experiment agreed with Newman et al. (2002b) that total fat accretion was not altered by dietary supplementation. However, decreased lean tissue accretion and breast muscle yield in

broilers fed the fish oil diet in the current experiment suggest that 13.68% fish oil supplementation alters metabolic muscle activity in growing broilers. In the event that nutrient partitioning was occurring, there appears to be an upper limit for dietary inclusion of polyunsaturated fatty acids in broiler diets and once that limit is reached growth performance may become impaired.

A similar decrease in broiler performance was reported by Ferrini et al. (2008) who fed 10% tallow, 10% flaxseed oil, 10% sunflower oil, or 10% mixed fat (55% tallow, 35% flaxseed oil, and 10% sunflower oil resulting in equal parts saturated, mono- and polyunsaturated fatty acids) diets to broilers from 8 to 42 d of age. No differences were observed in feed intake among broilers fed the treatment diets, which was in agreement with the results from the current experiment (Ferrini et al., 2008). However, feeding a 10% mixed fat diet to broilers resulted in 7% increased final BW, 50% increased abdominal fat pad weight, and 10% increased skin weight compared to broilers fed the 10% flaxseed oil and 10% sunflower oil diets ($P < 0.05$; Ferrini et al., 2008). In agreement with Ferrini et al. (2008), feeding the fish oil diet in the current experiment, highest in polyunsaturated fatty acids, to broilers resulted in reduced muscle accretion and reduced BW compared to the diets containing a mixture of saturated and unsaturated fatty acid sources. These findings support that the dietary balance of saturated and unsaturated fatty acids is more important for overall broiler performance than prophylactic EPA and DHA supplementation.

Although it is common to encounter lameness and bone deformities in commercial broiler flocks (Sørensen et al., 2000; Bizeray et al., 2002; Grupioni et al., 2015), the broilers in this experiment did not exhibit clinical signs of leg weakness, abnormal gait, dyschondroplasia, or rotation deformities. The experiment was designed to investigate if

dietary omega-3 fatty acid supplementation fed to broilers would prophylactically improve the bone characteristics of an "at risk" model prone to lameness and bone lesions. Lameness may be caused by increased competition for feeder space based on stocking density (Sørensen et al., 2000), use of abrasive straw bedding (Su et al., 2000), or opportunistic bacterial infections (Dumas et al., 2011) in commercial environments. Further investigation while actively inducing lameness or stress may elucidate if dietary omega-3 fatty acid supplementation may serve a therapeutic purpose for real world applications.

Dietary flaxseed oil and fish oil supplementation in broilers still serve a useful role for creating value-added enriched meats; however, the results from the current experiment did not demonstrate improved broiler bone health with therapeutic levels of omega-3 fatty acid supplementation. Due to the potential for performance losses when using fish oil, further investigation is recommended to evaluate the stability of fish oil sources prior to commercial applications in broiler diets.

## 4.6 Acknowledgements

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## TABLES

**Table 4.1.** Lameness and bone conformation scale with definition used to score Ross 308 male broiler chickens fed palm oil control, flaxseed oil, or fish oil supplemented diets[1] for 28 consecutive d.[2]

| Score | Measure | Definition[3] |
|---|---|---|
| 0 | **Lameness** | Bird walked normally without limping and took 10 uninterrupted steps within a 1-m distance |
| | **Bone** | Bird had normal keel (sternum), leg, and wing bones |
| 1 | **Lameness** | Bird walked with some difficulty and took between 6 and 9 uninterrupted steps within a 1-m distance |
| | **Bone** | One of these aforementioned bones was mildly curved |
| 2 | **Lameness** | Bird walked with much difficulty and took ≤ 5 uninterrupted steps within a 1-m distance |
| | **Bone** | One of these aforementioned bones was moderately curved |
| 3 | **Lameness** | Bird was non-ambulatory and was removed from the trial |
| | **Bone** | Bone was sigmoid, severely curved, or broken |

[1]Experimental diets were applied for a 28 consecutive d dietary supplementation period (6 to 34 d of age). Broiler birds were fed a palm oil control diet (basal diet + no supplemental omega-3 fatty acid source), flaxseed oil (basal + 4.1% alpha-linolenic acid), or fish oil (4.1% eicosapentaenoic acid and docosahexaenoic acid blend). Control and dietary treatments contained 80 broilers each with 20 replicate experimental units.

[2]A 5 d acclimation period was given to broiler chickens (n = 240).

[3]Lameness definition located on the top row in relation to a score and bone conformation definition located below lameness within a given score. Lameness definitions were adapted from previously published work by Almeida Paz et al. (2010) and keel bone conformation definitions were adapted from Tarlton et al. (2013).

**Table 4.2.** Calculated and analyzed values for palm oil control, flaxseed oil, and fish oil experimental starter diets[1] fed to Ross 308 broiler chickens for a 28 consecutive d dietary supplementation period to evaluate the effects of dietary alpha-linolenic (ALA) or eicosapentaenoic acid (EPA) and docosahexaenoic (DHA).

| | Experimental diet (%) | | |
|---|---|---|---|
| **Ingredients** | **Palm oil control** | **Flaxseed oil** | **Fish oil** |
| Corn | 26.60 | 26.61 | 26.61 |
| Soybean meal 48% crude protein | 50.00 | 50.00 | 50.00 |
| Wheat middlings | 5.00 | 5.00 | 5.00 |
| Palm oil | 14.00 | 6.53 | 0.33 |
| Flaxseed oil | 0.00 | 7.48 | 0.00 |
| Fish oil | 0.00 | 0.00 | 13.68 |
| Dicalcium phosphate | 1.76 | 1.76 | 1.76 |
| Calcium carbonate[2] | 1.06 | 1.07 | 1.07 |
| Sodium chloride | 0.50 | 0.50 | 0.50 |
| Methionine hydroxy analogue | 0.32 | 0.30 | 0.30 |
| Choline chloride | 0.10 | 0.10 | 0.10 |
| L-threonine | 0.03 | 0.03 | 0.03 |
| V and M premix[3] | 0.63 | 0.63 | 0.63 |
| **Calculated values** | | | |
| Calcium | 1.00 | 1.00 | 1.00 |
| Available phosphorus | 0.45 | 0.45 | 0.45 |
| **Analyzed values** | | | |
| Crude fat | 13.61 | 15.15 | 13.21 |

[1]Experimental diets used a common basal diet combined with 14% palm oil, 7.48% flaxseed oil, or 13.68% fish oil. Calculated ME for each diet was 3,400 kcal/kg. Control and dietary treatments contained 80 broilers each with 20 replicate experimental units.

[2]Calcium carbonate added was a 50/50 mixture of small (< 2 mm) and large (≥ 2 mm) particle.

[3]Vitamin and mineral premix provided per kg of diet: selenium 250 μg; vitamin A 8,250 IU; vitamin $D_3$ 2,750 IU; vitamin E 17.9 IU; menadione 1.1 mg; vitamin $B_{12}$ 12 μg; biotin 41 μg; choline 447 mg; folic acid 1.4 mg; niacin 41.3 mg; pantothenic acid 11 mg; pyridoxine 1.1 mg; riboflavin 5.5 mg; thiamine 1.4 mg; iron 282 mg; magnesium 125 mg; manganese 275 mg; zinc 275 mg; copper 27.5 mg; iodine 844 μg.

**Table 4.3.** Dietary fatty acid analysis for palm oil control, flaxseed oil, and fish oil experimental starter diets[1] fed to Ross 308 broiler chickens for a 28 consecutive d dietary supplementation period used to evaluate the effects of dietary alpha-linolenic (ALA) or eicosapentaenoic acid (EPA) and docosahexaenoic (DHA).

| | Experimental diet (%) | | |
|---|---|---|---|
| **Fatty acid (C:double bond)** | **Palm oil control** | **Flaxseed oil** | **Fish oil** |
| Myristic acid (14:0) | 1.04 | 0.67 | 7.99 |
| Palmitic acid (16:0) | 39.34 | 21.46 | 19.96 |
| Palmitoleic acid (16:1) | 0.00 | 0.33 | 8.63 |
| Margaric acid (17:0) | 0.00 | 0.00 | 0.42 |
| Stearic acid (18:0) | 3.82 | 3.48 | 3.56 |
| Oleic acid (18:1) | 35.49 | 25.49 | 11.61 |
| Linoleic acid (18:2) | 18.06 | 18.81 | 11.74 |
| Alpha-linolenic acid (18:3) | 1.91 | 28.39 | 2.32 |
| Arachidic acid (20:0) | 0.33 | 0.22 | 0.24 |
| Arachidonic acid (20:4) | 0.00 | 0.00 | 1.07 |
| Eicosapentaenoic acid (20:5) | 0.00 | 1.16 | 19.55 |
| Behenic acid (22:0) | 0.00 | 0.00 | 0.00 |
| Docosapentaenoic acid (22:5) | 0.00 | 0.00 | 2.04 |
| Docosahexaenoic acid (22:6) | 0.00 | 0.00 | 10.87 |
| Crude fat[2] | 13.61 | 15.15 | 13.21 |
| Saturated fat | 44.53 | 25.83 | 32.17 |
| Unsaturated fat | 55.47 | 74.17 | 67.83 |
| Total omega-3 fatty acid[3] | 1.91 | 29.55 | 32.73 |
| Total omega-6 fatty acid[4] | 18.06 | 18.81 | 12.82 |
| Omega-6:omega-3 ratio | 9.46 | 0.64 | 0.39 |

[1]Experimental diets used a common basal diet combined with 14% palm oil, 7.48% flaxseed oil, or 13.68% fish oil. Control and dietary treatments contained 80 broilers each with 20 replicate experimental units.

[2]Crude fat was the analyzed fat content of pool feed samples. The fatty acid values represent the percent composition of the crude fat for each diet.

[3]Total omega-3 fatty acid was the sum of alpha-linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid.

[4]Total omega-6 fatty acid was the sum of linoleic acid and arachidonic acid.

**Table 4.4.** Bone health measurements of Ross 308 male broiler chickens fed palm oil control, flaxseed oil, or fish oil experimental diets[1] for a 28 consecutive d dietary supplementation period to evaluate the effects of dietary alpha-linolenic (ALA) or eicosapentaenoic acid (EPA) and docosahexaenoic (DHA).[2]

| | Experimental diet[3, 4] | | | SEM | *P*-value | | |
|---|---|---|---|---|---|---|---|
| | **Palm oil control** | **Flaxseed oil** | **Fish oil** | **Diet** | **Diet** | **Time** | **Diet*Time** |
| **Lameness score** | 0.05 | 0.04 | 0.03 | 0.039 | 0.83 | 0.98 | 1.00 |
| **Conformation score** | 0.04 | 0.01 | 0.02 | 0.034 | 1.00 | 0.96 | 1.00 |
| **BMC (g)** | 27.53 | 29.56 | 28.60 | 0.810 | 0.21 | - | - |
| **BMD (g/cm$^2$)** | 0.157$^{ab}$ | 0.164$^{a}$ | 0.149$^{b}$ | 0.0041 | 0.04 | - | - |

[1]Experimental diets were applied for a 28 consecutive d dietary supplementation period (6 to 34 d of age). Broiler birds were fed a palm oil control diet (basal diet + no supplemental omega-3 fatty acid source), flaxseed oil (basal + 4.1% ALA), or fish oil (4.1% EPA and DHA blend). Control and dietary treatments contained 80 broilers each with 20 replicate experimental units.

[2]A 5 d environmental acclimation period was given to broiler chickens (n = 240).

[3]One bird per experimental unit (EU) was measured for bone mineral content (BMC) and bone mineral density (BMD) after the 28 consecutive d dietary supplementation period using dual-energy x-ray absorptiometry.

[4]Individual birds per EU were scored every 7 d for lameness in gait (scale; 0 = normal to 3 = severe, non-ambulatory) and palpated for bone conformation (scale of 0 = normal to 3 = severe curvature or broken).

[a-b]Least square means without a common superscript differ significantly within a row ($P \leq 0.05$).

**Table 4.5.** Performance of Ross 308 male broiler chickens fed palm oil control, flaxseed oil, or fish oil experimental diets[1] for a 28 consecutive d dietary supplementation period to evaluate the effects of dietary alpha-linolenic (ALA) or eicosapentaenoic acid (EPA) and docosahexaenoic (DHA).[2]

| | Experimental diet[3] | | | | |
|---|---|---|---|---|---|
| | **Palm oil control** | **Flaxseed oil** | **Fish oil** | **SEM** | ***P*-value** |
| **BW (kg; 34 d of age)**[4] | 2.480[a] | 2.510[a] | 2.246[b] | 0.0318 | ≤ 0.01 |
| **ADG (kg)** | 0.083[a] | 0.084[a] | 0.075[b] | 0.0011 | ≤ 0.01 |
| **FI (kg/bird)** | 4.292 | 4.217 | 4.240 | 0.0413 | 0.43 |
| **FCR** | 1.857[b] | 1.791[b] | 2.023[a] | 0.0358 | ≤ 0.01 |
| **Fat accretion (kg)** | 0.139 | 0.146 | 0.135 | 0.0160 | 0.87 |
| **Lean accretion (kg)** | 2.245 | 2.307 | 2.124 | 0.0563 | 0.07 |
| **Breast yield (%)** | 13.894[a] | 13.522[ab] | 12.593[b] | 0.3553 | 0.05 |

[1]Experimental diets were applied for a 28 consecutive d dietary supplementation period (6 to 34 d of age). Broiler birds were fed a palm oil control diet (basal diet + no supplemental omega-3 fatty acid source), flaxseed oil (basal + 4.1% ALA), or fish oil (4.1% EPA and DHA blend). Control and dietary treatments contained 80 broilers each with 20 replicate experimental units.

[2]A 5 d environmental acclimation period was given to broiler chickens (n = 240).

[3]Values represent measurements after 28 consecutive d dietary supplementation period. Body weight (BW) and feed disappearance were measured every 7 d for feed intake (FI), feed conversion ratio (FCR), and average daily gain (ADG) calculations. Fat mass and lean accretion were obtained using dual-energy x-ray absorptiometry imaging.

[4]Starting BW at 6 d of age was not different among the broilers fed experimental diets ($P$ = 0.42).

[a-b]Least square means without a common superscript differ significantly within a row ($P \leq$ 0.05).

# CHAPTER 5. DIETARY FISH OIL REDUCED INFLAMMATORY CYTOKINE GENE EXPRESSION AND INCREASED BONE MINERAL RETENTION IN BROILERS SUBJECTED TO A REPEATED INFLAMMATORY CHALLENGE

A manuscript to be submitted to *Poultry Science*

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## 5.1 Abstract

Growing broilers are susceptible to pathogenic infection due to their naïve immune systems. Broilers are efficient production animals genetically selected for prolific growth rates, which has resulted in a model at risk for stress and leg bone pathology. The omega-3 fatty acids: alpha-linolenic (ALA), eicosapentaenoic (EPA), and docosahexaenoic (DHA) acids possess anti-inflammatory properties and modulate bone mineral deposition *in vitro*. Dietary omega-3 fatty acid supplementation in broiler diets may be able to mitigate performance losses due to inflammation or bone health issues. Experimental palm oil control, flaxseed oil (4.1% ALA), and fish oil (2.6% EPA and 1.5% DHA) diets were fed for a 28 d dietary supplementation period (6 to 34 d old; n = 120). To evaluate the protective anti-inflammatory and bone modulating effects of dietary flaxseed and fish oils on performance and health under stress, a repeated inflammatory challenge was induced by administering

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lipopolysaccharide injections (LPS) every 48 h at 35, 37, 39, and 41 d of age. Broilers received intraperitoneal injections of LPS solution in increasing dosage (1.0 to 1.7 mg/kg) or equal volume sterile saline assigned randomly by pen (2 broilers/pen = experimental unit). Performance was evaluated during the repeated inflammatory challenge with liver, muscle, and whole carcass dual-energy x-ray absorptiometry images collected at d 7 of the LPS challenge for body composition and mRNA gene expression. Data were analyzed using PROC MIXED in SAS with significant means separated using Tukey's Test ($P \leq 0.05$). Repeated LPS injection in broilers reduced BW by 0.133 ± 0.0183 kg and FI by 0.102 ± 0.0198 kg compared to saline ($P \leq 0.01$). Feeding broilers fish oil increased total lean tissue body composition by 4.3 and 7.2 ± 0.97% compared to control and flaxseed oil ($P \leq 0.01$). Feeding fish oil to broilers reduced liver IL-1β, IL-6, IL-18 and IFNγ expression by ≥ 0.21-fold compared to the control ($P \leq 0.05$). Feeding broilers flaxseed oil or fish oil increased total bone mineral content and accretion by ≥ 0.2 ± 0.03 g and ≥ 3.625 ± 0.9263%, respectively, compared to control ($P \leq 0.01$). Although ALA or EPA and DHA provided anti-inflammatory effects and bone health improvements to broilers, it did not mitigate performance losses during the repeated inflammatory challenge. Therefore, flaxseed oil and fish oil supplementation in broiler diets is recommended for enriching broiler muscle tissue and providing secondary health benefits to the bird during periods of inflammation.

Key words: ALA, EPA, DHA, lipopolysaccharide, DEXA

## 5.2 Introduction

Growing broilers have a naïve immune system and are susceptible to environmental stress and infection by opportunistic bacteria and pathogens (Saif et al., 2008; Langkabel et

al., 2015). Furthermore, broiler varieties used for meat production have been genetically selected for rapid muscle growth, which has resulted in increased reports of lameness observed in commercial flocks (Knowles et al., 2008; Grupioni et al., 2015). Previous *in vitro* studies demonstrated that omega-3 fatty acids, specifically eicosapentaenoic acid (EPA; C20:5) and docosahexaenoic acid (DHA; C22:6) found in fish oil, exhibited anti-inflammatory properties and increased bone mineral retention (Sun et al., 2003; Tur et al., 2012; Kim et al., 2017). Alpha-linolenic acid (ALA; C18:3), the omega-3 fatty acid precursor to EPA and DHA, found in flaxseed oil has been linked to reducing keel bone breakage in free-range laying hens (Tarlton et al., 2013; Toscano et al., 2015). Omega-3 fatty acid supplementation in broiler diets may potentially provide commercial broilers anti-inflammatory protection and improved bone health during periods of production stress.

Omega-3 fatty acid supplementation in broiler diets may be able to mitigate the intense inflammatory reaction and subsequent performance losses that result from infection or due to environmental stress (de Pablo and de Cienfuegos, 2000; Serhan, 2014; Shoda et al., 2015). Omega-3 fatty acids, specifically EPA and DHA, perform multiple anti-inflammatory functions at a cellular level, such as displacing pro-inflammatory omega-6 fatty acids from cell membranes (Newman et al., 2002a), acting as intracellular second messengers (de Pablo and de Cienfuegos, 2000), and acting as a substrate for generating pro-resolving lipid metabolites (Serhan, 2014). When incorporated into cell membranes, EPA and DHA decrease cell membrane fluidity, lipid raft formation and size, which results in reduced inflammatory cell receptor activation and cytokine signaling (Rockett et al., 2011; Hou et al., 2015; Shoda et al., 2015). Dietary omega-3 fatty acid supplementation in broiler diets may be

able to provide some form of relief to commercial broilers under production stress or disease challenge through multimodal anti-inflammatory action.

Chapter 4 of this dissertation demonstrated that prophylactic supplementation of 4.1% dietary ALA or EPA and DHA did not improve broiler bone characteristics when fed from 6 to 34 d of age. However, other work reported that feeding broilers a 3% flaxseed oil and 2 % palm oil diet from 0 to 42 d of age increased tibial bone mineral density by 0.1 g/cm$^2$ compared to broilers fed a 5% lard diet ($P < 0.05$; Zhong et al., 2014). The slight increase in broiler bone mineral density was detected using 3% flaxseed oil supplementation (Zhong et al., 2014), therefore a higher dietary concentration was used in the current experiment in order to generate a therapeutic response in broiler bone activity. It appeared that osteoclastic bone resorption was inhibited by ALA supplementation in the study by Zhong et al. (2014), allowing for increased osteoblastic bone mineral deposition (Nakanishi and Tsukamoto, 2015; Kim et al., 2017). The *in vitro* effects of EPA and DHA include inducing mature osteoclasts to undergo apoptosis and attenuating osteoclastic precursor cells from proliferation and differentiation, DHA attenuating osteoclasts more than EPA (Rahman et al., 2008; Kim et al., 2017). However, poultry are only able to metabolize ≤ 5% dietary ALA to EPA and DHA because endogenous elongases and desaturases become overwhelmed (Burdge and Calder, 2006; Zivkovic et al., 2011). Even though prophylactic use of dietary omega-3 fatty acid supplementation did not definitively improve bone mineral density in a research setting, it may be providing protective effects only detectable during times of physical or biological stress.

In order to study the protective effects of dietary omega-3 fatty acids on broiler health during a stressful period, a controlled inflammatory challenge was induced. Previous

research has established that lipopolysaccharide (LPS) administration can generate a controlled acute phase inflammatory response in broilers and laying hens through the Toll-like receptor 4 (TLR4) pathway (Korver et al., 1998; Leshchinsky and Klasing, 2001; Munyaka et al., 2013). Inflammation generates clinical signs such as fever, swelling, and redness that are modulated by pro-inflammatory cytokines synthesized by stimulated immune cells (MacKay and Lester, 1992; Nakamura et al., 1998; Xie et al., 2000). Many LPS models are challenged using a short time frame with measurements taken from 1 to 72 h after a single LPS dose (Xie et al., 2000; Cheng et al., 2004; Kaiser et al., 2012). In the few broiler experiments where repeated LPS injections were administered, the repeated inflammatory challenges lasted for ≤ 7 d with a constant LPS dosage for each injection (Korver and Klasing, 1997; Korver et al., 1998; Jiang et al., 2010). Administering repeated LPS injections to broilers using the same dosage resulted in a reduced inflammatory response, which suggests immunotolerance with repeated exposure (De Boever et al., 2008). A novel approach used in the current experiment to prevent immunotolerance was increasing the LPS dosage with each successive LPS injection to ensure an inflammatory reaction occurred throughout a repeated LPS inflammatory challenge. Although different from pathogenic infection and commercial stress, a repeated LPS inflammatory challenge can be used on growing broilers to generate stress in order to help evaluate the protective health benefits of dietary omega-3 fatty acid supplementation.

The objective of this experiment was to feed growing broilers a flaxseed oil (4.1% ALA source) or fish oil (4.1% EPA and DHA source) supplemented diet and induce a repeated LPS inflammatory challenge using 20% increasing LPS dosage (1.0 to 1.7 mg/kg) with each successive injection in order to investigate the protective effects on growth

performance, liver inflammatory gene expression, muscle metabolic gene expression, and bone mineral characteristics. The hypothesis of this experiment was that bioactive EPA and DHA supplementation from fish oil would mitigate broiler performance losses, improve bone health, and provide anti-inflammatory protection during a repeated inflammatory challenge better than ALA when fed at equivalent 4.1% concentrations.

## 5.3 Materials and Methods

### 5.3.1 Animals and Housing

The current experiment was a continuation of the previously described experiment in Chapter 4 of this dissertation. Therefore, the descriptions of the animals and housing, omega-3 fatty acid supplementation period from 6 to 34 d of age, production performance, and fatty acid analysis in these materials and methods were identical or adapted from those described in Chapter 4. The protocol for this experiment was approved by the Iowa State University Institutional Animal Care and Use Committee. Broilers were cared for in accordance with the FASS Guide for the Care and Use of Agricultural Animals in Research and Teaching (2010). Two hundred and forty male Ross 308 hatching-day broiler chicks were obtained from a commercial hatchery (Bancroft, IA) and transported to the Poultry Research and Teaching Unit at Iowa State University. The poultry house was enclosed with cross ventilation and divided in half, resulting in 2 rooms containing 30 pens in each half of the house. Chicks were housed in floor pens (121.9 cm length × 121.9 cm width; 3,600 $cm^2$/bird at placement) with a supplemental 125-watt clear incandescent heat bulb in each pen. Each pen contained 10 cm of pine shavings placed on the concrete floor. Rooms were heated to 29°C at time of placement using a vented gas-fired heater (Modine High Efficiency, Modine Manufacturing

Co., Racine, WI) and room temperature was reduced by 2.5°C every 7 d until 21°C was attained (by experimental d 15). The light program was 23L:1D for the first 7 d of life (experimental d -6 to 1) and then adjusted to 20L:4D for the duration of the experiment. Light intensity was 30 to 40 lux at bird level, as recommended by the Ross Broiler Management Manual (2009). Feed was provided *ad libitum* in one circular hanging feeder (30.5 cm diameter × 29.2 cm height) per pen (Model HF30, Brower Equipment, Houghton, IA). The feeders were placed at bedding level at the start of the experiment, and then were raised as the birds grew (7 cm at 10 d of age, 10 cm at 17 d of age, 13 cm at 21 d of age, and 15 cm from 28 to 43 d of age). Water was provided via Ziggity 360° nipple waterlines with 5 nipples per pen (Ziggity System Inc., Middlebury, IN) set to 7 cm of column pressure and suspended 12 cm above bedding level from 0 to 14 d of age. Column pressure was increased to 10 cm from 14 to 28 d of age and then increased to 15 cm column pressure for the remainder of the experiment. Waterlines were raised to 22 cm above bedding level 14 to 21 d of age, 25 cm from 21 to 28 d of age, and 29 cm respectively for the remainder of the experiment.

### 5.3.2 Omega-3 Fatty Acid Supplementation Period

Broilers were given a 5 d environmental acclimation period (0 to 5 d of age) prior to starting the experiment. At 6 d of age (baseline; experimental d 0) each bird was wing banded (Tab End Wing Band, Style 898, National Band & Tag Co., Newport, KY into the chick's right wing web), weighed on a digital gram scale (Defender 3000, Ohaus Corp., Parsippany, NJ), sorted by 5 g increments, distributed equally by body weight (BW) to pens, and randomly assigned an experimental diet. Broilers were fed diets for 28 consecutive d,

from 6 to 34 d of age, which served as the dietary supplementation period allowing for omega-3 fatty acid incorporation into the broiler tissues.

The experimental design was a randomized complete block design where pen was the experimental unit (EU) consisting of 4 broilers/pen with 20 EU/diet. Each half of the poultry house was a block with equal assignment of experimental diets. In order to evaluate broiler health responses to omega-3 fatty acid supplementation, 4.1% dietary ALA or EPA and DHA was supplemented into the treatment diets, which was a 50% increase in omega-3 fatty acid concentration from previous work (Lopes et al., 2013; Tarlton et al., 2013; Zhong et al., 2014). Palm oil was used as the control fat source because it was composed of 50% saturated fatty acids, 42% monounsaturated fatty acids, and ≤ 8% polyunsaturated fatty acids with little to no omega-3 or omega-6 fatty acids. Addition of palm oil to experimental diets had little impact on omega-6:omega-3 fatty acid ratios, due to its fatty acid profile. Three experimental diets were compared using a corn-soybean meal-wheat middlings basal diet (Table 5.1) formulated to meet or exceed NRC (1994) requirements: 1) palm oil control (no supplemental omega-3 fatty acids; BW 0.157 ± 0.0010 kg), 2) flaxseed oil (4.1% ALA; BW 0.157 ± 0.0010 kg), and 3) fish oil (2.5% EPA and 1.6% DHA; BW 0.158 ± 0.0010 kg) diet. A starter diet was formulated for 0 to 21 d of the experiment and a grower-finisher formulation was used from 22 to 36 d. All feeders were manually agitated daily to keep feed flow uniform across experimental pens.

### 5.3.3 Post-supplementation Inflammatory Challenge

In order to evaluate the protective anti-inflammatory effects of dietary ALA or EPA and DHA supplementation fed to growing broilers, a repeated inflammatory challenge was

applied over a 7 d inflammatory challenge period (35 to 42 d of age). Following the 28 d dietary supplementation period, 10 pens/diet each containing 2 broilers were injected intraperitoneally with 1 mg/kg LPS (L2630, *E. coli* 0111:B4 LPS, Sigma-Aldrich Co. LLC., St. Louis, MO) resuspended in sterile saline. A further 10 pens/diet each containing 2 broilers were injected with the equivalent volume of sterile saline. Broilers were injected every 48 h (35, 37, 39, and 41 d of age) with each successive dosage being increased by 20% (from 1 to 1.7 mg/kg). Twenty-four h post-final injection, birds were euthanized via $CO_2$ asphyxiation following the AVMA Panel on Euthanasia (2013). Right liver lobe and left pectoralis major sections were harvested (2 $cm^3$) and flash frozen in liquid nitrogen and stored at -80°C for further analysis.

### 5.3.4 Production Performance

All broilers were observed twice daily in accordance with university policy. Broilers were weighed individually on a digital gram scale at the start of the inflammatory change (35 d of age) and then every 48 h for 7 consecutive d. Feed disappearance was measured over the 7 d challenge for feed intake (FI), feed conversion ratio (FCR), and average daily gain (ADG) was calculated as follows: FI = start feed weight kg – end feed weight kg; FCR = FI ÷ (end BW kg – start BW kg); and ADG = (end BW kg – start BW kg) ÷ d.

At the start of the inflammatory challenge (35 d of age), 1 broiler/EU was euthanized by $CO_2$ asphyxiation for imaging using dual-energy x-ray absorptiometry (DEXA; Discovery A, Hologic Inc., Marlborough, MA) following methods previously described by Mitchell et al. (1997). Baseline DEXA imaging provided starting fat mass (kg), lean mass (kg), and total mass (kg). After the 7 d inflammatory challenge, 1 broiler/EU (120 = 40 broilers/diet) was

euthanized and imaged to obtain the end DEXA performance values. The difference between the end and baseline values equated to tissue accretion for the 7 d inflammatory challenge period (35 to 42 d of age).

### 5.3.5 Fatty acid analysis

Feed samples from each experimental diet were collected for every generated batch and stored at -20°C, and fatty acid analysis was performed using methods previously described by Nam et al. (2001) and Sun et al. (2013). Samples were ground through a 0.5 mm screen (Thomas-Wiley Laboratory Mill Model 4, Arthur H. Thomas Company, Philadelphia, PA) and fatty acid profiles were analyzed using gas chromatography (HP 6890, Hewlett Packard Co., Palo Alto, CA). Fatty acid profiles from frozen muscle samples (3 EU) from each factorial arrangement of experimental diet by inflammatory challenge (diet*challenge) were analyzed post-inflammatory challenge period (35 to 42 d of age).

### 5.3.6 Quantitative Real-Time PCR

Flash frozen liver samples were stored at -80°C. RNA extraction and DNase cleanup followed manufacturer directions (RNeasy Mini Kit, Qiagen, Valencia, CA). RNA samples from 5 EU for each factorial arrangement (diet*challenge) were analyzed in triplicate by quantitative real-time PCR (qRT-PCR; DNA Engine Opticon 2 System, Bio-Rad Lab. Inc., Hercules, CA) using 1-step SYBR Green master mix (QuantiTect SYBR Green RT-PCR, Qiagen, Valencia, CA) adapted from methods described by Kaiser et al. (2006). Relative RNA expression levels were measured for interleukin-1 beta (IL-1β), IL-6, IL-18, interferon gamma (IFNγ), serum amyloid A (SAA), IL-10, and peroxisome proliferator-activated

receptor gamma (PPARγ) using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference gene. Forward and reverse primers were generated (Integrated DNA Technologies, Coralville, IA) from sequences listed in Table 5.2 with referenced source. Adjusted cycle threshold (Adj. Ct) value and normalized gene expression level (fold change) were calculated using the following adapted formulas (Eldaghayes et al., 2006; Kaiser et al., 2012): Adj. Ct = $40 - [\text{Mean } Ct_{\text{test gene}} + (\text{Median}_{GAPDH} - \text{Mean } Ct_{GAPDH})] \times (\text{Slope}_{\text{test gene}} \div \text{Slope}_{GAPDH})$; Fold change = $2^{(-(Ct_{\text{test gene treatment}} - Ct_{\text{GAPDH treatment}}) - (Ct_{\text{test gene control}} - Ct_{\text{GAPDH control}}))}$.

Flash frozen muscle samples were stored at -80°C. RNA extraction followed manufacturer directions (Ambion TRIzol Reagent, Life Technologies, Carlsbad, CA). RNA samples from 5 EU for each factorial arrangement (diet*challenge) were analyzed in triplicate by qRT-PCR (MyiQ Optical Module with iCycler, Bio-Rad Lab. Inc., Hercules, CA) using 1-step SYBR Green master mix as previously described. Relative RNA expression levels were measured for IL-6, SAA, eukaryotic translation initiation factor 4E binding protein 1 (4EBP1), ribosomal protein S6 kinase 1 (RPS6K1), ubiquitin, calpain-2 (CAPN2), and proteasome 26S subunit ATPase 2 (PSMC2) using ribosomal 28S subunit (28S) as a reference gene (Table 5.2). Adjusted Ct and fold change were calculated as previously described using 28S.

### 5.3.7 Bone Mineral Absorptiometry

Baseline DEXA imaging as previously described in 5.3.4 Production Performance provided starting bone mineral content (BMC; g) and bone mineral density (BMD; g/cm$^2$). BMC was measured to evaluate total mineral content within bone and BMD was measured to evaluate the distribution of mineral in an aerial plane. At the end of the 7 d inflammatory

challenge, 1 broiler/EU (120 = 40 broiler/diet) was euthanized and imaged to obtain end BMC and BMD values. The difference between the end and baseline values equated to BMC accretion and BMD change for the 7 d inflammatory challenge period (35 to 42 d of age).

### 5.3.8 Statistical Analysis

Data were analyzed using the UNIVARIATE procedure in SAS 9.4 (SAS Institute Inc., Cary, NC) to test for normality by plotting the predicted residuals and the quantile-quantile values. The experiment was a randomized complete block design consisting of 3 experimental diets (palm oil control, flaxseed oil, and fish oil) and 20 EU replicates for each diet (n = 120 broilers). Each half of the poultry house was a block with experimental diets equally assigned to each block. For the repeated inflammatory challenge, broilers from 10 EU replicates for each diet were injected with LPS and the broilers from the remaining 10 EU replicates for each diet were injected with equal volume of sterile saline. Performance (BW, FI, FCR, and ADG), fatty acid, qRT-PCR, and DEXA data (fat accretion, total fat, lean accretion, total lean, mass accretion, total mass, BMC, and BMD) were analyzed using the MIXED procedure in SAS. The statistical model included inflammatory challenge injection (saline vs. LPS) and experimental diet (palm oil control vs. flaxseed oil vs. fish oil) as fixed effects in a factorial arrangement (diet*challenge) with baseline 35 d of age starting BW and starting BMD as covariates. The random effect of pen nested within treatment was included in the model. The PDIFF option was used to identify significant differences. Tukey's Honestly Significant Difference (HSD) Test was used to separate means. A $P$-value $\leq 0.05$ was considered to be significant.

## 5.4 Results

### 5.4.1 Production Performance

No diet*challenge interactions occurred for performance measured during the repeated inflammatory challenge period from 35 to 42 d of age ($P \geq 0.26$); therefore, the main effects of inflammatory challenge (LPS and saline injection) and diet (palm oil control, flaxseed oil, and fish oil diet) were reported.

Lipopolysaccharide injection administered to broilers resulted in decreased end BW by 0.133 ± 0.0183 kg/bird compared to saline injection administered to broilers during the 7 d inflammatory challenge ($P \leq 0.01$). Lipopolysaccharide injection given to broilers, regardless of experimental diet during the 7 d challenge period, resulted in reduced FI by 0.102 ± 0.0198 kg and ADG by 0.019 ± 0.0024 kg/d compared to broilers that were injected with saline ($P \leq 0.01$). Broilers that were injected with LPS resulted in increased FCR by 0.189 ± 0.0404 compared to broilers that were administered saline injections ($P = 0.03$; Table 5.3).

Feeding the fish oil diet to broilers, regardless of challenge, resulted in increased lean accretion by 0.229 ± 0.0498 kg compared to broilers fed the flaxseed oil diet ($P = 0.04$). Feeding the fish oil diet to broilers resulted in increased total lean tissue composition by > 4% at 42 d of age compared to broilers fed either the palm oil control or flaxseed oil diets ($P \leq 0.01$; Table 5.4). The effect of diet did not affect the remaining performance parameters during the inflammatory challenge ($P \geq 0.11$; Table 5.4), other than 35 d of age starting BW used a statistical covariate in the model ($P \leq 0.01$; Table 5.3).

### 5.4.2 Fatty Acid Analysis

Flaxseed oil and fish oil complete diets both consisted of 4.48% total dietary omega-3 fatty acids when calculated using analyzed ALA, EPA, DHA, and dietary crude fat values (Table 5.5). The control diet contained 2 times more palmitic acid and ≥ 1.4 times more oleic acid than the treatment diets, which corresponded with the increased dietary composition of palm oil (Table 5.1). The flaxseed oil diet contained 14 times more ALA content compared to palm oil control and fish oil diets, while the fish oil diet contained 19 times more EPA and 10 times more DHA than the palm oil control and flaxseed oil diets. Neither the palm oil control nor flaxseed oil diets contained detectable amounts of DHA (Table 5.5).

Neither the inflammatory challenge nor the interaction of experimental diet by inflammatory challenge altered the fatty acid composition of broiler muscle tissue ($P \geq 0.11$). Feeding the fish oil diet to broilers resulted in increased myristic, palmitoleic, margaric, EPA, and DHA content in muscle tissue compared to muscle tissue from broilers fed the palm oil control or flaxseed oil diets ($P \geq 0.01$). Feeding the flaxseed oil diet to broilers resulted in the greatest concentration of ALA in muscle tissue compared to muscle tissue from broilers fed the palm oil control or fish oil diets ($P \geq 0.01$). Muscular total fat content was not different among broilers fed experimental diets (P = 0.66; Table 5.6).

### 5.4.3 Quantitative Real-Time PCR

An interaction was observed when investigating the inflammatory responses of broiler liver to the experimental diet and inflammatory challenge. It was found that feeding the flaxseed oil diet and administering LPS to broilers resulted in reduced liver IL-6 gene expression by 0.09-fold compared to broilers fed the palm oil control diet and injected with

saline ($P$ = 0.02). A similar response was observed for broilers fed the fish oil diet and injected with saline, which resulted in reduced liver IL-6 gene expression by 0.04-fold compared to broilers fed the palm oil control diet and injected with saline ($P$ = 0.02; Figure 5.1A). Lipopolysaccharide administration resulted in increased relative mRNA gene expression of liver pro-inflammatory cytokines IL-1β by 4.90 and IL-18 by 1.65-fold compared to broilers injected with saline ($P \leq 0.01$; Figure 5.1B). Feeding the fish oil diet to broilers resulted in reduced liver pro-inflammatory cytokines IL-1β, IL-6, IL-18, and IFNγ and anti-inflammatory IL-10 relative gene expression by 0.38, 0.21, 0.62, 0.62 and 0.41-fold, respectively, compared to broilers fed the palm oil control diet ($P \leq 0.05$; Figure 5.1C).

An interaction was observed when investigating the metabolic responses of broiler muscle tissue to the experimental diet and inflammatory challenge. It was discovered that feeding the palm oil control diet to broilers and administering a LPS injection resulted in reduced relative expression of muscle catabolic protein PSMC2 by 0.8-fold compared to muscle tissue from broilers fed the palm oil control diet and injected with saline ($P$ = 0.04; Figure 5.2A). No differences were observed in broiler pectoralis muscle relative mRNA gene expression due to the inflammatory challenge (Saline vs. LPS) or from feeding experimental diets ($P \geq 0.06$; Figures 5.2B and 5.2C, respectively). However, feeding the flaxseed oil diet to broilers resulted in a trending reduction in pro-inflammatory cytokine IL-6 ($P$ = 0.06) and acute phase protein SAA (P = 0.09) gene expression in muscle tissue compared to broilers fed the palm oil control diet (Figure 5.2C).

### 5.4.4 Bone Mineral Absorptiometry

Inflammatory challenge and interaction of inflammatory challenge by experimental diet did not affect broiler BMC or BMD ($P \geq 0.24$). Broilers fed the flaxseed oil and fish oil diets resulted in increased BMC accretion during the 7 d challenge period by an average of 10.217 ± 0.9263 g compared to broilers fed the palm oil control diet ($P \leq 0.01$). Feeding the flaxseed oil and fish oil diets to broilers resulted in increased total BMC by ≥ 0.2% and BMD by ≥ 0.020 g/cm$^2$ at 42 d of age compared to broilers fed the palm oil control diet ($P \leq 0.01$). Feeding the fish oil diet to broilers resulted in a 0.023 ± 0.0041 g/cm$^2$ increase in BMD compared to broilers fed the flaxseed oil diet, while feeding the palm oil control diet to broilers resulted in a loss in BMD g/cm$^2$ by -0.010 during the 7 d challenge period ($P \leq 0.01$; Table 5.7).

## 5.5 Discussion

The growth performance data from this experiment support that repeated LPS injections at 20% increasing dosage was effective at inducing inflammation in broilers through the 7 d inflammatory challenge. As expected, LPS injections regardless of diet resulted in depressed FI, reduced BW, and increased FCR. These phenotypic performance losses were in agreement with previous broiler LPS studies, which reported that LPS administration resulted in reduced FI and BW (Korver et al., 1998; Gonzalez et al., 2011; Morris et al., 2014). Although BW and total mass accretion were negatively affected by LPS administration, the tissue composition and amount accrued was directly influenced by the experimental diets fed.

During the repeated LPS inflammatory challenge from 35 to 42 d of age, EPA and DHA supplied by dietary fish oil supported anabolic activity in broiler muscle tissue. Feeding the fish oil diet to broilers resulted in significantly increased lean tissue by > 4% based on total body composition, regardless of LPS or saline injection. This agreed with previous work reported by Newman et al. (2002b) that dietary fish oil supplementation increased broiler breast muscle:fat pad ratio. However, neither dietary ALA or EPA and DHA was able to mitigate the overall loss of 0.133 ± 0.0183 kg BW due to LPS administration. Gene expression in muscle from the current experiment did not provide supportive evidence that anabolic activity was up regulated or catabolic activity was down regulated, as measured by 4EBP1, RPS6K1, ubiquitin, CAPN2, and PSMC2. The current experimental data suggests that dietary fish oil supplementation may not alleviate broiler performance losses during periods of stress, where the expectation is to produce lean meat efficiently.

Previous studies demonstrated that LPS administration to rats generated increased ubiquitin and proteasome subunit synthesis from tissue samples, although measured 24 h after a single LPS injection (Rossi et al., 2015). In the current experiment, repeated LPS administration and feeding experimental diets did not result in inflammatory or anabolic gene expression differences in broiler muscle tissue. However, feeding the palm oil control diet to broilers and administering LPS injections resulted in reduced gene expression of catabolic protein PSMC2 compared to their palm oil control-fed counterparts that were injected with saline. The protein PSMC2 is part of the proteasome complex which is responsible for degrading intracellular proteins marked for removal (Nakashima et al., 2009). Reduced PSMC2 gene expression with LPS administration suggests that protein degradation was suppressed during the repeated inflammatory challenge while dietary ALA or EPA and DHA

supplementation assisted in maintaining myocellular catabolic capabilities. Measuring additional metabolic genes would assist in describing how broiler muscle tissue responds to inflammatory stress.

Protective anti-inflammatory effects were observed in broilers due to EPA and DHA from dietary fish oil supplementation; however, feeding flaxseed oil to broilers and injecting them with LPS resulted in suppressed expression of IL-6, a pro-inflammatory cytokine responsible for inducing the acute phase inflammatory response and T lymphocyte activation (Staeheli et al., 2001). In all other instances, flaxseed oil supplementation had no detectable effect at this age. The lack of anti-inflammatory effect may be due to inefficient enzymatic synthesis, as seen in other species at this young age, which would impair ALA conversion to EPA and DHA (Burdge and Calder, 2006; Guilloteau et al., 2010).

Overall, feeding the fish oil diets to broilers resulted in consistent suppression of inflammatory genes IL-1β, IL-6, IL-18, and IFNγ in the liver throughout the repeated LPS inflammatory challenge. These results supported previous *in vitro* work suggesting that EPA and DHA interfered with inflammatory signaling and activation, most likely through cell membrane incorporation and receptor interference (Rockett et al., 2011; Hou et al., 2015; Shoda et al., 2015). The inflammatory cytokine IL-1β is an endogenous pyrogen responsible for stimulating acute phase protein production in the liver and IL-18 stimulates natural killer cells and $T_H1$ lymphocytes to produce IFNγ for pathogen clearance (Duque and Descoteaux, 2014; Wawrocki et al., 2016). Another study reported that feeding mice fish oil diets supplying EPA and DHA resulted in suppressed IFNγ signaling during a *Listeria monocytogenes* infection and mice were not able to clear the pathogen from infected cells (Irons and Fritsche, 2005). Muted pro-inflammatory responses or gene expression, as

reported in the current experiment, are beneficial for managing chronic inflammatory conditions or autoimmune disorders (Lofvenborg et al., 2014; Shoda et al., 2015). However, immunosuppression may allow for or prevent clearance of opportunistic infections and may not be ideal in all situations, specifically for commercial producers that are dealing with influenza, Gram-positive, and mycobacterial disease challenges (Irons and Fritsche, 2005; McMurray et al., 2011).

The repeated LPS inflammatory challenge was effective at inducing inflammation in broilers. All broilers administered the LPS injection experienced increased gene expression of IL-1β and IL-18 in the liver, confirming that the immune system was under stress (Duque and Descoteaux, 2014; Wawrocki et al., 2016). During periods of acute inflammation PPARγ becomes elevated, which acts as an agonist for IL-10 release (Ferreira et al., 2014). However, in the current experiment feeding the fish oil diets to broilers resulted in reduced liver expression of the anti-inflammatory gene IL-10 due to EPA and DHA. These results suggest that inflammatory resolution occurred and finished for fish oil supplemented broilers during the repeated inflammatory challenge. If inflammation resolved, IL-10 would no longer be needed, therefore, expression levels would be reduced.

Throughout the repeated inflammatory challenge, broilers fed flaxseed oil and fish oil diets resulted in increased total BMC and BMD compared to the control. These results agree with previous reports that dietary fish oil supplementation fed in swine and rats models increased BMC and BMD compared to their respective controls (Lukas et al., 2011; Andersen et al., 2013). The current experiment, using both ALA or EPA and DHA, also supported work done by Zhong et al. (2014) who demonstrated that dietary ALA was able to increase final BMD in growing broilers, although LPS was not used. Under stressful

conditions, dietary flaxseed oil and fish oil supplementation provided protective effects on broiler bone metabolism allowing for improved mineral deposition.

The results of this study demonstrated that fish oil supplementation in broiler diets provided anti-inflammatory protection and both flaxseed and fish oils improved bone characteristics, demonstrating a secondary benefit for producers that supplement their broiler rations. However, the experimentally high oil level of 14% used in the current experiment to attain the therapeutic omega-3 fatty acid concentrations in the broiler diets may not be easily applied in commercial feed mills due to infrastructural limitations. Further investigation is recommended to determine the minimum level of oil needed to produce beneficial health effects during inflammation.

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## TABLES

**Table 5.1.** Composition of experimental starter diets[1] fed to Ross 308 broilers for a 28 consecutive d dietary supplementation period followed by a repeated inflammatory challenge.[2]

| | Experimental diet (%) | | |
|---|---|---|---|
| **Ingredients** | **Palm oil control** | **Flaxseed oil** | **Fish oil** |
| Corn | 26.60 | 26.61 | 26.61 |
| Soybean meal 48% crude protein | 50.00 | 50.00 | 50.00 |
| Wheat middlings | 5.00 | 5.00 | 5.00 |
| Palm oil | 14.00 | 6.53 | 0.33 |
| Flaxseed oil | 0.00 | 7.48 | 0.00 |
| Fish oil | 0.00 | 0.00 | 13.68 |
| Dicalcium phosphate | 1.76 | 1.76 | 1.76 |
| Calcium carbonate[3] | 1.06 | 1.07 | 1.07 |
| Sodium chloride | 0.50 | 0.50 | 0.50 |
| Methionine hydroxy analogue | 0.32 | 0.30 | 0.30 |
| Choline chloride | 0.10 | 0.10 | 0.10 |
| L-threonine | 0.03 | 0.03 | 0.03 |
| V and M premix[4] | 0.63 | 0.63 | 0.63 |
| **Calculated values** | | | |
| Calcium | 1.00 | 1.00 | 1.00 |
| Available phosphorus | 0.45 | 0.45 | 0.45 |
| **Analyzed values** | | | |
| Crude fat | 13.61 | 15.15 | 13.21 |

[1]Palm oil control, flaxseed oil, and fish oil experimental diets used a common basal diet combined with 14% palm oil, 7.48% flaxseed oil, or 13.68% fish oil. Calculated ME for each diet was 3,400 kcal/kg.

[2]Broilers were injected intraperitoneally 4 times with LPS solution (1 mg/kg) or equal volume sterile saline every 48 h (35, 37, 39, 41 d of age) administering 20% increased dosage with each successive injection (1.0 to 1.7 mg/kg). An experimental unit (EU) consisted of 2 broilers sharing a pen with 20 EU for each experimental diet.

[3]Calcium carbonate added was a 50/50 mixture of small (< 2 mm) and large (≥ 2 mm) particle.

[4]Vitamin and mineral premix provided per kg of diet: selenium 250 μg; vitamin A 8,250 IU; vitamin $D_3$ 2,750 IU; vitamin E 17.9 IU; menadione 1.1 mg; vitamin $B_{12}$ 12 μg; biotin 41 μg; choline 447 mg; folic acid 1.4 mg; niacin 41.3 mg; pantothenic acid 11 mg; pyridoxine 1.1 mg; riboflavin 5.5 mg; thiamine 1.4 mg; iron 282 mg; magnesium 125 mg; manganese 275 mg; zinc 275 mg; copper 27.5 mg; iodine 844 μg.

**Table 5.2.** Forward (F) and reverse (R) primer sequences[1] for quantitative real-time PCR mRNA gene expression.

| Gene[2] | Primer Sequence | Accession number or referenced source |
|---|---|---|
| **IL-1β** | F 5'-GCTCTACATGTCGTGTGTGATGAG-3'<br>R 5'-TGTCGATGTCCCGCATGA-3' | AJ245728 |
| **IL-6** | F 5'-GCTCGCCGGCTTCGA-3'<br>R 5'-GGTAGGTCTGAAAGGCGAACAG-3' | AJ250838 |
| **IL-18** | F 5'-AGGTGAAATCTGGCAGTGGAAT-3'<br>R 5'-ACCTGGACGCTGAATGCAA-3' | AJ276026 |
| **IFNγ** | F 5'-GTGAAGAAGGTGAAAGATATCATGGA-3'<br>R 5'-GCTTTGCGCTGGATTCTCA-3' | Y07922 |
| **SAA** | F 5'-TTCTGTGGGCTAGGTTCCTG-3'<br>R 5'-GCAAGTCAGCAACAACCAGA-3' | Q9PSM7 |
| **IL-10** | F 5'-CATGCTGCTGGGCCTGAA-3'<br>R 5'-CGTCTCCTTGATCTGCTTGATG-3' | AJ621614 |
| **PPARγ** | F 5'-GGGCGATCTTGACAGGAA-3'<br>R 5'-GCCTCCACAGAGCGAAAC-3' | Sun et al., 2014 |
| **4EBP1** | F 5' -GCGAATGTAGGTGAAGAAGAG-3'<br>R 5' -AACAGGAAGGCACTCAAGG-3' | Deng et al., 2014 |
| **RPS6K1** | F 5'-CAATTTGCCTCCCTACCTCA-3'<br>R 5'-AAGGAGGTTCCACCTTTCGT-3' | Deng et al., 2014 |
| **Ubiquitin** | F 5'-CGCACCCTGTCTGACTACAA-3'<br>R 5'-GCCTTCACGTTCTCAATGGT-3' | Nakashima et al., 2009 |
| **CAPN2** | F 5'-ACATCATCGTGCCCTCTACC-3'<br>R 5'-GAGATCTCTGCATCGCTTCC-3' | Nakashima et al., 2009 |
| **PSMC2** | F 5'-AACACACGCTGTTCTGGTTG-3'<br>R 5'-CTGCGTTGGTATCTGGGTTT-3' | Nakashima et al., 2009 |
| **GAPDH** | F 5'-GTGCTGGCATTGCACTGAA-3'<br>R 5'CACAACACGGTTGCTGTATCC-3' | NM_204305.1 |
| **28S** | F 5'-GGCGAAGCCAGAGGAAACT-3'<br>R 5'-GACGACCGATTTGCACGTC-3' | X59733 |

[1]Primers were designed using the corresponding accession number or listed reference.
[2]Gene expression analyzed for interleukin-1 β (IL-1β), interleukin-6 (IL-6), interleukin-18 (IL-18), interferon γ (IFNγ), serum amyloid A (SAA), interleukin-10 (IL-10), peroxisome proliferator-activated receptor (PPARγ), eukaryotic translation initiation factor 4E binding protein 1 (4EBP1), ribosomal protein S6 kinase 1 (RPS6K1), ubiquitin, calpain-2 (CAPN2), proteasome 26S subunit ATPase 2 (PSMC2), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) reference gene, and ribosomal 28S subunit (28S) reference gene.

**Table 5.3.** Ross 308 broiler performance[1] when fed palm oil control, flaxseed oil, or fish oil experimental diets[2] followed by a repeated inflammatory challenge.[3]

| | Inflammatory challenge | | | *P*-value[4] | | |
|---|---|---|---|---|---|---|
| **Measure** | **Saline** | **LPS** | **SEM** | **Diet** | **Challenge** | **Diet*Challenge** |
| SBW (kg) | 2.420 | 2.426 | 0.0289 | ≤ 0.01 | 0.89 | 0.27 |
| EBW (kg) | 3.298 | 3.165 | 0.0183 | 0.95 | ≤ 0.01 | 0.26 |
| FI (kg) | 1.631 | 1.529 | 0.0198 | 0.46 | ≤ 0.01 | 0.62 |
| FCR | 1.909 | 2.098 | 0.0404 | 0.78 | 0.03 | 0.50 |
| ADG (kg/d) | 0.125 | 0.106 | 0.0024 | 0.89 | ≤ 0.01 | 0.37 |

[1]Performance measurements included starting body weight at 35 d of age (SBW), ending body weight at 42 d of age (EBW), feed intake (FI), feed conversion ratio (FCR), and average daily gain (ADG).

[2]Experimental diets were applied for a 28 consecutive d dietary supplementation period (6 to 34 d of age). Broiler chicks were fed a palm oil control diet (basal diet + no supplemental omega-3 fatty acids), flaxseed oil (basal + 4.1% alpha-linolenic acid), or fish oil (basal + 4.1% eicosapentaenoic acid and docosahexaenoic acid).

[3]Broilers were injected intraperitoneally 4 times with LPS solution (1 mg/kg) or equal volume sterile saline every 48 h (35, 37, 39, 41 d of age) administering 20% increased dosage with each successive injection (1.0 to 1.7 mg/kg). An experimental unit (EU) consisted of 2 broilers sharing a pen with 30 EU injected with LPS and the remaining 30 EU injected with sterile saline.

[4]Challenge = inflammatory challenge with 10 EU per experimental diet injected with LPS and the remaining 10 EU injected with sterile saline.

**Table 5.4.** Tissue absorptiometry[1] for Ross 308 broilers fed palm oil control, flaxseed oil, or fish oil experimental diets[2] followed by a repeated inflammatory challenge.[3]

| | Experimental diet | | | | *P*-value[4] | | |
|---|---|---|---|---|---|---|---|
| **Measure** | **Palm oil control** | **Flaxseed oil** | **Fish oil** | **SEM** | **Diet** | **Challenge** | **Diet*Challenge** |
| Fat accretion (kg) | 0.033 | 0.047 | 0.022 | 0.0156 | 0.63 | 0.75 | 0.71 |
| Total fat (%) | 5.5 | 5.9 | 5.0 | 0.49 | 0.61 | 0.96 | 0.60 |
| Lean accretion (kg) | $0.710^{ab}$ | $0.603^{b}$ | $0.832^{a}$ | 0.0498 | 0.04 | 0.18 | 0.88 |
| Total lean (%) | $92.1^{b}$ | $89.2^{b}$ | $96.4^{a}$ | 0.97 | $\leq 0.01$ | 0.37 | 0.87 |
| Mass accretion (kg) | 0.789 | 0.772 | 0.783 | 0.0507 | 0.97 | 0.05 | 0.77 |
| Total mass (kg) | 3.200 | 3.255 | 3.070 | 0.0507 | 0.11 | 0.05 | 0.77 |

[1]Absorptiometry measurements included fat accretion (kg), lean tissue accretion (kg), and total mass accretion (kg).

[2]Experimental diets were fed for a 28 consecutive d dietary supplementation period (6 to 34 d of age). Broilers were fed a palm oil control (basal + no supplemental omega-3fatty acids), flaxseed oil (basal + 4.1% alpha-linolenic acid), or fish oil (basal + 4.1% eicosapentaenoic acid and docosahexaenoic acid) diet. An experimental unit (EU) consisted of 2 broilers sharing a pen with 20 EU per experimental diet.

[3]Broilers were injected intraperitoneally 4 times with LPS solution (1 mg/kg) or equal volume sterile saline every 48 h (35, 37, 39, 41 d of age) administering 20% increased dosage with each successive injection (1.0 to 1.7 mg/kg).

[4]Challenge = inflammatory challenge with 10 EU per experimental diet injected with LPS and the remaining 10 EU injected with sterile saline.

[a-b]Least square means without a common superscript differ significantly within a row ($P \leq 0.05$).

**Table 5.5.** Dietary fatty acid profile for palm oil control, flaxseed oil, and fish oil experimental starter diets[1] fed to Ross 308 broiler chickens for a 28 consecutive d dietary supplementation period followed by a repeated inflammatory challenge.[2]

| | Experimental diet (%) | | |
|---|---|---|---|
| **Fatty acid (C:double bond)** | **Palm oil control** | **Flaxseed oil** | **Fish oil** |
| Myristic acid (14:0) | 1.04 | 0.67 | 7.99 |
| Palmitic acid (16:0) | 39.34 | 21.46 | 19.96 |
| Palmitoleic acid (16:1) | 0.00 | 0.33 | 8.63 |
| Margaric acid (17:0) | 0.00 | 0.00 | 0.42 |
| Stearic acid (18:0) | 3.82 | 3.48 | 3.56 |
| Oleic acid (18:1) | 35.49 | 25.49 | 11.61 |
| Linoleic acid (18:2) | 18.06 | 18.81 | 11.74 |
| Alpha-linolenic acid (18:3) | 1.91 | 28.39 | 2.32 |
| Arachidic acid (20:0) | 0.33 | 0.22 | 0.24 |
| Arachidonic acid (20:4) | 0.00 | 0.00 | 1.07 |
| Eicosapentaenoic acid (20:5) | 0.00 | 1.16 | 19.55 |
| Docosapentaenoic acid (22:5) | 0.00 | 0.00 | 2.04 |
| Docosahexaenoic acid (22:6) | 0.00 | 0.00 | 10.87 |
| Crude fat[3] | 13.61 | 15.15 | 13.21 |
| Saturated fat | 44.53 | 25.83 | 32.17 |
| Unsaturated fat | 55.47 | 74.17 | 67.83 |
| Total omega-3 fatty acid[4] | 1.91 | 29.55 | 32.73 |
| Total omega-6 fatty acid[5] | 18.06 | 18.81 | 12.82 |
| Omega-6:omega-3 ratio | 9.46 | 0.64 | 0.39 |

[1]Palm oil control, flaxseed oil, and fish oil experimental diets used a basal diet containing 14% palm oil, 7.48% flaxseed oil, or 13.68% fish oil.

[2]Broilers were injected intraperitoneally 4 times with LPS solution (1 mg/kg) or equal volume sterile saline every 48 h (35, 37, 39, 41 d of age) administering 20% increased dosage with each successive injection (1.0 to 1.7 mg/kg). An experimental unit (EU) consisted of 2 broilers sharing a pen with 20 EU per experimental diet.

[3]Crude fat was the analyzed fat content of pooled feed samples. The fatty acid values represent the percent composition of the crude fat for each diet.

[4]Total omega-3 fatty acid was the sum of alpha-linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid.

[5]Total omega-6 fatty acid was the sum of linoleic acid and arachidonic acid.

**Table 5.6.** Pectoralis major fatty acid analysis for Ross 308 broilers fed palm oil control, flaxseed oil, or fish oil experimental diets[1] for a 28 consecutive d dietary supplementation period followed by a repeated inflammatory challenge.[2]

| | Experimental diet (%) | | | | *P*-value[3] | | |
|---|---|---|---|---|---|---|---|
| **Fatty acid (C:double bond)** | **Palm oil control** | **Flaxseed oil** | **Fish oil** | **SEM** | **Diet** | **Challenge** | **Diet*Challenge** |
| Myristic acid (14:0) | 0.93[b] | 0.62[b] | 4.63[a] | 0.250 | ≤ 0.01 | 0.50 | 0.26 |
| Palmitic acid (16:0) | 29.03[a] | 20.57[b] | 22.81[b] | 0.590 | ≤ 0.01 | 0.20 | 0.26 |
| Palmitoleic acid (16:1) | 2.57[b] | 1.63[b] | 6.88[a] | 0.277 | ≤ 0.01 | 0.43 | 0.23 |
| Margaric acid (17:0) | 0.11[b] | 0.10[b] | 0.56[a] | 0.033 | ≤ 0.01 | 0.96 | 0.60 |
| Stearic acid (18:0) | 7.06 | 7.00 | 8.75 | 0.447 | 0.31 | 0.77 | 0.56 |
| Oleic acid (18:1) | 38.93[a] | 27.54[b] | 16.18[b] | 1.576 | ≤ 0.01 | 0.76 | 0.36 |
| Linoleic acid (18:2) | 15.32[a] | 16.87[a] | 8.88[b] | 0.459 | ≤ 0.01 | 0.61 | 0.15 |
| Alpha-linolenic acid (18:3) | 0.23[b] | 19.07[a] | 1.89[b] | 0.534 | ≤ 0.01 | 0.65 | 0.89 |
| Arachidic acid (20:0) | 0.12 | 0.10 | 0.07 | 0.030 | 0.50 | 0.54 | 0.43 |
| Arachidonic acid (20:4) | 4.30[a] | 1.59[b] | 2.17[b] | 0.240 | ≤ 0.01 | 0.60 | 0.11 |
| Eicosapentaenoic acid (20:5) | 0.29[b] | 1.49[b] | 11.55[a] | 0.589 | ≤ 0.01 | 0.49 | 0.52 |
| Docosapentaenoic acid (22:5) | 0.55[b] | 1.99[a] | 2.86[a] | 0.247 | ≤ 0.01 | 0.63 | 0.60 |
| Docosahexaenoic acid (22:6) | 0.56[b] | 1.43[b] | 12.77[a] | 0.876 | ≤ 0.01 | 0.85 | 0.18 |
| Muscular crude fat[4] | 2.80 | 2.62 | 2.11 | 0.392 | 0.66 | 0.70 | 0.86 |
| **Calculated values** | | | | | | | |
| Saturated fat | 37.25 | 28.39 | 36.81 | - | - | - | - |
| Unsaturated fat | 62.75 | 71.61 | 63.19 | - | - | - | - |
| Total omega-3 fatty acid[5] | 1.08 | 21.99 | 26.22 | - | - | - | - |
| Total omega-6 fatty acid[6] | 19.62 | 18.46 | 11.05 | - | - | - | - |
| Omega-6:omega-3 ratio | 18.10 | 0.84 | 0.42 | - | - | - | - |

[1]Muscle tissue was sampled from 3 experimental units per factorial arrangement (Diet*Challenge). An experimental unit (EU) consisted of 2 broilers sharing a pen with 20 EU per experimental diet. Experimental diets were applied for a 28 consecutive d dietary supplementation period (6 to 34 d of age). Broiler birds were fed a palm oil control (basal + no supplemental omega-3 fatty acids), flaxseed oil (basal + 4.1% alpha-linolenic acid), or fish oil (basal + 4.1% eicosapentaenoic acid and docosahexaenoic acid) diet.

**Table 5.6 continued.**

[2]Broilers were injected intraperitoneally 4 times with LPS solution (1 mg/kg) or equal volume sterile saline every 48 h (35, 37, 39, 41 d of age) administering 20% increased dosage with each successive injection (1.0 to 1.7 mg/kg).

[3]Challenge = inflammatory challenge with 10 EU per experimental diet injected with LPS and the remaining 10 EU injected with sterile saline.

[4]Crude fat was the analyzed fat content of muscle samples. The fatty acid values represent the percent composition of the crude fat for each treatment.

[5]Total omega-3 fatty acid was the sum of alpha-linolenic acid, eicosapentaenoic acid, and docosahexaenoic acid.

[6]Total omega-6 fatty acid was the sum of linoleic acid and arachidonic acid.

[a-b]Least square means without a common superscript differ significantly within a row ($P \leq 0.05$).

**Table 5.7.** Changes in bone absorptiometry[1] for Ross 308 broilers fed palm oil control, flaxseed oil, or fish oil experimental diets[2] followed by a repeated inflammatory challenge.[3]

| | Experimental diet | | | | *P*-value[4] | | |
|---|---|---|---|---|---|---|---|
| **Measure** | **Palm oil control** | **Flaxseed oil** | **Fish oil** | **SEM** | **Diet** | **Challenge** | **Diet*Challenge** |
| BMC accretion (g) | 6.127[b] | 9.752[a] | 10.682[a] | 0.9263 | ≤ 0.01 | 0.24 | 0.63 |
| Total BMC (%) | 1.0[b] | 1.2[a] | 1.3[a] | 0.03 | ≤ 0.01 | 0.96 | 0.28 |
| BMD change (g/cm$^2$) | -0.010[b] | 0.000[b] | 0.023[a] | 0.0041 | ≤ 0.01 | 0.69 | 0.74 |
| BMD (g/cm$^2$) | 0.145[b] | 0.165[a] | 0.174[a] | 0.0040 | ≤ 0.01 | 0.83 | 0.58 |

[1]Absorptiometry measurements included bone mineral content (BMC) accretion and changes in bone mineral density (BMD).

[2]Experimental diets were applied for a 28 consecutive d dietary supplementation period (6 to 34 d of age). Broilers were fed a palm oil control (basal + no supplemental omega-3 fatty acids), flaxseed oil (basal + 4.1% alpha-linolenic acid), or fish oil (basal + 4.1% eicosapentaenoic acid and docosahexaenoic acid) diet. An experimental unit (EU) consisted of 2 broilers sharing a pen with 20 EU per experimental diet.

[3]Broilers were injected intraperitoneally 4 times with LPS solution (1 mg/kg) or equal volume sterile saline every 48 h (35, 37, 39, 41 d of age) administering 20% increased dosage with each successive injection (1.0 to 1.7 mg/kg).

[4]Challenge = inflammatory challenge with 10 EU per experimental diet injected with LPS and the remaining 10 EU injected with sterile saline.

[a-b]Least square means without a common superscript differ significantly within a row ($P \leq 0.05$).

## FIGURES

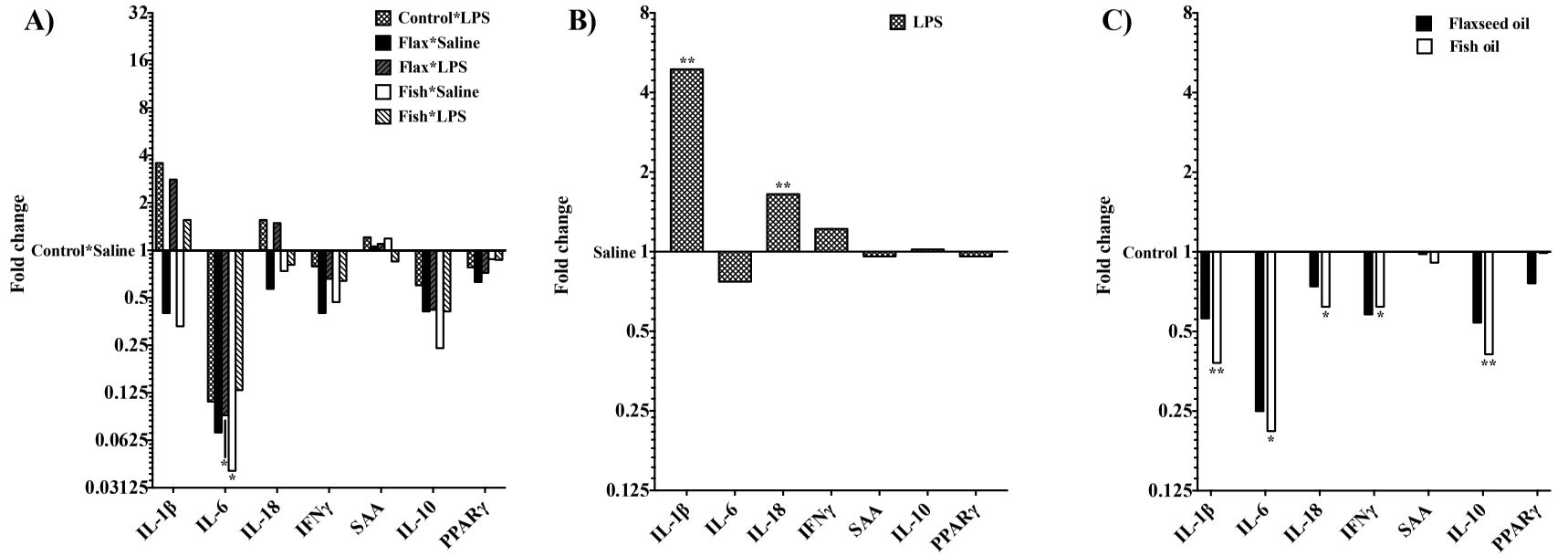


**Figure 5.1.** Liver relative mRNA gene expression in Log2 transformed fold change for broilers fed a palm oil control (basal + no supplemental omega-3 fatty acids), flaxseed oil (basal + 4.1% alpha-linolenic acid), or fish oil (basal + 4.1% eicosapentaenoic acid and docosahexaenoic acid) diet from 6 to 34 d of age followed by a repeated inflammatory challenge from 35 to 42 d of age. Fold change displayed as A) diet*challenge interaction for cytokines interleukin-1 β (IL-1β), IL-6, IL-18, interferon γ (IFNγ), serum amyloid A (SAA), IL-10, and peroxisome proliferator-activated receptor γ (PPARγ) using glyceraldehyde 3-phosphate dehydrogenase reference gene, B) inflammatory challenge injection (LPS vs. Saline), and C) diet (Palm oil control vs. Flaxseed oil vs. Fish oil). $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$ in reference to baseline 1-fold change, unless otherwise noted. A) Baseline 1-fold change = Control*Saline, B) baseline 1-fold change = Saline, and C) baseline 1-fold change = Control.

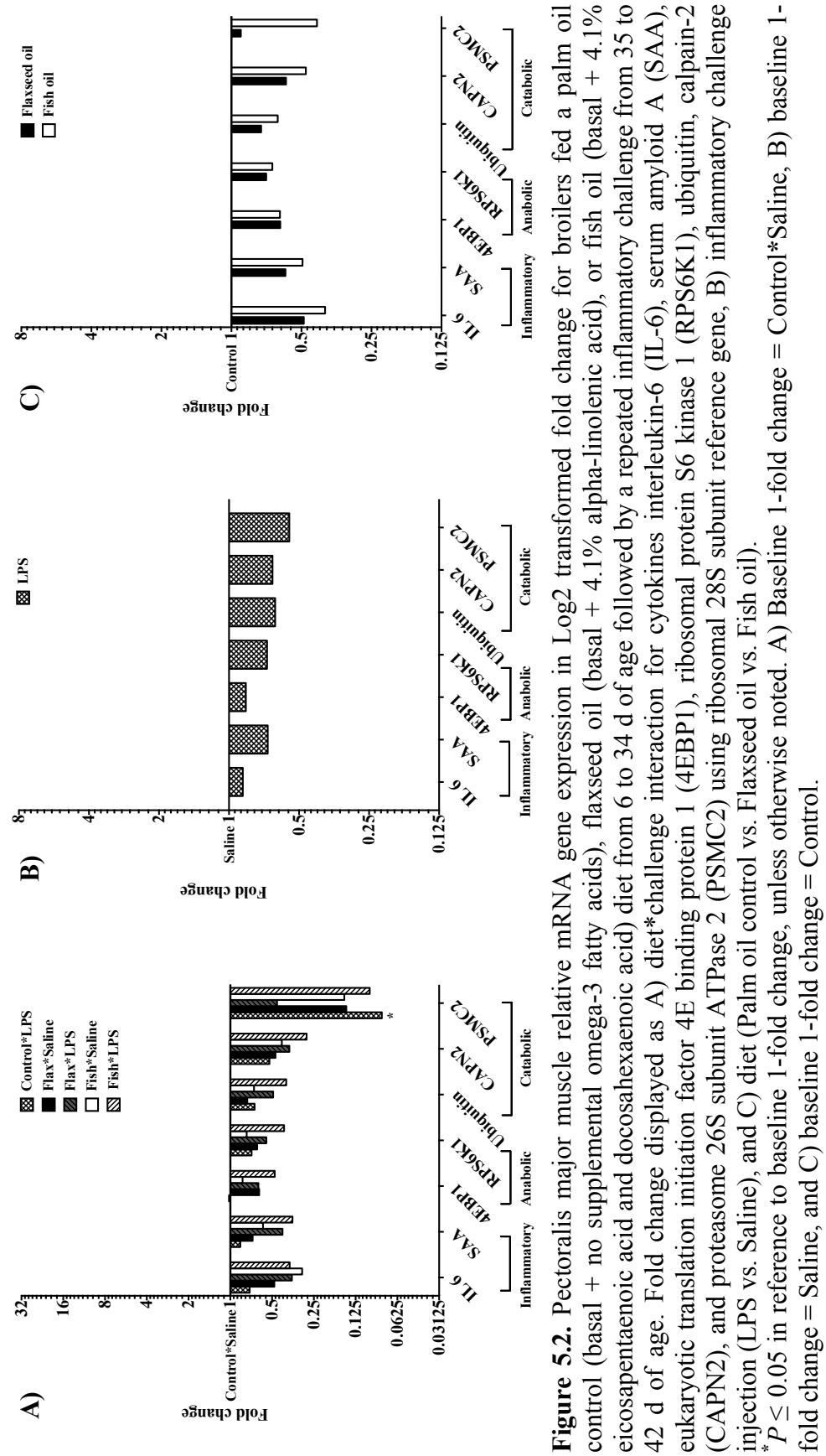


**Figure 5.2.** Pectoralis major muscle relative mRNA gene expression in Log2 transformed fold change for broilers fed a palm oil control (basal + no supplemental omega-3 fatty acids), flaxseed oil (basal + 4.1% alpha-linolenic acid), or fish oil (basal + 4.1% eicosapentaenoic acid and docosahexaenoic acid) diet from 6 to 34 d of age followed by a repeated inflammatory challenge from 35 to 42 d of age. Fold change displayed as A) diet*challenge interaction for cytokines interleukin-6 (IL-6), serum amyloid A (SAA), eukaryotic translation initiation factor 4E binding protein 1 (4EBP1), ribosomal protein S6 kinase 1 (RPS6K1), ubiquitin, calpain-2 (CAPN2), and proteasome 26S subunit ATPase 2 (PSMC2) using ribosomal 28S subunit reference gene, B) inflammatory challenge injection (LPS vs. Saline), and C) diet (Palm oil control vs. Flaxseed oil vs. Fish oil).
* $P \leq 0.05$ in reference to baseline 1-fold change, unless otherwise noted. A) Baseline 1-fold change = Control*Saline, B) baseline 1-fold change = Saline, and C) baseline 1-fold change = Control.

# CHAPTER 6. GENERAL CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

Poultry diets are supplemented with omega-3 fatty acids to generate value-added eggs and meats. There is a gap in research regarding how ALA or EPA and DHA supplementation in poultry diets affect bird health and performance. The overarching goal of this dissertation was to determine the effects of omega-3 fatty acid supplementation on poultry health and performance with the hypothesis that dietary omega-3 fatty acid supplementation would provide a health benefit to the bird leading to a secondary benefit for the producer. In order to investigate the potential health benefits provided to poultry consuming omega-3 fatty acid supplemented diets, four experiments were performed addressing specific objectives.

The objective of Experiment 1 was to evaluate how dietary ALA sources affect the fatty acid transfer rate from laying hen diet to the egg yolk near peak production. The data gathered from the experiment allowed for the creation of omega-3 fatty acid transfer equations, which can be used by producers to accurately formulate flaxseed oil supplemented diets for generating value-added omega-3 fatty acid enriched eggs with specific label claims. The results demonstrated that flaxseed oil was a more efficient and cost effective functional ingredient for producing value-added ALA enriched eggs compared to ground flaxseed, therefore it was applied to the future experiments. It took the hens up to 6 wk to acclimate to the supplemented diets and reach a transfer plateau, therefore a minimum of 6 wk was used in the following laying hen experiment.

The objective of Experiment 2 was to determine if dietary supplementation of ALA or EPA and DHA improves performance and bone health of aged laying hens with mature

immune systems, at risk for osteoporosis, and whether protective anti-inflammatory effects would be exerted during acute inflammation. Contrary to the hypothesis, dietary ALA or EPA and DHA did not improve aged laying hen performance or bone strength prophylactically. The lack of bone health response may have been due to the older age of the laying hens. Supplementation at this stage may have been too late to generate a bone health response whereas supplementation at an earlier age may have been beneficial. Under stress, dietary ALA or EPA and DHA fed to the laying hens provided anti-inflammatory protection, which is advantageous with a healthy flock of birds in a clean environment. However, immune suppression may not be advantageous in disease challenged commercial settings, such as with *Mycoplasma* spp. infected populations. This experiment demonstrated that omega-3 fatty acid supplementation in laying hen diets yielded value-added eggs and provided anti-inflammatory protection, but the protection may not be a practical benefit for producers faced with endemic disease challenges. Due to the age of the laying hens and lack of bone health response, a broiler model with growing bones was used in the following experiment and administered a therapeutic oil inclusion level (14% oil; 4% total omega-3 fatty acid).

The objective of Experiment 3 was to investigate if dietary supplementation of ALA or EPA and DHA impact bone health and performance in growing broilers, at risk for lameness and leg bone pathology. Dietary ALA or EPA and DHA at therapeutic levels did not improve broiler bone characteristics when provided prophylactically. It is possible that broilers require a full 6 wk to respond to the effects of ALA or EPA and DHA from their growing bones, similar to the time needed for dietary acclimation and transfer rate plateau of laying hens from Experiment 1. Fish oil supplementation negatively affected broiler

performance while the flaxseed oil diet containing 6.53% palm oil did not. Fatty acid utilization may have been affected by oxidative stability, where the fish oil may have been less stable than flaxseed oil. These results suggested that palm oil, providing saturated fatty acids, might be required in combination with ALA or EPA and DHA to maintain broiler performance. No secondary benefit of improving bone health or performance was demonstrated in this experiment when broilers were supplemented with flaxseed oil or fish oil; producers should consider the stability of the oil ingredient when selecting an omega-3 fatty acid supplement for their poultry diet to prevent potential performance losses. In a continuation of this experiment, these broilers were used for Experiment 4 in a repeated inflammatory challenge.

The objective of Experiment 4 was to examine the protective anti-inflammatory and bone modulating effects of ALA or EPA and DHA on performance and health of growing broilers with naïve immune systems at risk for lameness during a period of repeated inflammation, in contrast to Experiment 2 where aged laying hens with a developed immune system were challenged to a single acute inflammatory challenge. As hypothesized, broilers subjected to repeated inflammatory stress and fed therapeutic levels of dietary ALA or EPA and DHA improved bone health, providing a secondary benefit. Dietary fish oil supplementation provided anti-inflammatory protection, as discussed previously immune suppression may not be a benefit in certain disease challenge scenarios. Dietary ALA or EPA and DHA did not result in performance differences due to repeated inflammation. Producers should be aware that the beneficial effects in this experiment were attained using therapeutic levels of dietary oil (14%) in the feed, which may not be possible to manufacture in a commercial feed mill.

Overall, dietary ALA or EPA and DHA supplementation in poultry diets provided secondary benefits related to animal health, but these experiments did not demonstrate as many as hypothesized. Further investigation is needed in order to understand how immune suppression via ALA or EPA and DHA affects pathogen clearance in a living biological system (*in vivo* infectious model using laying hens and broilers) compared to published cell culture and *in vitro* research to determine the level of secondary benefit that the supplementation is providing.

# APPENDIX. EFFECTS OF PEROXIDIZED CORN OIL ON PERFORMANCE, $AME_N$, AND ABDOMINAL FAT PAD WEIGHT IN BROILER CHICKS[1]

A research note published in *Poultry Science*

I. J. Ehr[2,3], B. J. Kerr[4,5], and M. E. Persia[3,6]

## Abstract

There is a trend to use more alternative lipids in poultry diets, either through animal-vegetable blends, distillers corn oil, or yellow grease. This has resulted in the use of lipids in poultry diets with a higher concentration of unsaturated fatty acids, which have a greater potential for peroxidation. The objective of this experiment was to determine the effects of peroxidized corn oil on broiler performance, dietary $AME_n$, and abdominal fat pad weight. The same refined corn oil sample was divided into 3 subsamples, 2 of which were exposed to different peroxidative processes. The 3 diets contained the unperoxidized corn oil (UO), a slowly peroxidized corn oil (SO; heated for 72 h at 95°C with compressed air flow rate of 12

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L/min), or a rapidly peroxidized corn oil (RO; heated for 12 h at 185°C with compressed air flow rate of 12 L/min). Diets were fed from 0 to 14 d of age with each lipid fed at a 5% inclusion rate, continuing on from 15 to 27 d of age with each lipid fed at a 10% inclusion rate. There were 6 Ross 708 broiler chicks per cage with 10 replicates for each of the 3 dietary treatments. Abdominal fat pad and excreta collection was performed on d 27. Body weight gain, feed intake and feed efficiency were measured for the 0 to 14 and 0 to 27 d periods. The increased level of peroxidation reduced $AME_n$ in broiler diets (UO = 3,490 kcal/kg; SO = 3,402 kcal/kg; RO = 3,344 kcal/kg on an as-is basis; SEM = 12.9, $P \leq 0.01$). No significant treatment differences were observed among oil supplemented birds for BW gain, feed intake, feed efficiency, or abdominal fat pad weight. In conclusion, corn oil peroxidation status resulted in a decrease in dietary $AME_n$, but had minimal effects on broiler performance or fat pad weights.

Key words: broiler, energy, lipid peroxidation, performance

## Introduction

Dietary energy is a major cost component in feed formulation with lipids added to diets due to of their energy density. Sources of lipids include refined oils, rendered animal fats, acidulated soap stocks, and yellow grease to name a few. In addition, improved extraction technologies in the ethanol industry has allowed for the increased removal of corn oil from dried distiller's grains with solubles resulting in increased availability of distillers corn oil (Singh and Cheryan, 1998; Winkler-Moser and Breyer, 2011; Kim et al., 2013). However, lipids high in polyunsaturated fatty acids such as corn oil are sensitive to peroxidation (Holman, 1954; Liu et al., 2014) where it has been shown that consumption of

peroxidized lipids have the potential to adversely affect broiler performance (Cabel et al., 1988; Engberg et al., 1996), physiological oxidative status (Dibner et al., 1996; Zhang et al., 2011), and lipid deposition (Lin et al., 1989; Sheehe et al., 1994; Pesti et al., 2002). In a review of the literature Hanson (2014) reported that overall responses for pigs and broilers fed diets with peroxidized lipids had an 11% reduction in daily gain, 7% reduction in daily feed intake, and a 4% reduction in feed efficiency relative to animals fed diets with unperoxidized lipids. Dietary peroxide value (PV) was negatively correlated with gain in broilers, but other measures of lipid peroxidation were not measured making correlations to other measures of lipid peroxidation impossible.

The objective of this experiment was to determine the effects of slowly (SO) and rapidly peroxidized (RO) corn oil versus unperoxidized (UO) corn oil in diets fed to broilers on bird performance, dietary $AME_n$, and abdominal fat pad deposition.

## Materials and Methods

### Animals and Housing

The Institutional Animal Care and Use Committee at Iowa State University (Ames, IA) approved the experimental protocol. Commercial Ross × Ross 708 hatching-day male broiler chicks (Aviagen Group, Huntsville, AL) were procured from a local commercial hatchery. The experimental unit consisted of a cage of 6 broiler chicks with 10 replicates for each of the 3 treatments. On arrival, chicks were individually wing banded, weighed and sorted for placement into battery cages (Petersime Incubator Co., Gettysburg, OH) with an equal BW (treatment initial body weight was between 41.8 and 42.8 g/chick: $P = 0.31$) at a density of 567 cm/bird. Birds were supplied with continuous light and supplemental heat was

provided from brooders in the battery cages. The initial house temperature was set at 29°C, reduced by 2.5°C weekly until 21°C was attained. Birds had ad libitum access to experimental diets and water for the duration of the 27 d experimental period. Treatments were applied to cages in completely randomized design.

**Diets**

Formulation of the basal diet met or exceeded breeder recommendations (Ross Nutritional Supplement, 2012; Aviagen Group, Huntsville, AL) of the broiler chicks with the caloric energy formulation based on UO refined corn oil values. Titanium dioxide marker was included at 0.25% for $AME_n$ calculation (Leone, 1973). Diets were formulated to contain an UO, SO, or RO corn oil treatment (Table 1). The SO treatment was generated by heating refined corn oil at 95°C for 72 h while the RO treatment was generated by heating refined corn oil at 185°C for 12 hr, with both heating process accompanied by bubbling air into the heating vessel at a flow rate of 12 L/min (Table 2). To acclimate the birds to the higher than normal level of corn oil in the experimental diets, birds were fed at a 5% inclusion rate from 0 to 14 d of age and 10% from 15 to 27 d of age (Table 1). The increased oil inclusion rates used were higher than typical commercial oil additions for broiler chickens, but were used to maximize the opportunity to detect differences in dietary $AME_n$ among the oil treatments. The heat treatment of the corn oil (UO, SO, and RO) was the only difference between the treatment diets.

**Data Collection**

Broiler chicks were monitored twice daily over the duration of the experiment. The birds were individually weighed on d 0, 14, and 27 and BW gain was calculated over the 0 to 14 and 0 to 27 d periods. Feed disappearance was measured on d 14 and 27 to calculate d 0 to 14 and d 0 to 27 period feed intakes. Feed efficiency was corrected for mortality and reported as g BW gain per kg feed intake. Excreta collection pans located under the raised-wire cages were cleaned of all excreta, feed, and feathers on d 26 and fresh excreta were collected on d 26 and 27 for $AME_n$ determination. At the end of the experiment, d 27, all remaining broilers were euthanized via carbon dioxide asphyxiation. An incision was made into the abdominal cavity of each bird to allow access to the abdominal fat pad for removal and immediate wet weight determination. The removal of the abdominal fat pad was completed by a single individual to reduce variation in the collection process.

**Chemical Analysis**

Corn oil samples were analyzed for fatty acid profile (methods Ce 2–66; AOCS, 2009 and 996.06; AOAC, 2010), free fatty acids (method Ca 5a–40; AOCS, 2009), moisture (method Ca 2c–25; AOCS, 2009), impurities (method Ca 3a–46; AOCS, 2009), unsaponifiables (method Ca 6a–40; AOCS, 2009), peroxide value (method Cd 8–53; AOCS, 2009), thiobarbituric acid reactive substances, (TBARS, modified method Cd 19– 90; AOCS, 2009; using malonaldehyde as a standard as described by Pegg, 2001), and hexanal (Elisia and Kitts, 2011) at the University of Missouri Agricultural Experiment Station Chemistry Laboratory (AESCL, Columbia, MO). The p-ansidine value (method Cd 18– 90; AOCS,

2009) and oxidative stability index at 110°C (method Cd 12b–92; AOCS, 2009) were determined at Barrow-Agee Laboratories (Memphis, TN).

Feed samples were ground through a 0.5-mm screen and subsequently dried for 24 h at 100°C for DM determination. Pooled excreta samples were dried at 75°C in a convection oven for 3 d and subsequently ground through a 1.0-mm screen. Ether extract content of the feed samples was determined by Soxhlet extraction using diethyl ether at the University of Missouri Agricultural Experiment Station Chemistry Laboratory (method 920.39; AOAC, 2010; AESCL, Columbia, MO). For the feed and excreta samples, N concentration was determined by thermocombustion (TruMac N Analyzer, LECO Corp., St. Joseph, MI), GE determined using an adiabatic oxygen bomb calorimeter (Parr Instrument Co., Moline, IL), and Ti concentration were determined using the method outlined by Leone (1973). All samples of excreta and feed were analyzed in duplicate. The diet $AME_n$ determination was calculated as: $AME_n = GE_{Diet} - (GE_{Excreta} \times Ti_{Diet} \div Ti_{Excreta}) - 8.22 \times (N_{Diet} - N_{Excreta} \times Ti_{Diet} \div Ti_{Excreta})$.

**Statistics**

The experimental unit was defined as a cage of 6 birds and data were analyzed using the GLM procedure of SAS (SAS Institute Inc., Cary, NC) with means reported as LSMeans. Fisher's LSD test was used to separate treatment means if significance was detected ($P \leq 0.05$). In addition, the overall effect heating (SO and RO) was compared to the unheated lipid, UO, using a contrast statement.

## Results and Discussion

Lipid peroxidation is a complex process that is affected by several factors including the degree of lipid saturation, temperature, presence of oxygen, and contamination with transition metals, undissociated salts, or water (Frankel, 2005; Schaich, 2005). The process of lipid peroxidation is classically broken down into 3 phases, which include initiation, propagation, and termination, with each step "consuming" and "producing" primary, secondary, and tertiary compounds, respectively (Belitz et al., 2009). Because there is no single method accepted by the livestock community that adequately characterizes or predicts the peroxidation status of a lipid, we chose to analyze several compounds that represent a compound from each of these peroxidation phases and the remaining peroxidation potential. Peroxide value represented the initiation phase, TBARS and p-anisidine value representing the propagation phase, hexanal representing the termination phase, and oil stability index representing the remaining peroxidation potential. As shown in Table 2, there was a slight reduction in the relative concentration of C18:2 and C18:3; and slight increase in the relative concentration of C18:0 due to heating of the corn oil (SO and RO) relative to the unheated corn oil. This is not surprising given that the susceptibility of a lipid to peroxidation is directly related to the number of double bonds (Holman, 1954) and can subsequently affect fatty acid composition (Choe and Min, 2007). The SO and RO heating processes had a large impact on the peroxidation status of the corn oil as indicated by their elevated PV, TBARS, p-anisidine, and hexanal values, and the reduction in their oxygen stability index. This was expected and similar to other recent research on corn oil (Liu et al., 2014).

Regardless of the increased peroxidation of corn oil by either the SO or RO heating process, there were no significant differences observed for BW gain, feed intake, or feed

efficiency for the 0 to 14 or 0 to 27 d period (Table 3). Averaged across both heating processes, lipid peroxidation reduced BW gain by 4.0% (P = 0.05), feed intake by 1.8% (P = 0.34), and feed efficiency by 2.4% (P = 0.11) compared to birds fed the unheated corn oil control from d 0 to 14. From d 0 to 27, lipid peroxidation reduced BW gain by 3.4% (P = 0.11), feed intake by 1.2% (P = 0.49), and feed efficiency by 2.2% (P = 0.05) compared to birds fed the unheated corn oil control. In a review of the literature on experiments that measured growth performance in broilers, Hanson (2014) indicated that the magnitude of reduction from feeding diets with peroxidized lipids relative to diets with unperoxidized lipids was 11.1% for BW gain and 6.6% for feed intake. Our results were less than these values, but reflect the same directional impact on bird performance.

Supplementation of 10% peroxidized corn oil reduced the dietary $AME_n$ (P < 0.01) compared to birds fed the UO corn oil (Figure 1). Birds fed the diet containing UO corn oil resulted in a dietary $AME_n$ of 3,490 kcal/kg, with birds fed the diet containing the RO corn oil resulting in a dietary $AME_n$ of 3,344 kcal/kg, with birds fed the SO corn oil resulting in an intermediate $AME_n$ of 3,402 kcal/kg. On average, the heating of corn oil by the SO or RO process reduced dietary $AME_n$ by 3.4% (P < 0.01). The slight change in corn oil composition (Table 2) may be partially responsible for the reduced ability of the broilers to metabolize and utilize energy from the peroxidized oils to the same capacity as the unheated oil as reflected in the lower C18:2 and C18:3, or total PUFA, content compared to the UO corn oil. In addition, both SO and RO corn oil had greater primary, secondary, and tertiary oxidation values that would increase the oxidative stress on the animal, which may impact the dietary $AME_n$ values (Engberg et al., 1996). The fact that the reduction in $AME_n$ did not affect performance was not unexpected as it has been shown that small differences in lipid energy

values can be difficult to demonstrate an impact on bird performance (Pesti et al., 2002; Firman et al., 2008).

Differences in $AME_n$ should affect the ability of birds to utilize and store the energy from their feed (Pesti et al., 2002), and this was indeed the case, albeit differences in abdominal fat pad weights were not significantly different among treatments (Figure 2). On average, birds fed the peroxidized corn oil treatments had abdominal fat pads that were 9.7% lighter than that of birds fed the UO corn oil treatment ($P = 0.08$), suggesting an impact on lipid digestion or utilization in the body. Due to excreta collection, birds were housed in grower batteries that prohibited determination of performance and abdominal fat pad weight at older ages where cumulative effects may have become significant. However, it is worthy to note that Engberg et al. (1996) showed that intake of peroxidized vegetable oil caused a reduction in whole body lipid and energy retention when birds were fed for 28 d.

It is worthy to note that in the review by Hanson (2014), that dietary PV had the highest negative correlation to BW gain. Pesti et al. (2002) also reported correlations between various measures of lipid quality (active oxygen method, iodine value, initial PV, moisture plus insolubles plus unsaponifiables, and free fatty acids) and bird performance, but measures of lipid peroxidation are not independent of the fatty acid composition of the lipid (Liu et al., 2014), and therefore lipid peroxidation correlations to animal performance when evaluated across lipids with differing fatty acid composition, must be viewed with caution. In the current experiment, the lowest dietary $AME_n$ was noted with the RO corn oil was which had a lower PV compared to the SO corn oil. We have no explanation for this apparent discrepancy, but this supports the concept that the numerous lipid peroxidation compounds generated during the peroxidation process may have differing effects on lipid metabolism

(Liu et al., 2014). This implies that a single peroxidation variable may be inadequate in determining the "true" peroxidation status of a lipid relative to animal performance and their oxidative status (Liu et al., 2014). Consequently, a multivariate analysis, such as TOTOX, which encompasses both primary and secondary products of lipid peroxidation (TOTOX = [2 × PV] + p-anisidine), may be more predictive of the peroxidation status of a lipid (List et al., 1974; Shahidi and Wanasundara, 2008). It is important to note, however, that even this calculated value does not include tertiary lipid peroxidation compounds such as 2, 4-decadienal or 4-hydroxynonenal, both of which are known to damage DNA (Seppanen and Csallany, 2002; Wu et al., 2001; Zarkovic, 2003).

Overall, the data indicate that corn oil with oxidative damage results in a lower dietary $AME_n$ value when included at 10% in the diet. This lower caloric availability should be taken into account for commercial broiler ration formulations as it may affect bird performance and lipid deposition as well. A larger more encompassing study is deemed necessary to solidify the impact of lipid peroxidation on bird performance, whole body lean and lipid accretion rates, and measures of oxidative status. In addition, a comprehensive list of lipid peroxidation measures are also necessary to determine which measure or measures should be put into a model, independent of the FA composition of the lipid, to accurately characterize and quantify the "true" peroxidation status of a lipid to growing broilers.

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## TABLES

**Table 1.** Composition of basal broiler diets containing unheated, slowly heated, or rapidly heated 5% (0 to 14 d) and 10% (15 to 27 d) corn oil.

| Ingredient, % | 0 to 14 d | 15 to 27 d |
|---|---|---|
| Corn | 54.79 | 55.93 |
| Soybean meal, 48% CP | 33.72 | 27.86 |
| Meat and bone meal | 2.50 | 2.50 |
| Corn oil[1] | 5.00 | 10.00 |
| Sodium chloride | 0.40 | 0.40 |
| DL-Met, 99% | 0.32 | 0.27 |
| L-Lys, 50% | 0.35 | 0.30 |
| L-Thr, 98.5% | 0.06 | 0.04 |
| Dicalcium phosphate | 1.18 | 1.02 |
| Limestone | 0.70 | 0.70 |
| Choline chloride, 60% | 0.10 | 0.10 |
| Vitamin premix[2] | 0.63 | 0.63 |
| Titanium dioxide | 0.25 | 0.25 |
| Calculated composition | | |
| Metabolizable energy, kcal/kg | 3,232 | 3,561 |
| Crude protein, %[3] | 22.5 | 19.7 |
| Ether extract, %[4] | 7.8 | 12.8 |
| Digestible TSAA, % | 0.92 | 0.81 |
| Digestible Lys, % | 1.26 | 1.08 |
| Digestible Thr, % | 0.82 | 0.70 |

[1]Refined corn oil was unheated, slowly heated at 95°C for 72 hr, or rapidly heated at 185°C for 12 hr; each heat treatment accompanied with 12 L/m air flow.

[2]Provided per kg of diet: vitamin A, 8,250 IU; vitamin $D_3$, 2,750 IU; vitamin E, 17.9 IU; menadione, 1.1 mg; vitamin $B_{12}$, 12 µg; biotin, 41 µg; choline, 447 mg; folic acid, 1.4 mg; niacin, 41.3 mg; pantothenic acid, 11 mg; pyridoxine, 1.1 mg; riboflavin, 5.5 mg; thiamine, 1.4 mg; Cu, 27.5 mg; Fe, 282 mg; I, 844 µg; Mg, 125 mg; Mn, 275 mg; Se, 250 µg; Zn, 275 mg.

[3]Crude protein analyzed 21.2, 21.1, and 21.5% for the unheated, slowly heated, and rapidly heated treatments, respectively, for the 0 to 14 d diets and 19.5, 19.6, and 19.5% for the unheated, slowly heated, and rapidly heated treatments, respectively, for the 15 to 27 d diets.

[4]Ether extract analyzed 6.8, 4.4, and 5.5% for the unheated, slowly heated, and rapidly heated treatments, respectively, for the 0 to 14 d diets; and 10.2, 8.5, and 8.3% for the unheated, slowly heated, and rapidly heated treatments, respectively, for the 15 to 27 d diets.

**Table 2.** Characterization of corn oil.

| Item | Lipid peroxidation status[1] | | |
|---|---|---|---|
| | Unheated | Slow | Rapid |
| Fatty acid, % of total fat | | | |
| C16:0 | 11.5 | 12.5 | 11.2 |
| C16:1 | 0.1 | 0.1 | 0.1 |
| C18:0 | 1.1 | 2.1 | 1.6 |
| C18:1 | 29.8 | 32.1 | 27.1 |
| C18:2 | 53.9 | 50.6 | 49.3 |
| C18:3 | 1.0 | 0.8 | 0.7 |
| Free fatty acids, % | 0.3 | 1.0 | 0.5 |
| Moisture, % | 0.2 | 0.5 | 0.2 |
| Impurities, % | ND | ND | ND |
| Unsaponifiables, % | 1.7 | ND | 1.5 |
| Peroxide value, mEq/kg | 1.7 | 134.9 | 5.7 |
| TBARS[2], mg MDA eq/kg | 9.0 | 19.0 | 26.7 |
| p-anisidine value[3] | 5.3 | 150.0 | 138.0 |
| Hexanal, $\mu$g/g | 1.5 | 3.9 | 5.9 |
| Oxygen stability index, h | 10.8 | 1.7 | 2.2 |

[1]Refined corn oil was unheated, slowly heated at 95°C for 72 hr, or rapidly heated at 185°C for 12 hr; each heat treatment accompanied with 12 L/m air flow.
[2]Thiobarbituric acid reactive substances.
[3]There is no unit of measure for anisidine value.

**Table 3.** Performance of broiler chickens fed diets containing unheated, slowly heated, or rapidly heated 5% (0 to 14 d) and 10% (15 to 27 d) corn oil.

| Corn oil[2] | 0 to 14 d[1] | | | 0 to 27 d | | |
|---|---|---|---|---|---|---|
| | BW gain, g/bird | Feed intake, g/bird | G:F | BW gain, g/bird | Feed intake, g/bird | G:F |
| Unheated | 362 | 461 | 787 | 1,071 | 1,506 | 711 |
| Slow | 346 | 448 | 774 | 1,032 | 1,478 | 699 |
| Rapid | 349 | 457 | 763 | 1,037 | 1,497 | 692 |
| SEM | 5.9 | 6.7 | 8.8 | 17.7 | 21.3 | 5.9 |
| Model *P*-value | 0.14 | 0.38 | 0.19 | 0.27 | 0.65 | 0.11 |
| Contrast *P*-value[3] | 0.05 | 0.34 | 0.11 | 0.11 | 0.49 | 0.05 |

[1]Gain and feed intake reported on a g/bird basis. Gain:feed reported as g of BW gain per kg of feed consumed.
[2]Refined corn oil was unheated, slowly heated at 95°C for 72 hr, or rapidly heated at 185°C for 12 hr; each heat treatment accompanied with 12 L/m air flow.
[3]Contrast of unheated, UO, versus heated, SO and RO, corn oil. For each treatment there were 10 pens per treatment with 6 birds per pen.

## FIGURES

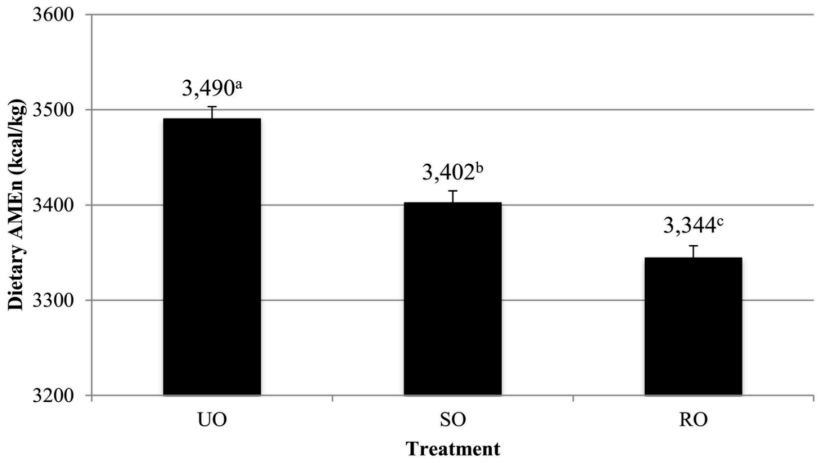


**Figure 1.** Broiler $AME_n$ (kcal/kg) of diets (as-is basis) containing 10% (15 to 27 d) refined unheated (UO), slowly heated at 95°C for 72 hr (SO), or rapidly heated at 185°C for 12 hr (RO) corn oil, with each heat treatment accompanied with 12 L/m air flow. Excreta samples collected on d 26 to 27 of the experiment to determine $AME_n$. Columns are mean values of 10 pens per treatment with 6 birds per pen and bars are defined as the overall pooled SEM (12.9).
[a-c]Superscript values without common letters are significantly different ($P \leq 0.05$). Contrast of unheated, UO, versus heated, SO and RO, corn oil; $P < 0.01$.

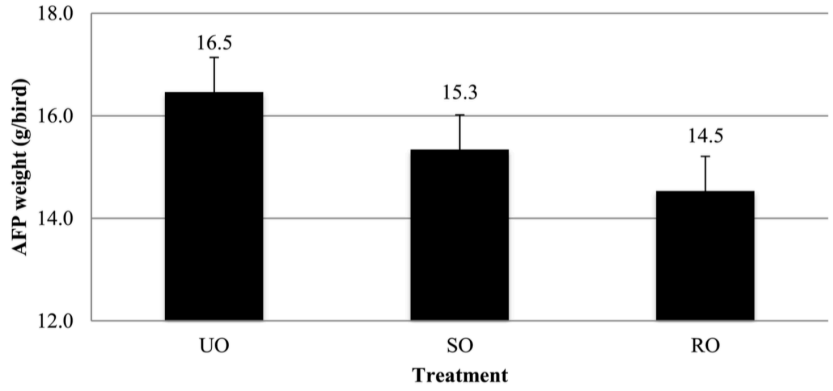


**Figure 2.** Abdominal fat pad (AFP) weight (g/bird) of broilers, collected on d 27, feed diets containing 5% (0 to 14 d) and 10% (15 to 27 d) refined unheated (UO), slowly heated at 95°C for 72 hr (SO), or rapidly heated at 185°C for 12 hr (RO) corn oil, with each heat treatment accompanied with 12 L/m air flow. Columns are mean values of 10 pens per treatment with 6 birds per pen and bars are defined as the overall pooled SEM (0.68) for AFP weight. Overall treatment P-value was 0.15. Contrast of unheated, UO, versus heated, SO and RO, corn oil; $P = 0.08$.