2019

Revealing the Role of Short Chain and Polyunsaturated Fatty Acids as Regulators of Metabolic Activity and Gene Expression in Ovarian Cancer

Abigayle Ochs
ochsam96@siu.edu

Follow this and additional works at: https://opensiuc.lib.siu.edu/uhp_theses

REVEALING THE ROLE OF SHORT CHAIN AND POLYUNSATURATED

FATTY ACIDS AS REGULATORS OF METABOLIC ACTIVITY AND GENE

EXPRESSION IN OVARIAN CANCER

Abigayle Ochs

A thesis submitted to the University Honors Program

in partial fulfillment of the requirements for the

Honors Certificate with Thesis

Southern Illinois University Carbondale

May 2019

I. Abstract

Previous research using the chicken model has provided evidence that a flaxseed-supplemented diet decreases both the severity and the incidence of ovarian cancer. Flaxseed is a source of omega-3 (OM3) polyunsaturated fatty acids (PUFA), particularly α-linolenic acid (ALA). ALA is converted into longer chain OM3s, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which inhibit prostaglandins, thereby inhibiting oxidative stress, inflammation, angiogenesis, and proliferation. The dietary fiber component of flaxseed can be fermented in the gut to produce short chain fatty acids (SCFA). Butyric acid, a commonly studied SCFA, has been shown be an important metabolic regulator by acting as a histone deacetylase (HDAC) inhibitor, a transcriptional modulator, as well as an anti-inflammatory molecule. In order to determine whether or not PUFA and butyric acid are upregulated by flaxseed supplementation in the chicken ovaries, the concentrations of each were quantified using gas chromatographic analysis. The amount of fatty acids in each sample were normalized to total protein content. Hens on a flaxseed-supplemented diet displayed an increase in the ratio of OM3:OM6 PUFA when compared to the control diet. Likewise, the concentrations of butyric acid were also increased in ovaries of hens fed a flaxseed-supplemented diet in comparison to hens that were fed a control diet. Moreover, butyric acid was significantly decreased in both the ovaries and ceca by ovarian cancer. These observations give us insight into the possible role of flaxseed in modulating metabolic activity through an upregulation of SCFA and OM3 PUFA in the ovaries.

II. Introduction

With nearly 22,000 new cases each year, and 14,000 of those cases leading to death, epithelial ovarian cancer (EOC) is the most lethal among all other gynecologic malignancies ("Ovarian Cancer Statistics," 2019). Ovarian cancer usually reaches stage 3 or 4 by the time it is diagnosed due to insufficient screening mechanisms and lack of symptoms early in the disease. For this reason, ovarian cancer has been termed the 'silent killer,' and is treated, perhaps most effectively, through prevention. Advancement in the treatment and prevention of ovarian cancer has been hindered in the past due to the lack of a functional animal model, but has recently begun to move forward with the introduction of the chicken model. EOC develops spontaneously in the White Leghorn Laying Hen and closely resembles the human form of the disease. Studies performed using the chicken model provide the opportunities to conduct large scale dietary intervention studies in a timely manner due to the fact that 2-year-old White Leghorn Hens ovulate 300-400 times, nearly the same as a perimenopausal woman, and similarly to humans, the risk of developing the disease and the severity of the disease for the laying hens increases with age.

In previous study conducted in Dr. Hales' lab, hens fed a 10% flaxseed-supplemented diet for four years displayed a significant decrease in incidence and severity of ovarian cancer (Eilati, Bahr et al. 2013), pointing at the possibility of flaxseed acting as a regulator of metabolic activity and gene expression. Flaxseed is a dietary fiber made up of two major components: omega-3 (OM3) polyunsaturated fatty acids (PUFA) and phytoestrogen lignin (SDG). OM3 PUFA provide anti-oxidant, anti-inflammatory, anti-angiogenic, and apoptotic effects, thereby preventing cancer development by inhibiting the actions of prostaglandins. Similarly, SDG is converted into enterodiol (ED) and enterolactone (EL), which prevent cancer development by blocking proliferation and mutagenesis via estrogen inhibition. This study was designed specifically to

explore the role of the fatty acid component of flaxseed by measuring OM3 PUFA levels in comparison to omega-6 (OM6) PUFA, as well as exploring the effects of flaxseed-supplementation on prostaglandin synthesis. The dietary fiber component of flaxseed was also investigated by measuring short chain fatty acid (SCFA) levels in the ovaries to demonstrate possible mechanisms of flaxseed acting as a metabolic regulator in ovarian cancer.

OM3 PUFA are considered essential fatty acids because they cannot be produced in the body, and therefore must be consumed by the diet. OM3 PUFA include stearadonic acid (SDA) (C18:4), alpha-lenolenic acid (ALA) (C18:3), and eicosapentanoic acid (EPA) (C20:5) and docosahexanoic acid (DHA) (C22:6), which are two byproducts of ALA. ALA serves a variety of purposes throughout the body, including reducing inflammation, promoting blood vessel health via incorporation into cell membranes, and being converted into the longer chain OM3 PUFA-EPA and DHA (Licastro, Candore et al. 2005). The ability for ALA to be converted into EPA and DHA is dependent upon diet. By reducing inflammation, ALA helps prevent and manage type 2 diabetes, heart disease, kidney disease, and many other chronic diseases, including some cancers, and by promoting blood vessel function, it also reduces the risk of heart attacks and stroke (Licastro, Candore et al. 2005). In addition, ALA produces energy for muscles by undergoing β-oxidation, can be used to make ketone bodies, and is stored in adipose tissue to serve as a reserve supply of energy.

ALA itself, however, does not have as many well described health benefits as EPA and DHA, but once consumed by the diet, ALA can be converted into these longer chain PUFA through a series of steps occurring in the endoplasmic reticulum, which involve alternating desaturations and elongations. Desaturase enzymes add double bonds to the fatty acids by removing hydrogen, while elongase enzymes add two additional carbons. ALA metabolism begins with desaturation

via delta-6-desaturase enzyme, forming SDA. This step is rate limiting because it is dependent on nutrition, hormones, and metabolism. The process of desaturation is very slow in comparison to the rapid process of elongation, so SDA is usually found in very low concentrations because once it is formed, it is quickly elongated to 20:4n3 (Licastro, Candore et al. 2005). 20:4n3 is then desaturated by delta-6-desaturase enzyme to produce EPA, which is also quickly elongated, forming docasapentaenoic acid (DPA) (C22:5). DPA is found in very trace amounts, as it is also quickly elongated to form 24:5n3. 24:5n3 then gets desaturated again, forming 24:6n3, which is finally β-oxidized to produce the end product of ALA metabolism: DHA (Licastro, Candore et al. 2005). Because this is the final step, DHA is often present in larger concentrations than the other OM3 PUFA. Following the metabolism of ALA, EPA and DHA can be incorporated throughout the body and further contribute to the viscosity of cell membranes, alterations in immune and tumor cell function, anti-inflammatory actions, and treatment or prevention of various diseases, such as cancer and cardiovascular disease (Swanson, Block et al. 2012). However, ALA metabolism does not always make it all the way to DHA. Some factors that can affect the conversion of ALA to EPA, DPA, and DHA include gender, smoking, and diet (Licastro, Candore et al. 2005). Cigarette smoke has been shown to reduce the conversion of ALA to EPA and DHA. Men also show a reduced metabolism of ALA compared to women, who are likely more sensitive to diet than women due to their unique hormonal profile. Women's increased ability to convert ALA to DHA is very important during pregnancy and breast feeding (Licastro, Candore et al. 2005). Lastly, a diet that is rich in OM6 PUFA significantly decreases the conversion of ALA, in addition to diets that are high in saturated fats, oleic acid, trans fatty acids, and cholesterol, which disrupt the actions of desaturase and elongase enzymes (Licastro, Candore et al. 2005). These factors suggest that ALA conversion is very dependent on one's individual metabolic capacity. If

dietary flaxseed increases the levels of OM3 PUFA compared to OM6 PUFA, and therefore increases the levels of DHA converted from ALA, this could demonstrate one mechanism in which flaxseed works to improve overall metabolism could be revealed.

OM6 PUFA, like OM3, are essential fatty acids and must be consumed from the diet. However, because these two groups of fatty acids cannot interconvert between one another, there is competition between the metabolism of the two (Licastro, Candore et al. 2005). Excess of one can affect the metabolism of the other because they use similar mechanisms, involving the same desaturase and elongase enzymes. OM6 PUFA are typically consumed much more abundantly than OM3, and primarily include linoleic acid (LA) (C18:2) and arachidonic acid (AA) (C20:4). LA consumed from the diet is desaturated to produce gamma-linolenic acid (GLA) (C18:3). GLA is elongated, forming dihomo-gamma-linolenic acid (DGLA) (C20:3). This fatty acid is desaturated to make AA, which is elongated and desaturated to eventually form DPA following β-oxidation. AA is known to be the primary source for prostaglandins. Prostaglandins consist of a 20-carbon, unsaturated fatty acid chain and are synthesized via the cyclooxygenase (COX1 and COX2) pathway (Chen, Wang et al. 2019). Prostaglandins can be harmful by causing inflammation, oxidative stress, angiogenesis, and proliferation. The action of prostaglandins can be inhibited by increasing the levels of OM3 PUFA, which will use the enzymes needed by OM6
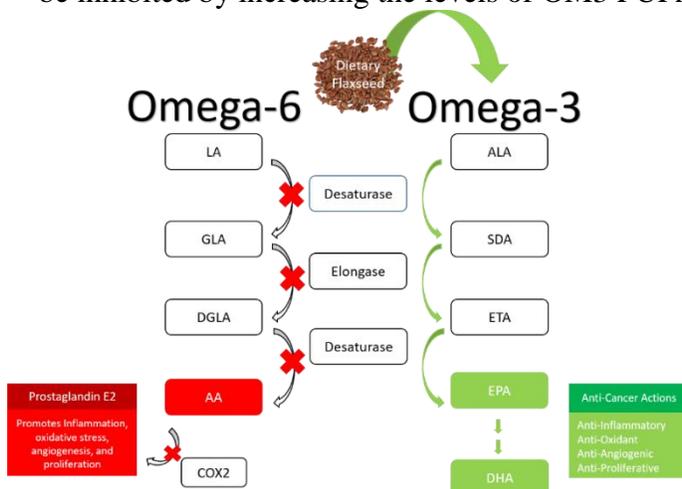
PUFA to produce AA. By decreasing the amount of AA converted from LA, the levels of prostaglandins will also be reduced. By showing a negative correlation between dietary flaxseed and AA / prostaglandin synthesis, another mechanism in which

flaxseed has the ability to improve metabolic activity, specifically by regulating inflammation and oxidative stress, could be observed.

The relationship between diet and gut microbiota strongly influences metabolic health by the bacterial synthesis of various metabolites, such as SCFA, which are fermented by bacteria in the gut from dietary fibers. Different dietary fibers are fermented differently based on fiber structure, which could favor or disfavor growth, and type of bacteria, which have varying metabolites that alter the gut microenvironment in different ways (Bishehsari, Engen et al. 2018). Pleiotropic effects, including immune system modulation, anti-inflammatory effects, and alterations in pathogen susceptibility, have been associated with an upregulation of SCFA, such as acetate, propionate, and butyrate (Shubham, Chakravarty et al. 2019). For this reason, SCFA are being studied as a possible preventative mechanism for various diseases, and in this case, ovarian cancer.

One SCFA in particular, butyric acid (C4:0), has been previously reported to have an exceptionally beneficial role in immunity. The SCFA facilitates an immune response by altering various catabolic processes in human cells and by modulating gene expression by acting as an HDAC inhibitor (Shubham, Chakravarty et al. 2019). HDAC inhibitors favor histone acetylation, resulting in an open and transcriptionally active form of chromatin. Through its HDACi activity, butyric acid has been shown to improve insulin signaling by upregulating the expression of IRS1, protect against oxidative stress linked to the over-expression of FOXO3a and PGC-1α, (Chriett, Dabek et al. 2019). In addition, butyric acid upregulates the activity of various mitochondrial genes, including the mitochondrial sirtuins (SIRT3, 4, and 5) responsible for regulation of mitochondrial activity, SOD2, and CPT1b, and also contributes to the expression of catalase, which functions in cell defense, especially against oxidative stress (Chriett, Dabek et al. 2019). All

of these genes are upregulated by butyric acid and function to improve cellular immunity, protect against oxidative stress, and regulate mitochondrial activity, enhancing overall metabolic activity and protecting the body from various diseases. Thus, identifying whether SCFA, in particular butyric acid, are upregulated by a flaxseed supplemented diet could provide insight into one mechanism for preventing or reducing the severity of ovarian cancer.

The objective for the current study was to better understand the role of dietary flaxseed in regulating metabolic activity and gene expression by exploring the levels of PUFA and SCFA in the ovaries collected from White Leghorn laying hens. The 2-year old hens were placed on one of six diets, four of which were flax based diets, and are outlined below in **Table 1.** The other two diets included fish oil and corn oil to further investigate the effect of diet on fatty acid production. After 11 months, a necropsy was performed to collect various samples from the birds, including the ovaries. The ovarian tissues were then analyzed to determine the concentrations of PUFA and SCFA in each sample. A simple lipid extraction was performed first to separate the lipids from the other components of the sample. Fatty acid methyl ester (FAME) preparation was then performed to prepare the samples for gas chromatographic analysis. By performing gas chromatography, the concentrations of fatty acids in each sample was determined and the data was normalized to protein content. An enzyme linked immune-sorbent assay (ELISA) was performed on the same ovarian samples to measure the levels of prostaglandin in each sample. This data was normalized to the protein content. In order to better understand the synthesis and distribution of SCFA in the body, the ceca were also analyzed using the same method as the ovaries previously discussed. These concentrations were then compared to the concentrations of the SCFA in the ovaries. The analysis revealed that hens that were fed a flaxseed-supplemented diet had higher levels of OM3 PUFA compared to OM6 PUFA in both the ovaries and the ceca. The concentrations of SCFA were also

slightly increased in the ovaries and ceca of flax-fed hens compared to the control group. More significantly than the effect of diet, this analysis showed that ovarian cancer results in a significant increase of SCFA concentrations in the ovaries and the ceca. These observations give us insight into the possible role of flaxseed in modulating metabolic activity through an upregulation of SCFA and PUFA in the ovaries.

Table 1: Content of flaxseed diets

Control	15% Whole Flax (WF)	5% Flax Oil (FXO)	15% Defatted Flax Meal (DFM)
	SDG + ALA	ALA	SDG
Omega 3/6	Omega 3 Rich	Omega 3 Rich	Low Omega 3
No SDG	High SDG	No SDG	High SDG

III. Material and Methods

Lipid Extraction

Ovarian tissues were collected and 200 mg were aliquoted into glass test tubes. Samples were homogenized with 1mL of homogenate buffer (20x Protease Inhibitor and 10mM Tris Buffer) using Tissue Tearor Homogenizer (Biospec Products, Inc. USA) on dry ice. 200 µl of the samples were transferred to new test tubes, and the rest was stored at -20°C until further use. To the 200 µl, 30 µg/mL heptadecanoic acid (C17:0) was added as an internal standard. These samples were then extracted twice using chloroform and water, and were centrifuged at 3400 rpm, for 10 min., at 4°C, between each extraction. The organic layer was removed and stored at -20°C until further use. This procedure was also used to extract lipids from the ceca, but 2 mL of homogenate was used, rather than 1 due to increased thickness of these samples.

Fatty Acid Methyl Ester (FAME) Preparation

Extracted samples were dried under ultra-pure nitrogen (UPN), and acetyl chloride and methanol was used to convert fatty acids to methyl esters. The oxygen was then flushed out of the samples using UPN, and the samples were incubated in a dry over for 1 hr. Dried samples were then taken out to cool, and fatty acids were extracted using hexane and water. The samples were centrifuged at 3400 rpm, for 10 min, at 4°C. The hexane layer was removed and dried once again under UPN. 125 µl of hexane was then used to re-suspend the dried samples, and the resuspension was transferred to gas chromatography vials. The vials were capped and stored at -20°C until further use. The same procedure was used to prepare the ceca for gas chromatographic analysis.

Gas Chromatography

Quantification of FAME was performed using gas chromatography with flame ionization detection (GC-FID). GC was performed on a Shimadzu GC-2010 Gas Chromatograph equipped with a Supelco Omegawax 250 capillary column (30 m, 0.25 mm ID, 0.25 µm film thickness). Samples were injected into a split injector held at 250°C and with helium carrier gas running at a column flow of 1.25 mL/min with a 100:1 split. Analytes were detected using a flame ionization detector held at 260°C. All data were acquired and analyzed Shimadzu Chromatography Data System. All automatic peak assignments and integrations were manually verified.

Protein Analysis

The remainder of the homogenized samples saved during the lipid extraction phase of the experiment was thawed and used in a protein assay to normalize the data collected from the GC. First, a standard curve was established using known amounts of protein (BSA [2mg/mL]), diluted with buffer SDS buffer (0.1% in 1x PBS) in the first two columns of a 96-well plate, using the amounts shown below in **Table 2.**

Table 2: BSA Protein Assay standards performed in replicates. Ratios denote protein (BSA) to buffer (0.1% SDS in 1x PBS)

protein:buffer (µL)	Column 1	Column 2 (replicate)
A	0:10	0:10
B	1:9	1:9
C	2:8	2:8
D	3:7	3:7
E	4:6	4:6
F	6:4	6:4
G	8:2	8:2
H	10:0	10:0

After the protein and buffer was pipetted to create the standard curve, 2 µl of each unknown ovary samples were added in triplicates in the subsequent wells with 8 µl of 0.1% SDS buffer in 1x PBS. Enough of a 50:1 dilution of BCA protein assay reagents A and B (Thermo Fischer Scientific, USA) was then made to add 200 µl of the mixture to each well, including both the standards and unknown samples. After the AB reagent mixture has been added to each of the wells, the plate is incubated in the Synergy plate reader with slow shaking at 37°C for 30 min. Following the incubation, the plate was read at 562 nm, and absorbance values are used to calculate the concentration of protein in each sample, which is in turn used to determine the concentration of fatty acid/mg of protein. The R^2 value determined by the accuracy of the standard curve should be greater than 0.99 to yield the most accurate results. For the ceca, the samples were sonicated prior to increase liquefaction.
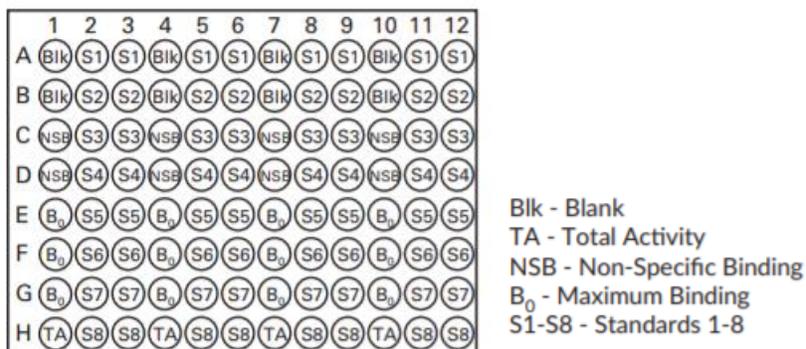
Enzyme Linked Immuno-Sorbent Assay (ELISA)

The same ovaries previously used were aliquoted again with 200 mg in glass test tubes. Samples were homogenized with 0.5 mL of homogenate buffer (20x Protease Inhibitor and 10mM Tris Buffer) using Tissue Tearor Homogenizer (Biospec Products, Inc. USA) on dry ice. These samples were then centrifuged at 3400 rpm, for 10 min., at 4°C, and the supernatant was collected. The

samples were then set aside for use in the ELISA kit (Cayman Chemical, USA) and the instructions provided in the kit were followed. First the ELISA and wash buffers were prepared. One vial of 10x ELISA buffer was diluted with 90 mL of UltraPure (UP) water. 2.5 mL of the 400x wash buffer was diluted to 1 mL with UP water and 0.5 mL of Polysorbate 20 was added. The standards were then prepared by adding 100µL of the ELISA buffer to the provided ELISA standard. Eight glass test tubes were then labelled and serial dilutions were performed as follows:
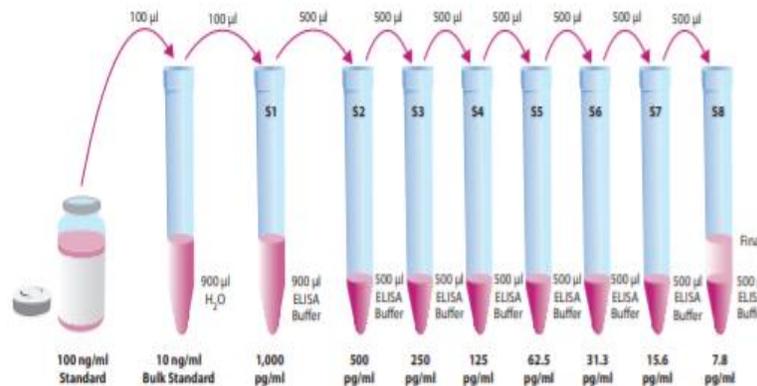
Acetylcholineesterase (AChE) Tracer and Antibody were then prepared by adding 6 mL of ELISA buffer to each of the 100 dtn vials. The 96-well plate was provided pre-coated with antibody. A sample of the plate format is shown below:

Blk - Blank
TA - Total Activity
NSB - Non-Specific Binding
B_0 - Maximum Binding
S1-S8 - Standards 1-8

First, 100 µL of ELISA buffer was added to the NSB wells, and 50 µL of ELISA buffer was added to the B_0 wells. 50 µL of the standards were then added in their coordinating wells beginning with

S8 and working up to S1. 50 µL of the samples that were previously prepared were then added to the remaining wells in duplicate. 50 µL of AChE Tracer was then added to all the wells except the TA and the Blk wells and 50 µL of AChE Antibody was added to each well except the TA, NSB, and Blk wells. The plate was then covered, and incubated for 18 hours at 4°C. After 18 hours the wells were emptied and washed 10 times with wash buffer. The 100 dtn Ellman's reagent was reconstituted in 20 mL of UP water right before use. 200 µL of this reagent was added to each well. 5 µL of AChE Tracer was added to the TA well, and the plate was incubated in the Synergy plate reader with slow shaking at 37°C for 60 minutes. The plate was read at 420 nm, and the concentrations of prostaglandin E2 (PGE2) were then calculated. A protein assay was again performed to normalize the data.

Statistical Analysis

Comparison of values between groups was performed by two-way ANOVA, using Tukey-Kramer's Post Hoc, $p < 0.5$ to identify significant values.

IV. Results
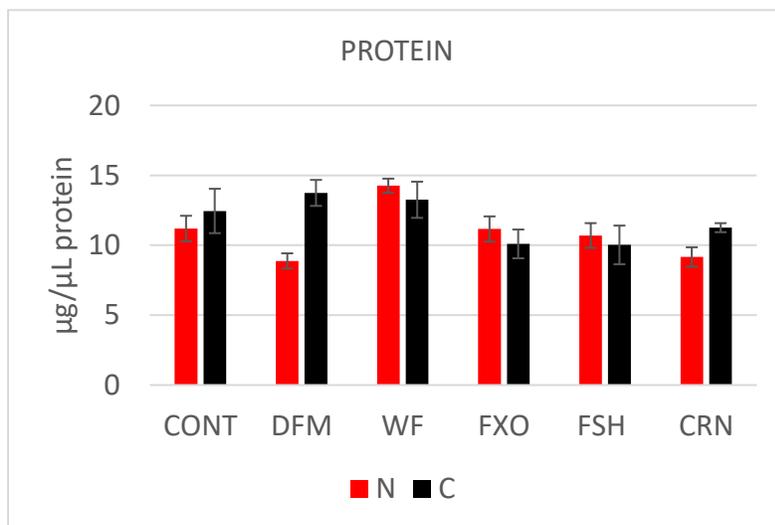
Figure 1: Concentrations of protein in ovarian tissues

A protein assay was used to determine the amount of protein in each sample. This was then used to normalize the gas chromatography data to determine the amount of fatty acid in each microgram of protein. The protein assay was performed in triplicates. $R^2 = 0.996$. Error bars represent SEM.
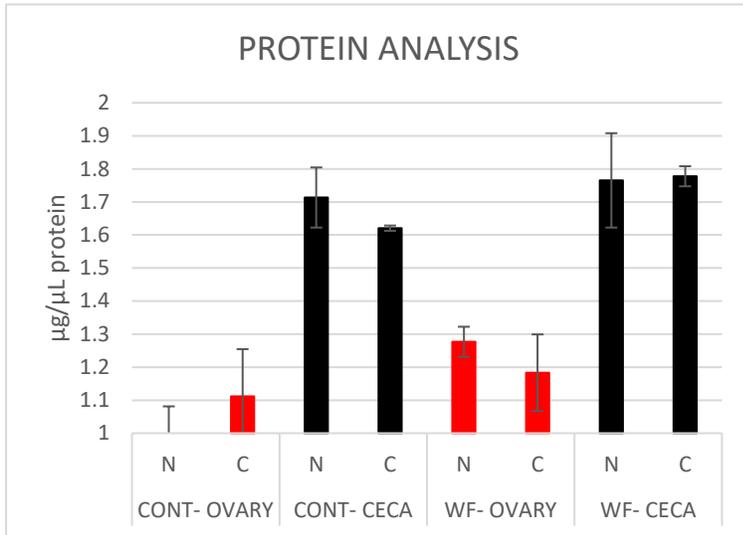
Figure 2: Concentrations of protein in ceca compared to ovarian tissues

A protein assay was used to determine the amount of protein in each ceca sample. This was then used to normalize the gas chromatography data to determine the amount of fatty acid in each microgram of protein. The protein content in the ceca samples were then compared to the protein content in the ovariean samples. The data was normalized to the amount of protein in the ovaries of healthy, control-fed hens. The protein assay was performed in triplicates. $R^2 = 0.998$. Error bars represent SEM.
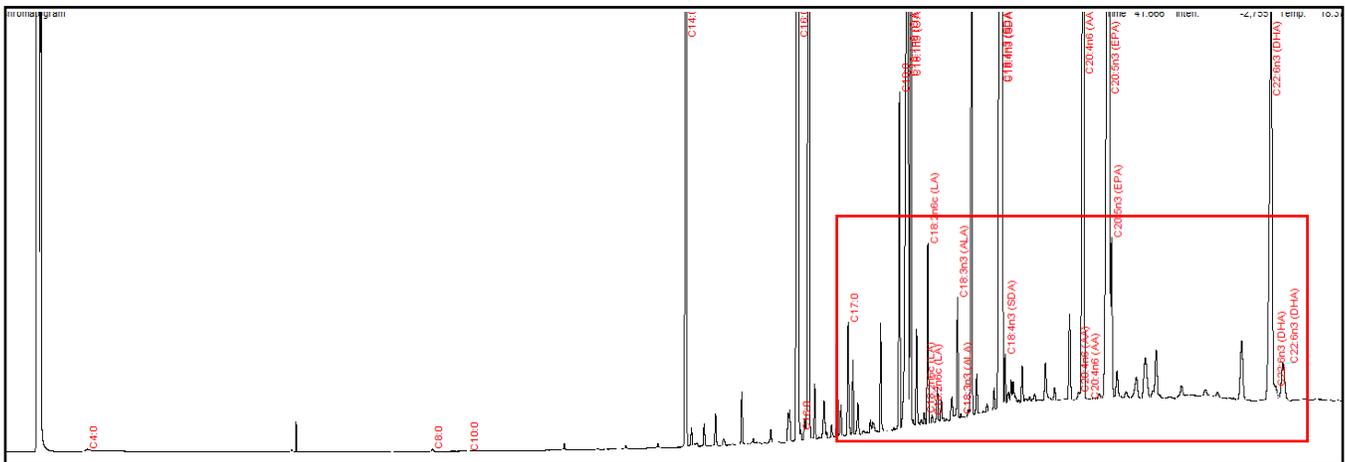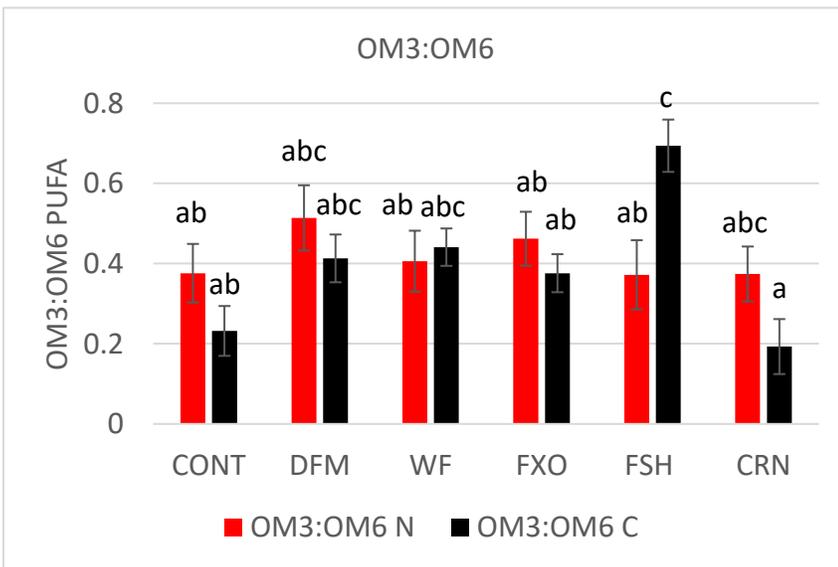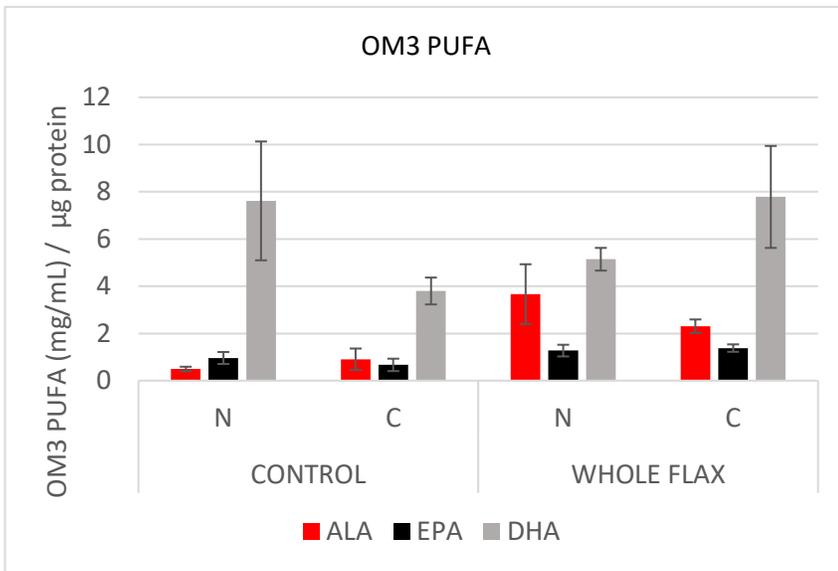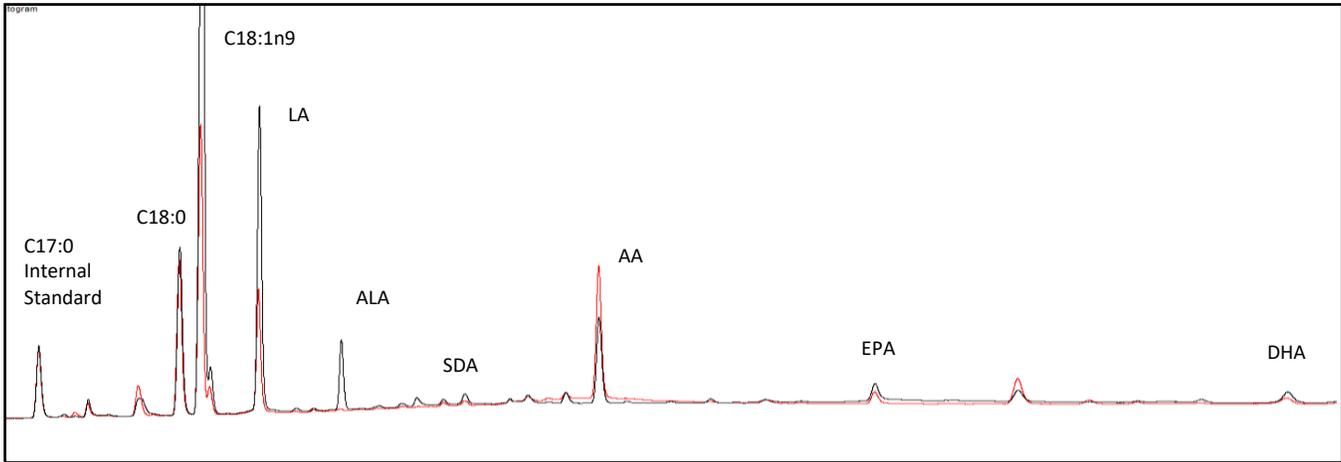
Figure 3: Whole Flax v. Control PUFA in cancerous ovarian tissues

Gas chromatographic analysis was performed to determine the concentrations of PUFA in ovarian tissues collected from hens fed 6 various diets. C4-C24 FAME mix was used to identify the fatty acids (shown in the first chromatograph). The next chromatograph compares whole flax (black) with control (red). C17:0 was used as an internal standard. The data was normalizaed using a protein assay, and the effects of the diet and disease on the ratio of OM3 to OM6 PUFA in the ovaries was examined. Error bars represent SEM. Tukey-Kramer's Post Hoc, $p < 0.05$.
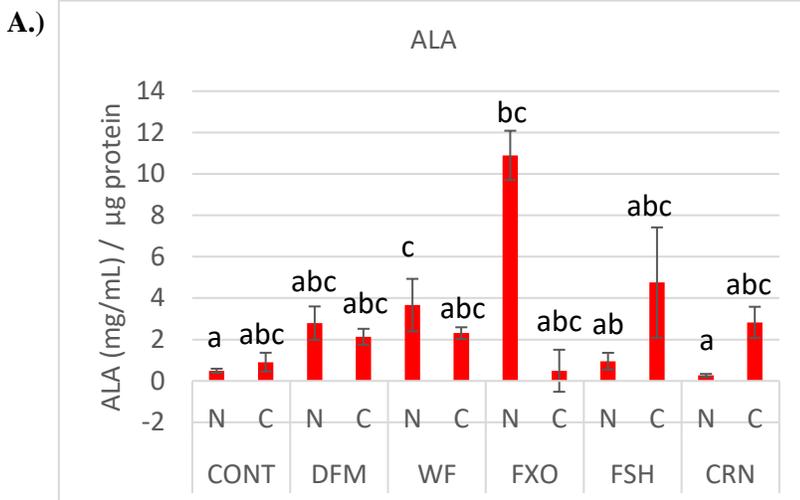
Figure 4: Effect of diet and disease on the conversion of ALA to DHA

ALA has various fates once it is introduced to the body. One of these involves being converted into longer chain fatty acids, such as EPA and DHA, by desaturase and elongase enzymes. **A.)** Compares the levels of ALA across all 6 diets in normal and diseased birds **B.)** Compares the levels of EPA alone. **C.)** Compares the levels of DHA alone. **D.)** Displays a chromatograph zoomed in on the peak representing DHA to observe how much DHA has been produced in WF-fed, healthy birds (red), WF-fed, cancerous birds (black), control-fed, healthy birds (blue), and control-fed, cancerous birds (green). The error bars indicate SEM. Tukey-Kramer's Post Hoc, $p < 0.05$.
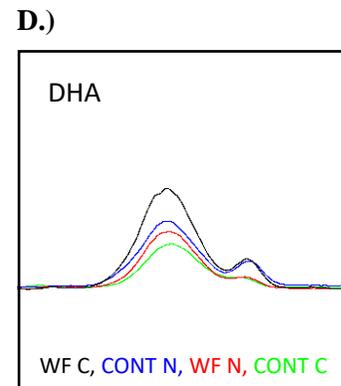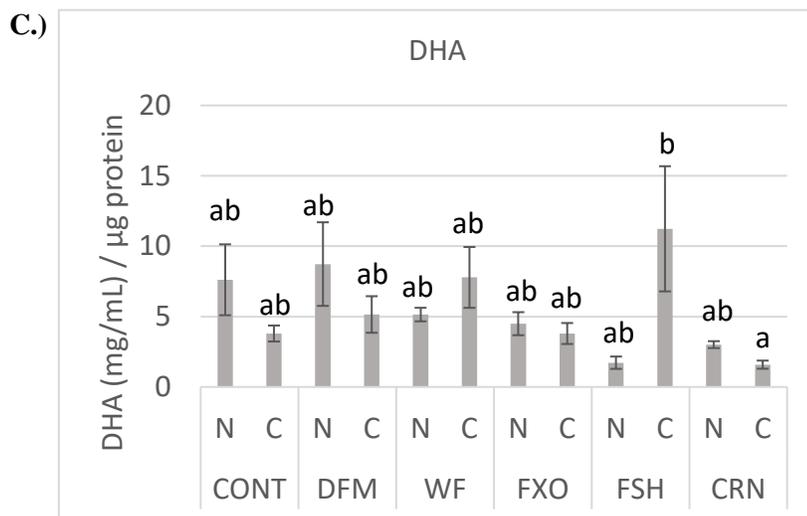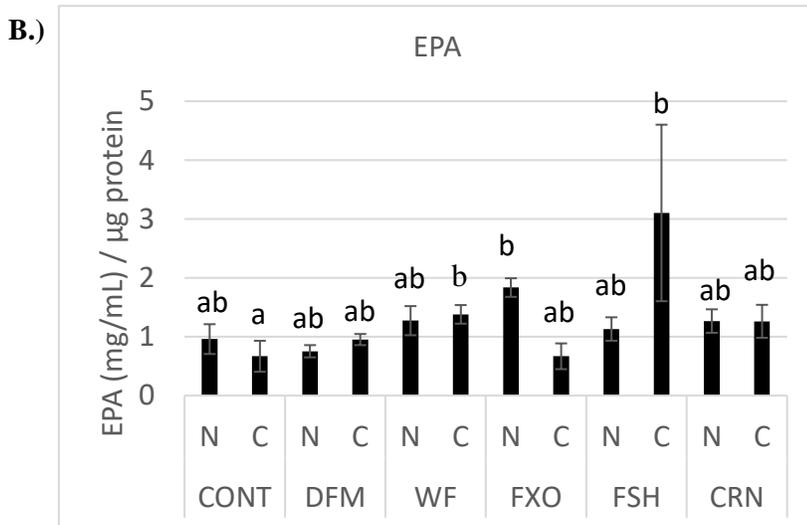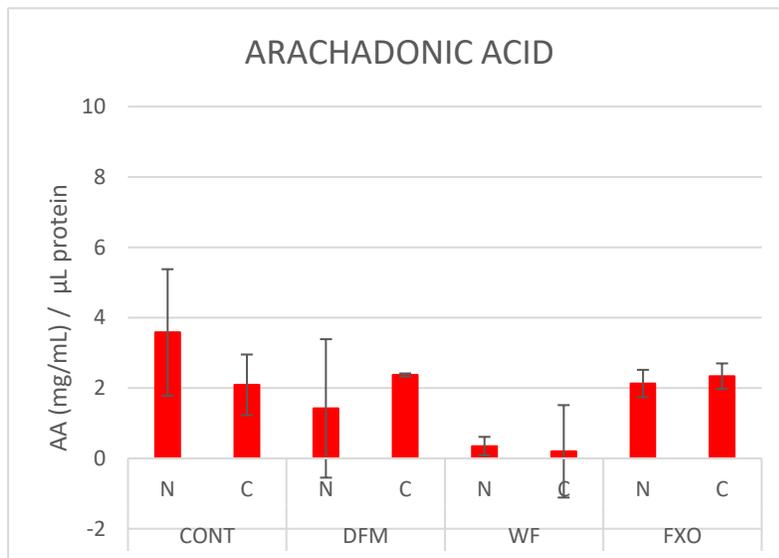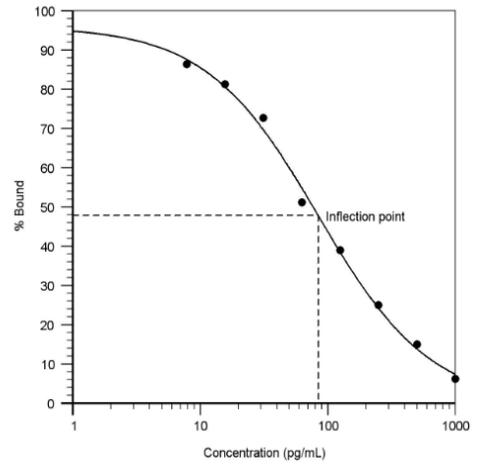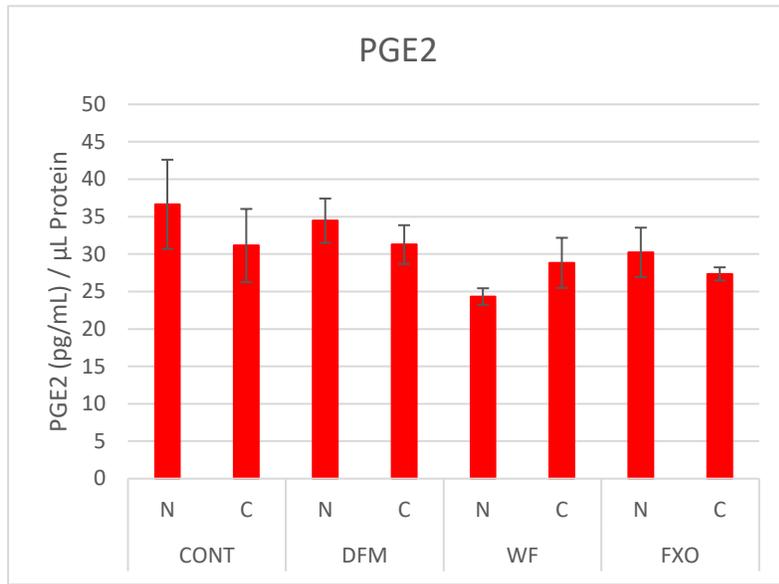
Figure 5: Effect of diet and cancer on levels of prostaglandin E2 and arachidonic acid in the ovaries

ELISA was performed on ovaries to also measure the levels of PGE2. $R^2 = 0.99$. Arachadonic acid concentrations were determined using gas chromatography. Data was quantified, and compared between the various diets and between healthy and diseased hens. Both of these concentrations were normalized to protein content. Error bars represent SEM.
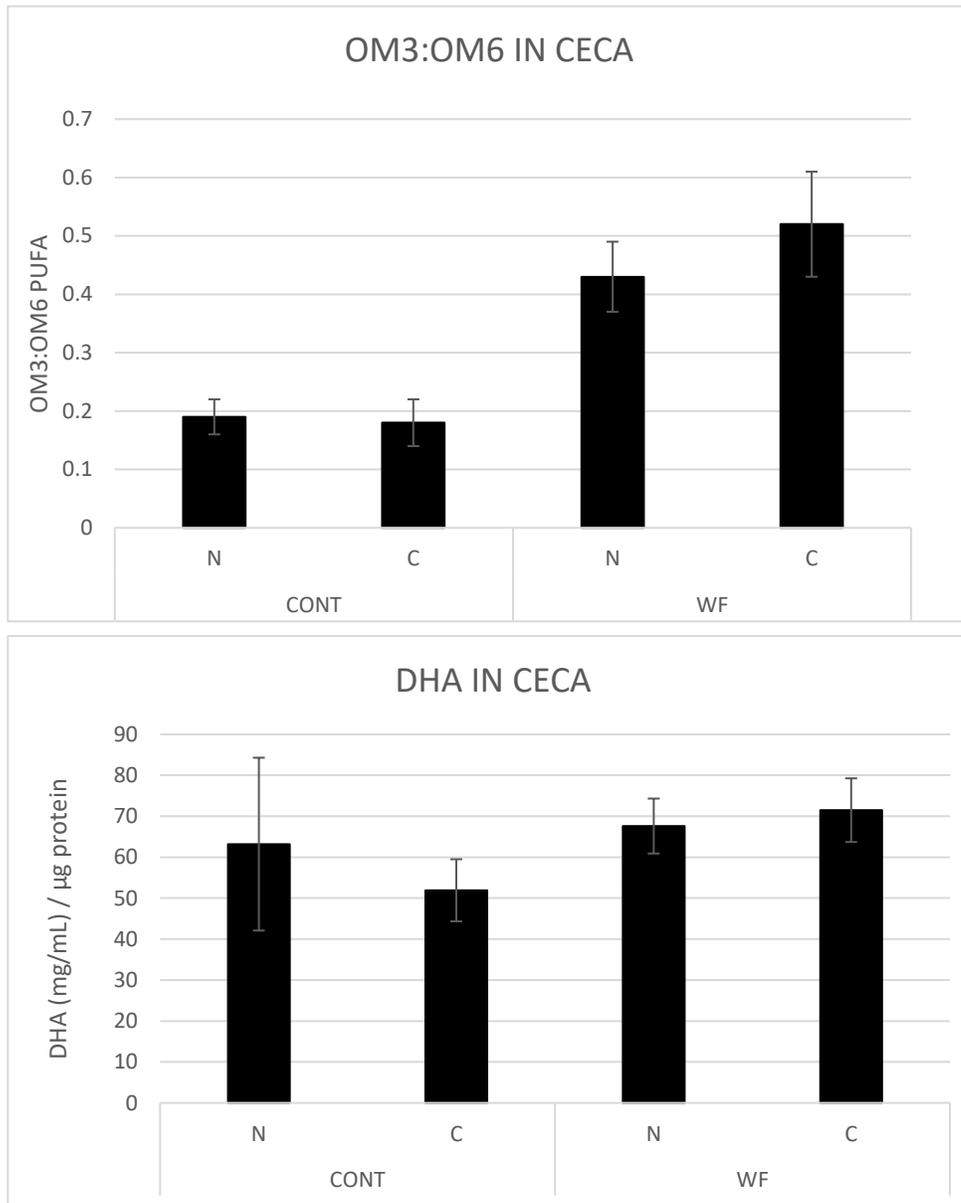
Figure 6: Ratio of OM3 to OM6 PUFA in hen ceca

PUFA concentrations were determined by gas chromatography. The levels of a OM3 and OM6 PUFA were quantified and the effects of diet and disease were then observed. Error bars represent SEM.
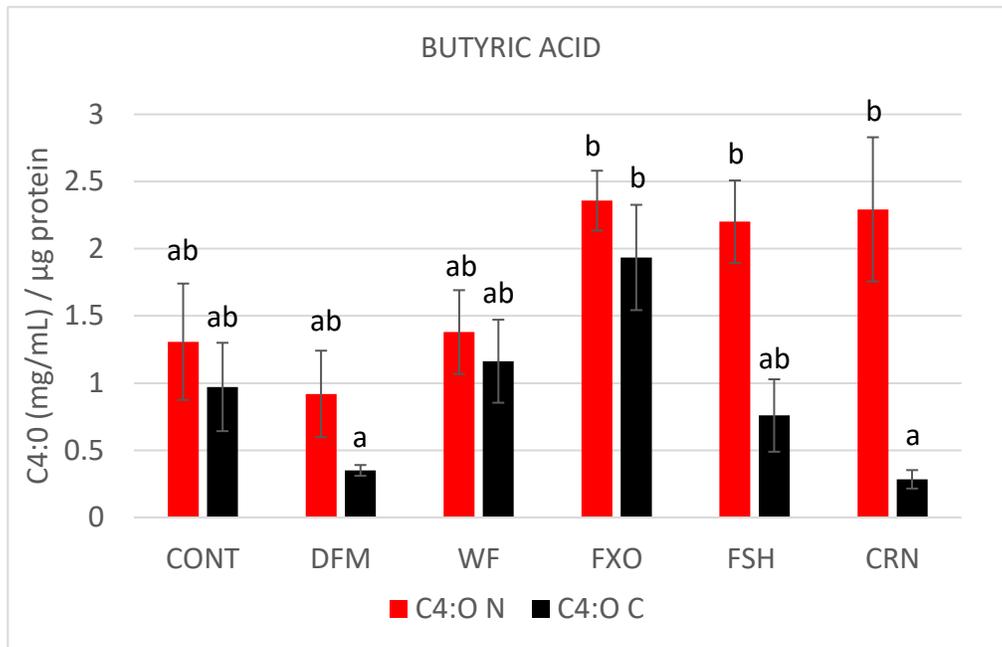
Figure 7: Diet and carcinogenic effects on butyric acid (C4:0) composition in ovarian tissues

SCFA concentrations were determined by gas chromatography. The levels of a common SCFA, butyric acid (C4:0), were quantified and compared between the six different diets and between healthy and diseased hens. Error bars represent SEM. Tukey-Kramer's Post Hoc, $p < 0.05$.
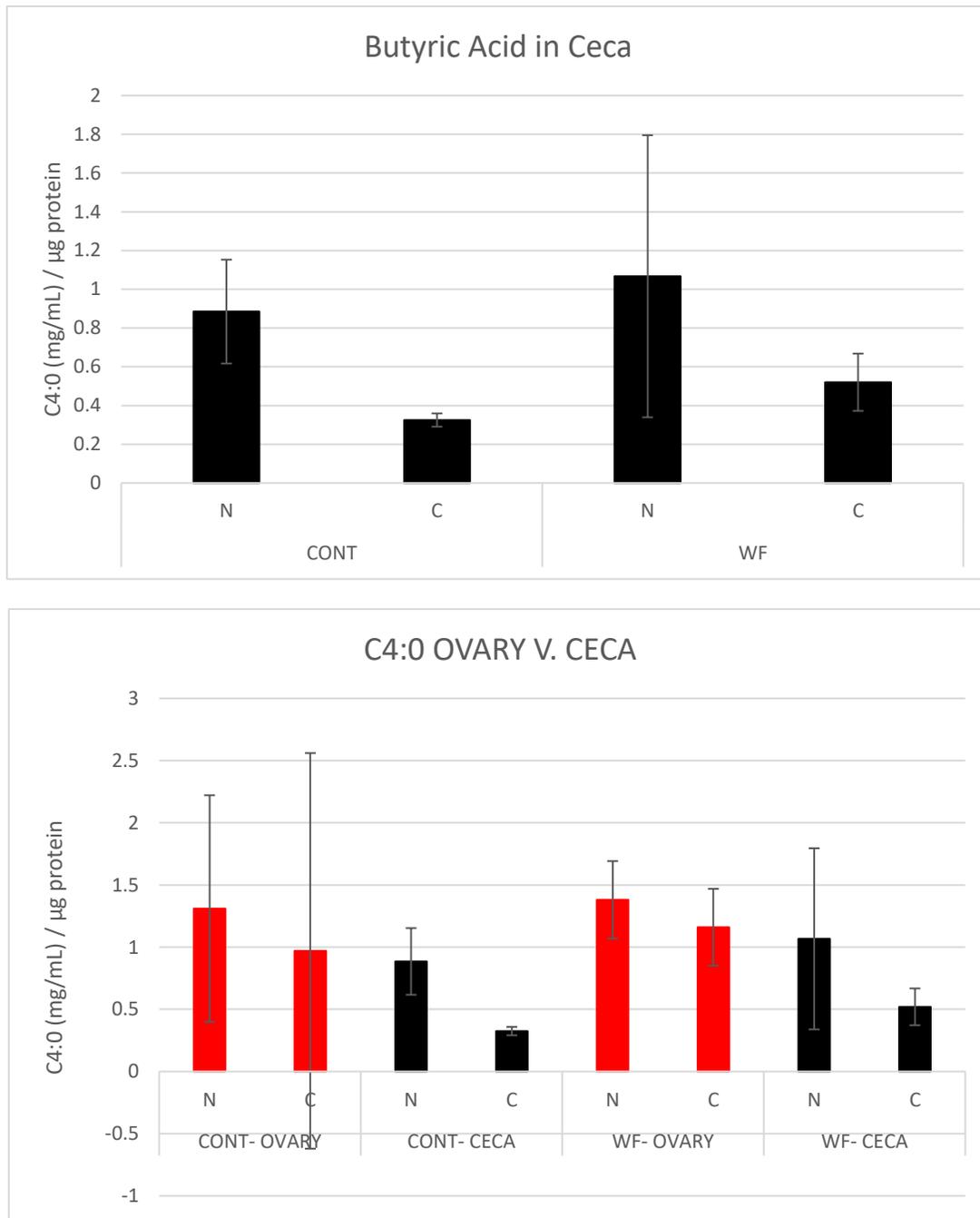
Figure 8: Effect of diet and cancer on C4:0 production in the ceca compared to levels in the ovaries

Butyric acid (C4:0) concentrations in chicken ceca were determined using gas chromatography. The levels were quantified and first compared between whole flax and control fed hens, and then between healthy birds and birds with advanced stage ovarian cancer. The data was normalized to protein content. The concentrations of C4:0 in the ceca were then compared to the data collected from the ovarian samples. Error bars represent SEM.

V. Discussion

The six diets explored in this study were designed to test the hypothesis that OM3 PUFA and SCFA are upregulated in the ovaries as a result of a flaxseed-supplemented diet, and that this upregulation may be one mechanism in which flaxseed can work to regulate metabolic activity. **Figure 1** indicates that protein concentrations in the ovaries are not affected by neither diet nor disease. **Figure 2** shows that diet and disease also have no effect on the protein content in the ceca. Although no effect of diet or disease was observed on either sample, the ceca samples had a much greater protein content compared to the ovarian samples.

In **Figure 3** it is shown that flax-supplemented diets slightly increase the mean levels of OM3:OM6 PUFA compared to control fed birds in both healthy and diseased states, although the quantities are not significantly different. This data also indicates that in control fed hens (hens that were not fed flaxseed as a source of OM3 PUFA), the disease may affect the ability to produce DHA shown by significantly reduced levels in the birds with ovarian cancer. **Figure 4** explored the efficiency of the conversion of ALA to the longer chain PUFA- EPA and DHA. The data show that birds with ovarian cancer that are fed a whole flax-supplemented diet more readily convert ALA to DHA than healthy birds and diseased birds fed a control diet. Levels of ALA vary across the diets but are lowest in the control and corn oil-fed hens, and levels of EPA remain mostly consistent. Next, the levels of PGE2 were examined in relation to the levels of AA. **Figure 5** indicates that no correlation was observed between these values, however, the levels of PGE2 were consistent with the trends observed in the ratios of OM3:OM6, indicating that when OM3 PUFA are increased, less PGE2 is able to be synthesized. It was also noted that AA was significantly reduced in whole-flax fed hens. The ratios of OM3:OM6 PUFA in the ceca were also determined, and in **Figure 6**, it was observed that a whole-flax diet significantly increased the levels of OM3,

in particular DHA. It was also shown in this data that the levels of DHA in the ceca, which average between 50 and 70 mg/mL per μg protein, are significantly higher compared to the levels in the ovaries, which are only between 5 and 10 mg/mL per μg protein. In addition, this data confirmed that disease state does in fact have an effect on DHA production because in the ovaries of control fed hens, birds with ovarian cancer had significantly less DHA than healthy birds (shown in **Figure 1**), but the DHA levels in the ceca had not been affected.

Butyric acid was increased in the oil based diets and flax-supplemented diets, although the values were not significantly different. Disease state showed to have a greater effect, as observed in **Figure 7**, butyric acid was significantly higher in healthy birds than ones with ovarian cancer, regardless of which diet the hen was fed. This observation signifies a possible impairment in enteric SCFA production and/or intra-ovarian tissue metabolism. To further explore whether this was an impairment in SCFA production or function, the levels of butyric acid were measured where they are produced, in the colon. In **Figure 8**, it is shown that the levels of butyric acid observed in the ovaries are consistent with the production in the ceca, showing a slight increase in the birds fed a whole-flax diet compared to control, and a significant decrease in birds with advanced stage ovarian cancer compared to healthy birds. However, the levels of butyric acid were higher in the ovaries than in the ceca, indicating that they likely do not stay in the ceca long before moving to the liver to be stored and/or to the target organ.

VI. Conclusion

Previously, our lab has shown that a flaxseed-supplemented diet reduces both the incidence and severity of ovarian cancer. This study aimed to investigate the possible mechanisms in which flaxseed, when consumed, is able to regulate metabolic activity and gene expression in ovarian cancer by first, exploring the role of the OM3 component of flaxseed. Because OM3 and OM6

PUFA cannot be produced by the body, production of these fatty acids is solely dependent on dietary intake. Gas chromatographic analysis revealed that ratios of OM3:OM6 PUFA are slightly elevated in the ovaries of birds fed a flaxseed-supplemented diet when compared to the control group. This data was consistent with the concentrations of AA measured in the ovaries, which were significantly reduced in flax-fed hens. This is significant because OM3 and OM6 PUFA compete to use the same elongase and desaturase enzymes. At higher levels of OM3, more DHA can be produced from ALA, rather than AA from LA, which would lead to increased prostaglandin synthesis. PGE2 levels were measured using ELISA, and were found to be slightly reduced in hens that were fed a whole flax or flax oil diets compared to the control group, which is consistent with the trends observed in the ratios of OM3:OM6, indicating that when OM3 PUFA are increased, less PGE2 is able to be synthesized. This data is also consistent with the levels of AA measured in the ovarian samples. These observations further indicate that by increasing levels of OM3 in the ovaries, a flaxseed-supplemented diet inhibits or significantly reduces the enzymatic activity available to convert LA into AA, which is required to produce PGE2. By decreasing the levels or PGE2, the negative side effects of inflammation and oxidative stress are also prevented.

In addition to a reduced overall ratio of OM3:OM6, the birds in the control group that were not fed a flaxseed-supplemented diet had slightly lower amounts of DHA in their ovaries compared to the flax-fed birds, and significantly lower amounts of DHA if the birds had ovarian cancer. However, the same effects were not observed in the ceca, indicating that the cancer likely had a direct effect on the ability for these hens to convert ALA to DHA at the ovarian tissue level. The levels of DHA in the flax-fed hens did not seem to be affected by the disease in the ovaries or the ceca, which suggests that hens that were fed a flaxseed-supplemented diet with ovarian cancer are more likely to benefit from the anti-cancer effects of DHA, which has greater anti-inflammatory

and antioxidant effects than ALA alone. Increases in DHA might suggest that flaxseed increases DHA by increasing Δ6-desaturase activity. The consistent levels of EPA that were observed are likely due to the fact that desaturase enzymes work very slowly to produce EPA from ALA, but once formed, EPA is very quickly elongated and converted to DHA by Δ6-desaturase enzymes, which contributes to why levels of DHA are consistently higher than ALA or EPA.

This study also investigated the role of flaxseed as a dietary fiber, which can be fermented in the gut to produce SCFA, specifically butyric acid. The flaxseed-supplemented diet exhibited a slight increase in butyric acid, which has been found to act as an HDAC inhibitor, acting on various genes responsible for improving cellular immunity, protecting against oxidative stress, regulating mitochondrial activity, and protecting the body from various diseases. The decreased level of SCFA observed in ovarian tumors as compared to healthy ovaries indicates impaired enteric production of SCFA (from dietary fibers) and/or enhanced oxidation of SCFA in ovarian tumors. This would reduce the effect of SCFA as HDAC inhibitors, transcriptional modulators, and anti-inflammatory molecules, in tumors versus healthy tissues. To further investigate the reduction of SCFA seemingly to be caused by ovarian tumors, butyric acid was also measure in the ceca where it is produced. The effects of diet and disease on the levels of butyric acid in the ceca was consistent with the effects observed in the ovaries- the birds fed a whole-flax diet had slightly higher levels of butyric acid in the ceca compared to the control group, and there was a significant decrease in butyric acid in birds with advanced stage ovarian cancer. This suggests that the cancer does not necessarily decrease the fermentation of SCFA or the activity of the gut microbiota, but instead, might increase the oxidation of SCFA in ovarian cancer cells because SCFA can readily diffuse across the mitochondrial membrane, in contrast to longer chain fatty acids, to be implemented as an energy source. If cancer cells are more quickly using up the stores of SCFA, this would

contribute to why the significant decrease in butyric acid is observed in both the ceca and ovaries collected from hens with ovarian cancer. Due to the significant increase in butyric acid levels measured in the ovaries compared to the ceca, it is concluded that the SCFA do not remain in the ceca for very long following fermentation, and are quickly transported to the liver to be stored and/or transported to the target organ.

Overall, this study suggests that dietary flaxseed could upregulate the conversion of ALA to DHA in ovarian cancer through an increase of OM3 PUFA over OM6. In addition, this increase in OM3:OM6 PUFA ratio contributes to a decrease in AA, and as a result, a slight decrease in PGE2. Through fermentation in the gut, the dietary fibers of flaxseed slightly increase the levels of butyric acid in the ovaries. However, these levels are significantly reduced in ovarian cancer, suggesting that SCFA are used as an energy source for ovarian cancer cells. These observations in OM3 PUFA and SCFA in the ovaries produced as a result of a flaxseed supplemented diet could provide insight into two possible mechanisms in which dietary flaxseed regulates metabolic activity and gene expression to prevent or reduce the severity of ovarian cancer.

VII. Future Directions

Future studies include further exploration into the role of SCFA by measuring their concentrations in the liver to acquire a better understanding of SCFA storage and movement within the body and increasing the sample size of the experiments already performed in order to obtain the most accurate results. Another future aim would include investigating the specific effects of flaxseed on the desaturase and elongase enzyme activity.

VII. Acknowledgements

IX. References

Bishehsari, F., et al. (2018). "Dietary Fiber Treatment Corrects the Composition of Gut Microbiota, Promotes SCFA Production, and Suppresses Colon Carcinogenesis." <u>Genes (Basel)</u> **9**(2).

Chen, Y., et al. (2019). "Positive acceleration adaptive training attenuates gastric ischemia-reperfusion injury through COX-2 and PGE2 expression." <u>Exp Ther Med</u> **17**(4): 2901-2906.

Chriett, S., et al. (2019). "Prominent action of butyrate over beta-hydroxybutyrate as histone deacetylase inhibitor, transcriptional modulator and anti-inflammatory molecule." <u>Sci Rep</u> **9**(1): 742.

Eilati, E., et al. (2013). "Long term consumption of flaxseed enriched diet decreased ovarian cancer incidence and prostaglandin E(2)in hens." <u>Gynecol Oncol</u> **130**(3): 620-628.

"Ovarian Cancer Statistics | How Common Is Ovarian Cancer." *American Cancer Society*, 2019, www.cancer.org/cancer/ovarian-cancer/about/key-statistics.html.

Licastro, F., et al. (2005). "Innate immunity and inflammation in ageing: a key for understanding age-related diseases." <u>Immun Ageing</u> **2**: 8.

Swanson, D., et al. (2012). "Omega-3 fatty acids EPA and DHA: health benefits throughout life." <u>Adv Nutr</u> **3**(1): 1-7.