5-2020

Developmental Regulation of Sirtuin Genes in Broiler Chicks

Heather L. Winter
hwinter3@vols.utk.edu

Developmental Regulation of Sirtuin Genes in Broiler Chicks

Heather Leigh Winter

Chancellor's Honors Program Thesis Project

Faculty Advisors: Dr. Brynn Voy PhD , Dr. John Stier PhD

University of Tennessee Knoxville

May 2020

Acknowledgements

I would like to acknowledge the support of my family, friends, and mentors that have made the completion of this project possible and have loved and challenged me along the way. I would like to thank my family for their love, guidance, support, and the countless number of late-night cups of tea that were brought to my room as I completed this project from home. To Dr. Voy, who agreed in 2015 to allow a curious high school student into her lab to get hands on experience in animal nutritional physiology and has miraculously put up with this student over the past five years, I cannot thank you enough for everything you have taught me about your field, researching, being a leader, and balancing in life. I want to thank Suchita Das whom I had the honor to learn under in the laboratory and who's enduring willingness to teach, and unending patience has guided me throughout college. I would also like to recognize the graduate students and friends that I have been able to work alongside and learn from. Thank you to Robert Mihelic, Kamille Piacquadio, Josh Powers, and Minjeong Kim for all that you have done to teach me and make my experiences researching so memorable and fun. Thank you to the family that I have found in the Department of Animal Science at the University of Tennessee, to my fellow classmates, and to Dr. Stier for his guidance and for keeping me on track throughout the writing process.

Abstract

A family of enzymes known as sirtuins have related, yet unique metabolic functions and have been associated primarily with longevity and aging processes (Lombard et al., 2007). Evidence suggests that this family of enzymes including *SIRT2, SIRT4, SIRT5, SIRT6,* and *SIRT7* is highly conserved across species (Greiss & Gartner, 2009).Despite wide study in species of yeast, flies, and mice, much less literature is available regarding these aging-related proteins in poultry. The objectives of this study are three-fold. The first objective is to examine the expression of *SIRT2, 4, 5, 6,* and *7* in subcutaneous adipose tissue of developing broiler chicks at five age points, two pre-hatch and three post-hatch ages. The adipose tissue of embryonic ages was sampled at embryonic days 13, 15, and 17, and at days 7 and 14 after the chicks hatched. The second objective is to determine if and how expression of these six genes vary across the following three tissue types: subcutaneous adipose, liver, and abdominal adipose in post hatch chicks, at the same 7 and 14-day time points. Finally, this study will determine if there were any significant correlations between elevated gene expression and abundance of specific fatty acids subcutaneous fat samples. It was expected that expression levels would differ greatly from embryonic ages to post hatch time points, however, our data show that such expression changes were unique to each of sirtuin genes. Data collection for this study took place as part of a larger study under direction of the Voy Laboratory.

Keywords: sirtuins, poultry metabolism, poultry development, gene expression, fatty acid analysis

Introduction

As demand for high quality protein is ever growing, there is an increasing need to optimize efficient and healthy conversion of feed in broiler chicks. This can be achieved by gaining a better understanding of the underlying metabolic processes that must take places to support a growing chick. In particular, a better understanding of the enzymes that catalyze metabolic reactions, including their substrates, products, location of activity, and abundance as measured by gene expression, is needed to determine how to most efficiently feed the developing birds.

A family of enzymes known as the sirtuin enzymes have related, yet unique metabolic functions and have been associated primarily with longevity and aging processes (Lombard et al., 2007). The family of enzymes includes *SIRT1, SIRT2, SIRT4, SIRT5, SIRT6,* and *SIRT7* and evidence suggests that the enzyme family is highly conserved across species (Greiss & Gartner, 2009). While this group of enzymes has been widely studied since its initial discovery in the 1970s, in species of yeast, flies, and mice, much less literature is available regarding these aging-related proteins in poultry. Sirtuin enzymes are nicotinamide adenine dinucleotide (NAD) dependent protein deacetylases and their primary function (North & Verdin, 2004) is to remove the acetyl group from acetylated peptides. They are unique from other deacetylases because they absolutely require NAD to function (Landry et al., 2000). This dependence on NAD links these age-related enzymes to metabolic function. NAD is an essential cofactor of cellular redox reactions. The fact that the sirtuin enzyme family relies on NAD indicates the enzymes may play an important metabolic role and further implies that sirtuin function depends on the metabolic state of the organisms in which it functions (Nakagawa & Guarente, 2011). This requirement might also suggest that metabolic activity is a principle regulator of the sirtuin enzymes. Studies by Nakagawa and others have in fact

shown that NAD regulates *SIRT3, 4,* and *5* in periods of nutrient stress. In addition to their role in metabolism, some of the sirtuin enzymes are linked to antioxidant and oxidative stress related processes like DNA repair and metabolic function (Singh et al., 2018).

The sirtuin family of enzymes play a role in aging as was shown with *Saccharomyces cerevisiae* yeast, but do so in various ways that will be further discussed (North & Verdin, 2004). In human cells and in yeast, the *SIRT2* protein is found in the cytoplasm and acts on α-Tubulin, causing a change to the cellular motility or to the ability of the cell to divide. This impact on division acts as a controller of the cell cycle (North & Verdin, 2004). Recent evidence indicates that *SIRT2* may function as a tumor suppressor gene because it was frequently deleted in instances of human glioma brain tumors (Hiratsuka et al., 2003).

SIRT3 on the other hand is located in the mitochondria, indicating that it may function in mitochondrial metabolism. Studies in mice have shown that the *SIRT3* gene is upregulated during periods of fasting and that mice who lack the *SIRT3* gene altogether exhibit hyperacetylation and consequent disorders. The signs associated with hyperacetylation include an intolerance to cold and lower overall ATP levels (Hiratsuka et al., 2003). Studies have indicated that increased *SIRT3* activity allows for further fatty acid oxidation of long chain fatty acids into their smaller components.

SIRT4 and *SIRT5*, like *SIRT3* are also localized to the mitochondria. *SIRT4* exhibits ADP-ribosyl transferase activity and functions to restrict another enzyme known as glutamate dehydrogenase (GDH). This restriction results in a limited use of amino acids in gluconeogenesis. It is also thought that during periods of fasting this restriction is alleviated and *SIRT4* regulates insulin secretion (Nakagawa, Lomb, Haigis, & Guarente, 2009). Much less is known about the exact function of *SIRT5* (Lombard et al., 2007). A study by Nakagawa and others suggests a very specific role of *SIRT5* in the initial step of the urea

cycle. By triggering the activity of the enzyme *CPS1*, *SIRT5* may help jump start the process of ammonia detoxification.

SIRT6 and *SIRT7* are associated with DNA repair when the genetic material is damaged as a result of oxidative stress and are thus centralized in the cell nucleus (Ren et al., 2017). *SIRT6*, in particular, is also associated with key metabolic functions. Mice lacking *SIRT6* are hypoglycemic, have low rates of β-oxidation, and accumulate fat in the liver (Nakagawa & Guarente, 2011). *SIRT7* is understood to play a role in the regulation of transcription. It has been shown to act as a positive regulator of RNA polymerase I and in studies using mice was found to be abundant in liver tissue compared to skeletal muscle and other tissues. In its absence, using *SIRT7*-knockout mice, it was observed that the mice underwent symptoms of premature aging as also seen in *SIRT6*-knockout mice. (Ford et al., 2006).

The sirtuin family of genes has been shown to be upregulated with sexual maturity in laying hens from 10 weeks of age to 35 weeks of age, particularly in liver tissue (Ren et al., 2017). However, there is still little available literature regarding the expression of sirtuin genes in broiler chickens. A better understanding of this metabolically pervasive family of enzymes is needed to gauge the demands of the rapidly developing broiler chick.

The objectives of this study are three-fold. The first objective is to examine the expression of *SIRT2, 4, 5, 6,* and *7* in subcutaneous adipose tissue of developing broiler chicks at five different age points, three pre-hatch or embryonic ages and two post-hatch ages. The adipose tissue of chicks still *in ovo* was sampled at embryonic days 13, 15, and 17, and at days 7 and 14 after the chicks hatched. The second objective is to determine if and how expression of these six genes vary across four different tissue types. The types of tissue examined are subcutaneous, abdominal, and crop adipose tissue, and liver in post hatch chicks, at the same 7 and 14-day time points. The final objective of the study is to determine

if there are any significant correlations between elevated gene expression and accumulation of short chain fatty acid compounds in the subcutaneous fat samples. Fatty acids will be measured via gas chromatography-mass spectrometry and will be reported as a percent of total fatty acids measured. The overarching goal of this study is to provide preliminary data as to baseline levels of the expression of sirtuin genes across various ages and tissue type in developing broiler chicks. It is expected that post hatch expression levels differ greatly from embryonic gene expression due to the major metabolic changes the chicks undergo upon hatch.

Materials and methods

Tissue sample collection

A commercial hatchery in Chattanooga, Tennessee supplied the fertilized eggs for this study. The fertilized eggs underwent standard incubation procedures at the University of Tennessee under the regulations of the University of Tennessee Animal Care and Use Committee. The samples for the study were collected at various time points to gauge variation in the embryo and hatchling development. Prior to hatch, embryos were sampled at days 13, 15, and 17, post-fertilization. These time points are further noted as E13, E15, and E17. From these embryos, subcutaneous adipose tissue was sampled. The remaining embryos were incubated until hatch and housed in standard conditions at the University of Tennessee. Post-hatch, chicks were fed a standard diet until sampling. Chicks were euthanized via carbon dioxide asphyxiation at either seven or fourteen days after hatch and will further be noted as 7d and 14d. The tissue samples collected from the 7d and 14d chicks included abdominal adipose, subcutaneous adipose from the thigh region, crop adipose from the neck region, and

liver tissue. All tissues were snap frozen in liquid nitrogen and stored at -80ºC for further processing (Mihelic et al., 2019).

RNA Isolation

RNA was isolated from the samples using Invitrogen™ TRIzol reagent (Invitrogen; Carlbad, CA) with approximately 50-100mg of tissue, depending on tissue type. Mechanical lysis of tissue was achieved using a polytron homogenizer and the isolation was carried out in accordance with the manufacturer's TRIzol isolation protocol. RNA quantitation was done using an Amersham Ultra Spec 1300 Pro Spectrophotometer at an optical density of 260nm. Gel electrophoresis on a 1% agarose non-denaturing gel was used to check the integrity of the isolated RNA. For downstream applications, cDNA was prepared from approximately 500ng of RNA template using Bio-Rad iScript cDNA synthesis kit (Bio-Rad Laboratories; Hercules, CA) in accordance with the Voy lab protocol (Mihelic et al., 2019).

Targeted RNA sequencing

The expression levels of the sirtuin genes were quantitated using a targeted RNA sequencing methodology. Gene primers were chosen to guarantee amplicons that spread from exon to exon and are approximately 90 to 100 base pairs in length. The Ensembl Genome Browser (Ensembl.org) was used to model the *Gallus gallus* genome (Gal gal V5.0) and served as the model from which the gene sequences for the five sirtuin genes of interest were taken. The five sirtuin genes profiled and their chromosomal locations are as follows: sirtuin 2 (*SIRT2*; Chr 32), sirtuin 4 (*SIRT4;* Chr 15), sirtuin 5 (*SIRT5*; Chr 2), sirtuin 6 (*SIRT6*; Chr 28), and sirtuin 7 (*SIRT7*; Chr 18). NCBI Primer Blast https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was utilized to design the primers for the stated genes, ensuring that the amplicons produced are between 90 and 100 base pairs. The

primer pairs were validated using PCR amplification of cDNA from a control poultry adipose sample.

In order to normalize the samples, housekeeping genes were included in the targeted RNA sequencing. These housekeeping genes included actin beta (*ACTB*), glucuronidase beta (*GUSB*), hypoxanthine guanine phosphoribosyl transferase (*HPRT*), ornithine decarboxylase antizyme 1 (*OAZ1*), peptidylprolyl isomerase A (*PPIA*), TATA-box binding protein (*TBP*), and tyrosine 3-onooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ*). Primers for the housekeeping genes were designed in the same way the gene of interest primers were designed. Of these genes, *ACTB*, *TBP*, and *YWHAZ*, were chosen to normalize the data due to their consistency across sample groups.

Using the cDNA prepared from the collected samples and the primer pairs, amplicons were produced using an optimized Hi-plex approach (MonsterPlex Technology; floodlightgenomics.com). The amplicons were then sequenced and mapped the *Gallus gallus* genome with CLC Genomic Workbench (Qiagen.com; Germantown, MD), with samples run in duplicates. The raw reads were then normalized using the mean of the three housekeeping genes mentioned above (*ACTB*, *TBP*, and *YWHAZ*) and the mean of the duplicates was taken (Mihelic et al., 2019).

Fatty Acid Analysis

A modified Folch method was used to extract fatty acids from the adipose tissue samples. To do so, the adipose samples were pulverized in a 2:1 methanol chloroform solution and pulverized for 2 minutes with a Qiagen TissueLyser set to 25hz. Following pulverization, the samples underwent a 15-minute sonication. Then 200µl of a 1:1 chloroform water solution was added to each sample to re-extract the fatty acids. The organic phase was separated and dried under a stream of nitrogen gas to reduce oxidation of the fatty acids.

Upon drying, the fatty acids were resuspended in 750µl of a 1:1 methanol chloroform solution (Roberts, West, Vidal-Puig, & Griffin, 2014).

The acid-catalyzed esterification procedure to produce the desired fatty acid methyl esters (FAMEs) was carried out by adding 125µl BF_3 methanol. The solution was incubated at 90°C for 90 minutes to allow the reaction to proceed to completion. Following incubation and allowing cooling of the samples, 150µl water and 300µl hexane were added. This was followed by vortexing for one minute and centrifuging for one minute at 4°C. The organic phase was then dried under a stream of nitrogen gas resuspended in 100µl analytic grade hexane. The samples were then processed in duplicates by gas chromatography-mass spectrometry analysis in the University of Tennessee Department of Chemistry.

Data processing was completed using Maven (Metabolomic Analysis and Visualization Engine) using the retention time and mass to charge ratio calculated from chemical formulas of 25 known FAMEs (Clasquin, Melamud, & Rabinowitz, 2012). An average of blank samples was used to correct the data and an average of the corrected duplicates was taken for statistical analysis (Mihelic et al., 2019). Values are reported as relative abundance and FAMEs are reported with fatty acid nomenclature.

Data Analysis

Analysis of the gene expression data was carried out using both Microsoft Excel and R Studio. After normalizing the raw counts from the targeted RNA sequencing, ANOVA tests were run in R Studio to determine statistical significance. Then, gene expression was compared across the developmental time points using means for each time point in Microsoft Excel. The same method was used again to examine variation in expression across tissue type in both 7d and 14d chicks. MetaboAnalyst was used to determine correlation between gene expression and fatty acid abundance (Chong, Wishart, & Xia, 2019).

Results

Objective 1: Effect of Age in Subcutaneous Adipose Tissue

The first objective of the study was to examine the developmental changes in expression of the sirtuin genes in the subcutaneous adipose tissue of broiler chicks. The variation in expression across the different time points was significant for all the sirtuin genes with the exception of *SIRT2*, using the p-value criteria of being less than or equal to 0.05. This was determined by ANOVA test in R Studio (*Table 1*.).

Expression levels of SIRT4 remain at a somewhat constant level across the three embryo sampling times, E17, E15, and E13 (*Figure 1*.). However, at day 7 post hatch an increase by approximately 31.5% is observed in the number of sequenced reads (*Table 2*.). This was followed at the 14-day time point by a decrease in expression of approximately 14.4%. Although the expression of *SIRT2* was not found to be significant across age group, the observation is made that like *SIRT4*, its highest expression level was seen at the first sampling post hatch, 7d.

SIRT5 localized in the mitochondria and *SIRT6* in the nucleus, both exhibited similar expression patterns across the age groups. The time of highest expression were E13 and E15 for *SIRT6* and *SIRT5*, respectively. These highest points were followed by large decreases in expression upon hatch, as observed at the 7d and 14d timepoints. It is noted, however, that the expression level of *SIRT6* at day 7, is extremely low in reference to the other days and this may in fact warrant further investigation. *SIRT5* and *SIRT6* are by large the most highly expressed sirtuins all time points in subcutaneous adipose tissue.

SIRT7 was the least abundantly expressed of the five genes measured. It peaked at embryonic day 15, followed by a decrease at E17. After hatch, however, the expression levels increase by approximately 94% followed by a major decrease to below pre-hatch levels.

Objective 2: Effect of Tissue Type for 7d and 14d Time Points

The goal of the second objective was to examine at the variation in expression across different tissue types at a singular time point. This was done for both post hatch time samples, 7d and 14d independently. The variation in expression across the four tissue types was significant for all the sirtuin genes, with the exception of *SIRT4*, that had a p-value of 0.59, thus violating the less than or equal to 0.05 criteria.

At day 7, *SIRT2* expression was on average, greatest in subcutaneous adipose, followed by abdominal fat, crop fat, and finally liver tissue. Day 14 expression levels of *SIRT2* varied extremely minimally across tissue type. During the second week of post-hatch development, tissue type seems to have very little correlation with the expression of *SIRT2*. It can also be noted that expression of SIRT2 in the crop fat and liver changed very minimally from day 7 to day 14. However, for both abdominal fat and subcutaneous fat, expression decreased significantly from day 7 to day 14 (*Figure 2*.). *SIRT2* had very low expression levels across liver, crop, and abdominal adipose tissues in comparison to *SIRT4*, 5, and 6.

In general, the expression levels of *SIRT4* are greater than that of *SIRT2*, however because the relationship between gene expression and tissue was deemed not statistically significant, little may be extracted from the visual distribution shown in *Figure 2*.

The liver tissue exhibited a significantly higher expression of *SIRT5* than any of the other tissues at both the 7d and 14d time points. Little variation is observed between *SIRT5* expression in the liver from 7d to 14d. 7d samples crop fat exhibited the next highest expression of *SIRT5* after liver, followed by abdominal fat, then subcutaneous fat. Day 14

samples differed slightly in that subcutaneous fat exhibited greater expression of the mitochondrial sirtuin than both crop and abdominal fat.

SIRT6 expression in 7d chicks is greatest in crop fat, followed by liver, then abdominal fat. The uncharacteristically low expression of SIRT6 in subcutaneous fat of 7d chick raises the question of a possible error in data for this metric. The 14d samples have SIRT6 expressed the greatest in abdominal fat and subcutaneous fat. A lesser expression of this nucleus-localized sirtuin is observed in crop fat and liver tissue.

SIRT7, which was noted in our temporal analysis of the first objective to exhibit the lowest expression levels in subcutaneous adipose, is also the lowest expressed of the five sirtuin genes in both crop and abdominal adipose. Development seems to have a correlation with *SIRT7* expression in abdominal fat and subcutaneous fat. However, for both crop adipose and liver tissue, little variation is detected between 7d and 14d sample averages.

Objective 3: Correlations of fatty acid abundance

The final object of this study was to determine if there are any significant correlations between elevated gene expression and fatty acid abundance in subcutaneous adipose tissue. Based on the results from the first objective, the analysis began by looking at correlates of SIRT5 and SIRT6, because they are the most abundantly expressed.

A moderately-strong positive correlation is seen between *SIRT5* and the following fatty acids, heptadecanoate (C17:0), pentadecanoate (C15:0), and myristate (C14:0) (*Figure 4.)* across all time points (E13-14d). Additionally, a moderate positive correlation is observed between *SIRT5* and the following long chain fatty acids, erucate (C22:1 n-9), nervonate (C24:1 n-9), and stearate (C18:0) (*Figure 4.)* It was interesting to note also the fatty acids with which *SIRT5* had a strong negative correlation. The following fatty acids were observed to have a correlation with *SIRT5* of less than -0.70, linoleate (C18:2 n-6), oleate (C18:1 n-9),

γ-linolenate (C18:3 n-6), palmitoleate (C16:1 n-9), linolenate (C18:3 n-3), cis-11-eicosenoate (C20:1 n-9), and cis-11-14-eicosadienoate (C20:2 n-6). *SIRT6* has moderate to weak positive correlations with stearate, erucate, arachidonate (C20:4 n-6), and cis-4-7-10-13-16-19-docosahexaenoate (C22:6 n-3), more commonly known as DHA.

SIRT7 and SIRT2 were observed to be highly positively correlated with one another and additionally positively correlated with some of the same fatty acids. SIRT7 was observed to have a moderate to weak correlation with palmitate (C16:0), heptadecanoate, pentadecanoate, and myristate. SIRT2 was found to have a moderate to weak correlation with heptadecanoate, pentadecanoate and myristate. SIRT4 was moderate to weakly-positively correlated with lignocerate (C24:0), cis-11-14-eicosadienoate, and cis-5-8-11-14-17-eicosapentaenoate (C20:5 n-3), more often referred to as simply EPA.

Discussion

Sirtuins, both the enzymes and the corresponding sirtuin genes that encode them, are commonly related to aging processes and in the last 20 years a great deal of research has yielded vast amounts of information about how and where these enzymes function and how the genes that encode them are regulated. Understanding how these enzymes change with age is crucial to understanding the metabolic roles that they play.

This family of metabolically diverse genes has been studied in reproductive age broiler and laying hens (Ren et al., 2017), however, less is known about expression levels in embryonic and early post hatch broiler chicks. Additionally, *SIRT1* and *SIRT3* remain the most widely studied of the seven sirtuin genes comprised in the *Gallus gallus* genome,

therefor this study focuses on *SIRT2*, *4*, *5*, *6*, and *7*, of which less information is currently available for broiler chicks.

The relatively constant expression levels of *SIRT4* across time points in subcutaneous adipose suggests there may be very little developmental regulation of this sirtuin. *SIRT4*, which is thought to regulate β-oxidation and insulin secretion in the liver did increase slightly at 7d in subcutaneous adipose (*Figure 1.*). Despite the relative consistency, this may illustrate the dietary changes that occur when the chick is first exposed to feed after hatch, and no longer relies on the yolk as a source of food. The expression of *SIRT2* was not found to be significant across age group so it will be omitted from further discussion.

SIRT5 located in the mitochondria and *SIRT6* located in the nucleus, were the most highly expressed sirtuins in subcutaneous adipose across the timepoints. Curiously, both genes exhibited highest expression levels in embryo, particularly at E13 and E15 followed by significant drops in expression after hatch. The role of *SIRT5* in the initiation of the urea cycle in rodents described by Nakagawa and others may offer some insight as to this observation in chick embryos which produce uric acid that must be absorbed in the allantois while *in ovo* (Nakagawa et al., 2009). It appears, from the data, that both *SIRT5* and *SIRT6* are downregulated upon hatch. More data may be necessary to confirm the validity of this observation. After hatch, the expression increases from 7d to 14d and it would be useful to continue this study to determine if *SIRT5* and *SIRT6* expression levels continue to increase to the highest prehatch levels.

SIRT6 and *SIRT7* alike play a role in DNA repair and are accordingly located in the nucleus (Ren et al., 2017). Despite expectations, these two sirtuins exhibited very unique expression patterns across the time window. *SIRT7* which along with *SIRT2* was the lowest expressed sirtuin genes did not appear to have a clear developmental pattern. With expression

peaks occurring at E13-15 and 7d it is difficult to make any conclusions about its developmental regulation.

Sirtuins have been widely studied in liver tissue due to their known importance in metabolic roles, β-oxidation and insulin release for example. With objective 2, this study sought to provide preliminary data regarding the expression across three adipose deposits (subcutaneous, abdominal, and crop) in addition to liver tissue. *SIRT5* was the only measured gene that had significantly higher expression in liver tissue compared to the other adipose tissues at both 7d and 14d. Because poultry do not possess the mitochondrial-dense brown adipose tissue, one might expect that the mitochondrial sirtuins would have higher expression in liver cells. While this was observed with SIRT5, the expression of SIRT4 did not exhibit this same phenomena. The expression of SIRT4 was greatest in crop adipose at 14d, followed by liver, abdominal fat and subcutaneous fat. At the 7-day time point, expression was greatest in abdominal fat, followed by liver, then subcutaneous fat, and finally crop fat.

At the 7d time point, the highest expression of *SIRT2* in both subcutaneous and abdominal fat. Studies have shown SIRT2 expression to be the highest expressed sirtuin in adipose tissue in mammals (Jing, Gesta, & Kahn, 2008). Our data suggests that in developing broiler chicks, it is one of the lower expressed sirtuins in the three measure adipose tissues. While *SIRT2* was expressed differentially across tissue types at 7d, the levels normalized at day 14 and little to no variation was seen across tissue type. This suggests that the role of *SIRT2*, possibly in cell division, is heightened immediately post hatch (within 7 days) but is reduced in the second week of life.

Much like *SIRT2*, *SIRT7* at 7d was most highly expressed in abdominal and subcutaneous fat and such heightened expression is dramatically reduced by day 14. The role of *SIRT7* as a transcription regulator offers less of an explanation than did the function of *SIRT2* for this trend. However, one possible explanation my lie in physiological changes

occurring within the adipose tissue at this two-week period. By visualization, *SIRT6* expression did not seem to vary dramatically by tissue type despite its statistical significance.

SIRT4 has been shown to downregulate beta-oxidation of fatty acids (Nasrin et al., 2010). Therefor one might expect to see that as *SIRT4* expression increases, the prevalence of shorter chain fatty acids produced as a result of beta-oxidation catabolism would decrease. Our data did not present any strong correlations, either positive or negative, between the expression of this mitochondrial sirtuin and the fatty acids measured. *SIRT4* was however moderately to weakly-positively correlated with long chain fatty acids such as lignocerate (C24:0) and EPA (C20:5 n-3). This correlation may support the claim that with increased *SIRT4* expression long chain fatty acids may be more prevalent and short chain fatty less abundant. It is possible that *SIRT4* may regulate oxidation of fatty acids outside the scope of this examination. *SIRT2*, *5*, and *7* were found to positively correlate with some of the same saturated fatty acids including palmitate (C16:0), heptadecanoate (C17:0), pentadecanoate (C15:0), and myristate (C14:0). Given the know variation in the metabolic roles and locations of actions of these 3 sirtuins the overlap between fatty acids correlates is interesting. *SIRT2* which is known to impact cellular division is found in the cytoplasm, *SIRT5* possibly involved in the initial steps of the urea cycle is in the mitochondria, and *SIRT7* involved in DNA repair, is located in the nucleus.

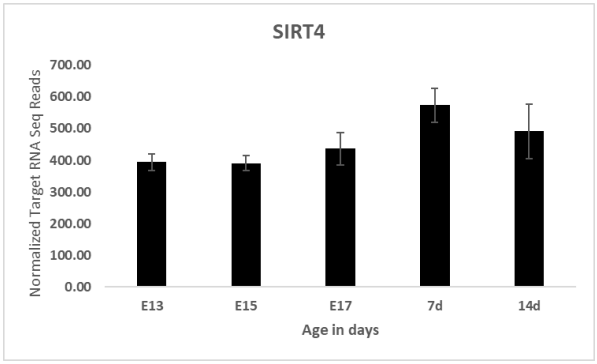
This study might be expanded to look at the interactions between the sirtuins genes and other metabolic genes as the broiler chicks hatch and begin rapid early development. Looking at correlated genes would provide more insight into the overall goal of better understanding embryonic and early post-hatch development. From this point, the next step would be to examine the changes in expression of the sirtuin genes and fatty acid abundance in different dietary conditions, for example fasting. The data and information provided here

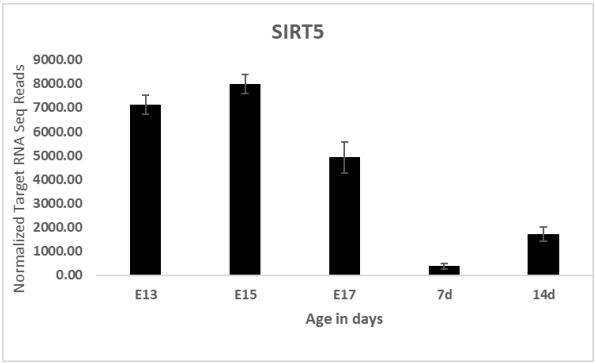
might serve as a baseline for which to compare another study focusing on the effects of diet in the developing birds.

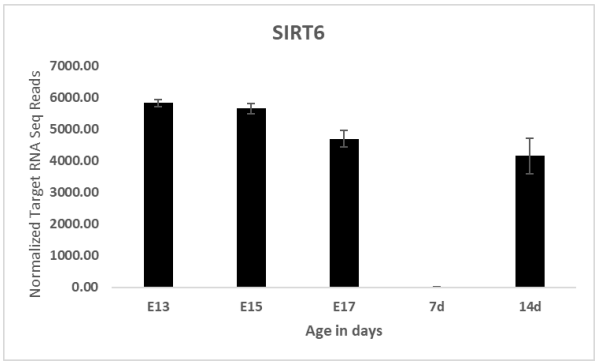
Conclusion

This three-objective study set out to examine the effects of age (1) and tissue type (2) on the expression of five sirtuin genes in developing broiler chicks while also examining correlations between fatty acids and the upregulation of these five genes (3). The overarching goal is to provide baseline data levels of the expression of sirtuin family of genes across various ages and tissue type in developing broiler chicks. While it was expected that post hatch expression levels would differ greatly from embryonic gene expression due to major metabolic changes upon hatch our data show that such expression changes were unique to each of the five measured sirtuin genes.

Figure 1. Gene expression of SIRT2, 4, 5, 6, and 7 showing developmental variation from time point E17 to post hatch day 14 in subcutaneous adipose tissue of the broiler chicks. Averages were taken for the different time points and plotted in excel and standard error for each time point were calculated. The error bars show the standard error with sample sizes ranging from 10 to 15 observations.







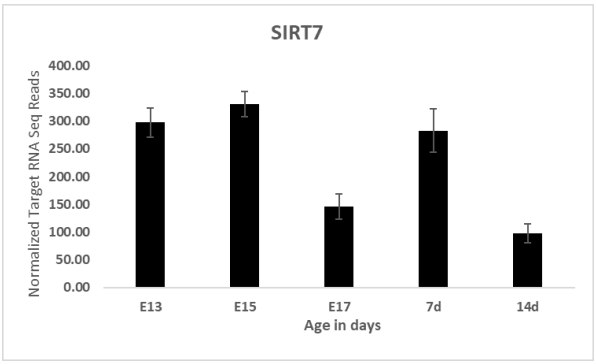


Table 1. Analysis of variance results from model predicting gene expression of 5 sirtuin genes from age group (E13, E15, E17, 7d, and 14d) in subcutaneous adipose tissue. Statistical significance denoted by * for p-values less than or equal to 0.05. ANOVA test run using R Studio.

gene	*p-value*
SIRT2	0.716
SIRT4	0.01289*
SIRT5	9.44E-14*
SIRT6	6.52E-08*
SIRT7	0.0388*

Table 2. Normalized gene expression data of SIRT2, 4, 5, 6, and 7 at time points E13, E15, E17, 7d, and 14d in subcutaneous adipose tissue of the broiler chicks. Standard error for each time point were calculated from sample sizes ranging from 10 to 15 observations.

SIRT2			**SIRT4**			**SIRT5**		
Age	**Mean**	**Standard Error**	**Age**	**Mean**	**Standard Error**	**Age**	**Mean**	**Standard Error**
E13	429.26	35.99	**E13**	394.57	25.98	**E13**	7123.82	396.69
E15	433.84	27.38	**E15**	391.13	24.52	**E15**	8001.77	394.68
E17	239.11	30.86	**E17**	436.10	51.38	**E17**	4921.35	644.38
7d	740.11	231.64	**7d**	573.34	54.63	**7d**	378.24	117.42
14d	128.06	21.72	**14d**	490.98	85.55	**14d**	1731.47	300.21

SIRT6			**SIRT7**		
Age	**Mean**	**Standard Error**	**Age**	**Mean**	**Standard Error**
E13	5832.62	105.10	**E13**	297.55	26.99
E15	5663.22	167.44	**E15**	331.20	22.71
E17	4701.67	264.55	**E17**	146.05	22.56
7d	28.57	5.67	**7d**	282.90	39.15
14d	4161.67	569.04	**14d**	97.28	16.94

Figure 2. Expression of SIRT2, 4, 5, 6, and 7 genes across four tissue types for 7d and 14d chicks. Averages were taken for the different tissue types, plotted in excel and shown with standard error bars for each tissue type and time point. Note the scale of gene expression varies for each bar chart.

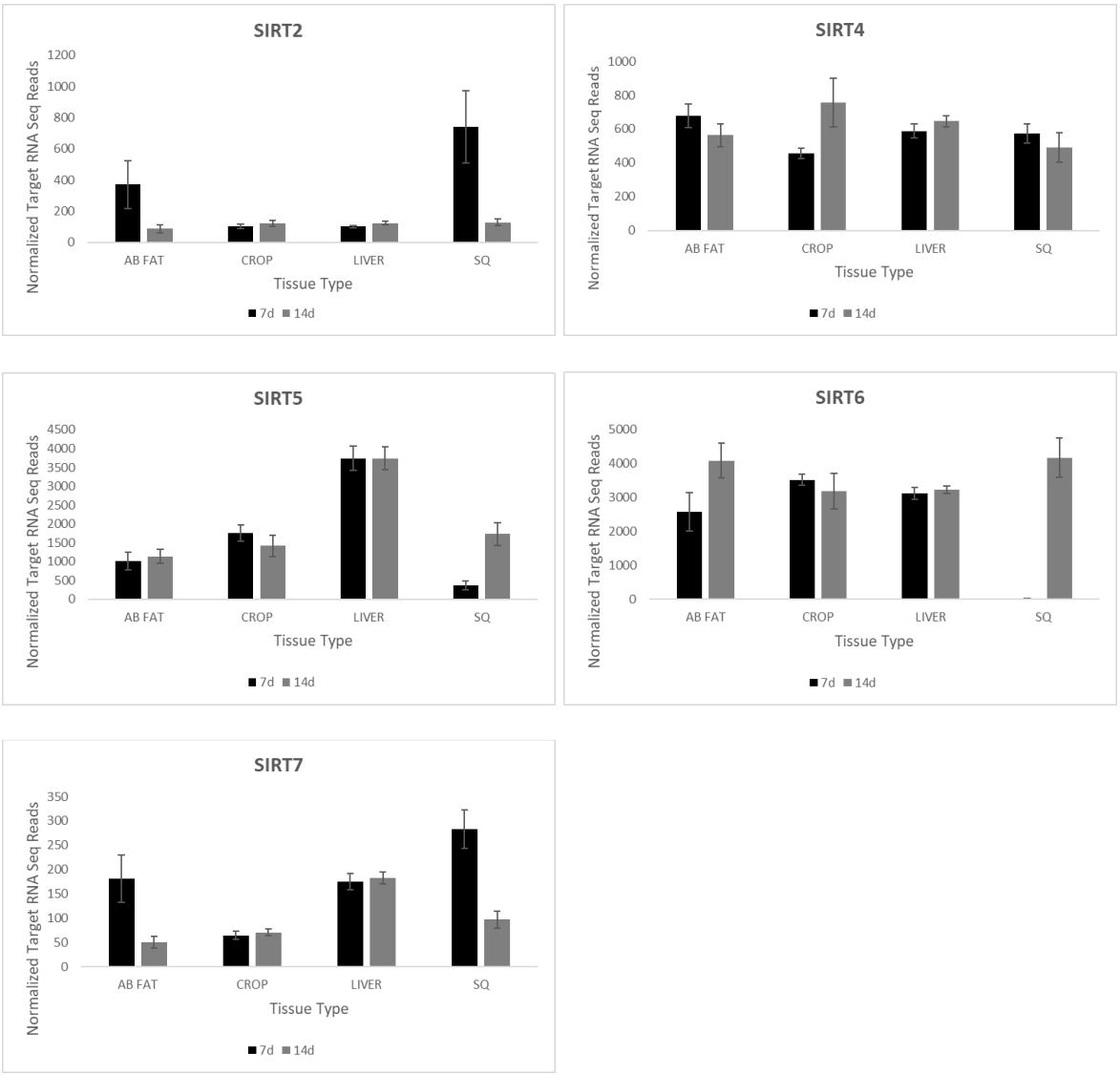
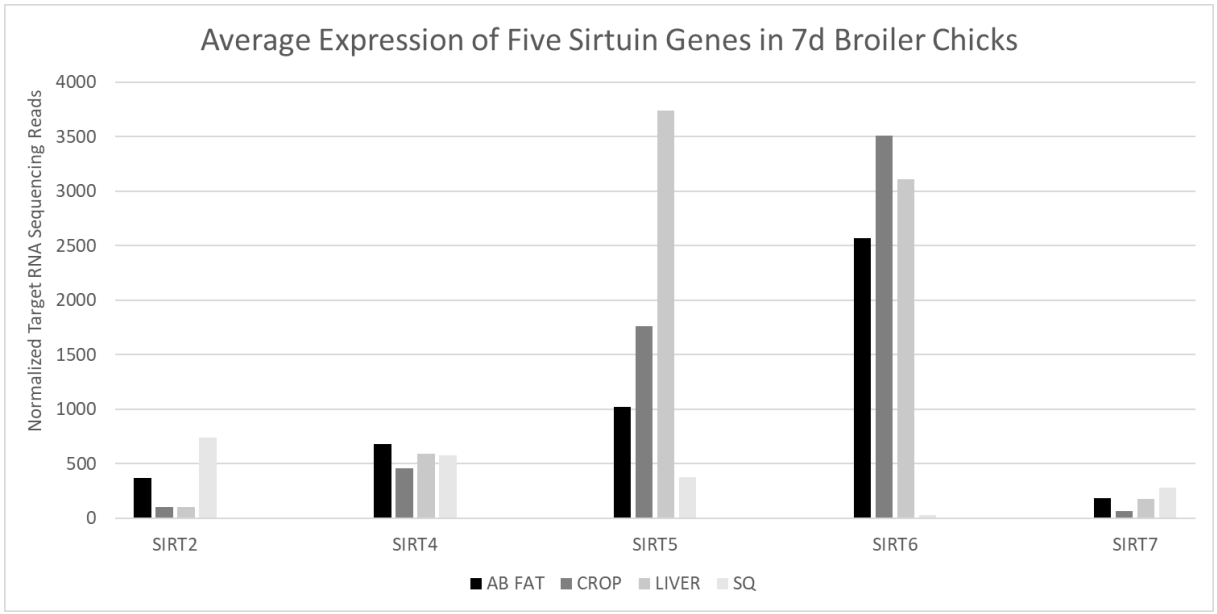


Figure 3. Expression of five sirtuin genes across four tissue types, abdominal fat, crop fat, liver, and subcutaneous fat. Data shown separately by day of sampling, either 7d or 14d. Another visualization of the data presented in Figure 2.



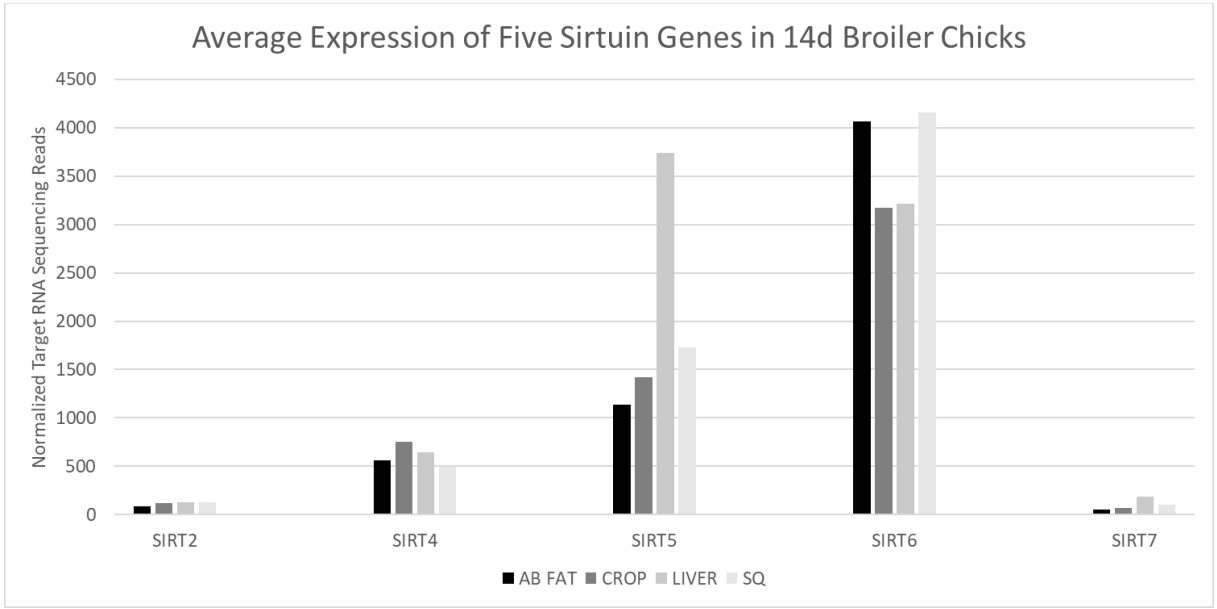


Table 3. Normalized gene expression data of SIRT2, 4, 5, 6, and 7 at time points 7d and 14d in four tissue types, abdominal fat (AB FAT), crop fat (CROP), liver (LIVER), and subcutaneous fat (SQ) of the broiler chicks. Standard errors for each time point and tissue type were calculated and are reported below as 7d SE and 14d SE.

SIRT2

Tissue Type	7d	7d SE	14d	14d SE
AB FAT	370.32	154.87	87.03	25.24
CROP	102.87	15.48	120.11	19.39
LIVER	102.12	7.84	123.88	9.88
SQ	740.11	231.64	128.06	21.72

SIRT4

Tissue Type	7d	7d SE	14d	14d SE
AB FAT	677.32	68.79	563.32	67.31
CROP	454.96	30.38	755.74	143.75
LIVER	588.37	42.03	646.03	32.16
SQ	573.34	54.63	490.98	85.55

SIRT5

Tissue Type	7d	7d SE	14d	14d SE
AB FAT	1018.03	236.61	1139.61	184.77
CROP	1759.56	219.54	1423.91	284.76
LIVER	3738.87	321.17	3738.57	299.99
SQ	378.24	117.42	1731.47	300.21

SIRT6

Tissue Type	7d	7d SE	14d	14d SE
AB FAT	2569.83	574.35	4068.06	509.81
CROP	3509.01	166.78	3171.46	515.45
LIVER	3108.88	176.34	3213.42	106.84
SQ	28.57	5.67	4161.67	569.04

SIRT7

Tissue Type	7d	7d SE	14d	14d SE
AB FAT	182.09	48.65	50.97	12.40
CROP	65.05	8.73	71.14	6.61
LIVER	175.32	16.79	182.94	12.75
SQ	282.90	39.15	97.28	16.94

Table 4. Analysis of variance results comparing gene expression in both 7d and 14d chicks across four tissue types. The interaction term between Tissue and Age is included in the regression model and denoted below as Tissue Type*Age. Statistical significance denoted by * for p-values less than or equal to 0.05. ANOVA test run using R Studio.

gene	Tissue Type p-value	Tissue Type*Age p-value
SIRT2	4.98E-03*	1.34E-02*
SIRT4	5.93E-01	1.82E-02*
SIRT5	2.20E-16*	7.60E-03*
SIRT6	6.92E-04*	2.59E-07*
SIRT7	1.31E-05*	4.07E-04*

Figure 4. Spearman rank correlation heat map of five sirtuin genes and 25 fatty acids produced in MetaboAnalyst 4.0. Normalized data was transformed using a logarithmic transformation.

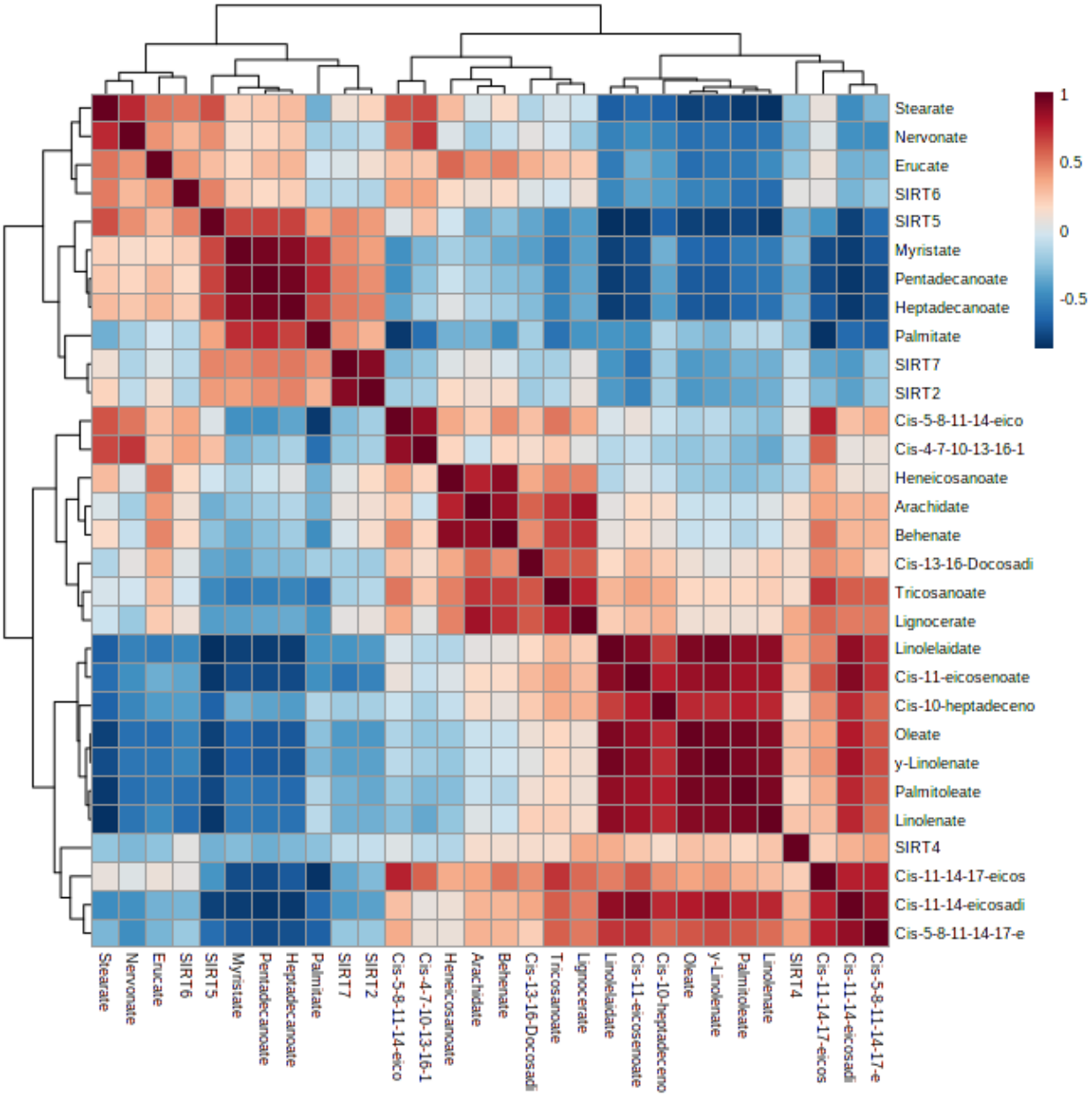


Table 5. Relative abundance of 25 fatty acids in subcutaneous adipose tissue. Data for each of the five time points was averaged to give a singular value for each time point.

Fatty Acid	Abbreviation	E13 Avg	E15 Avg	E17 Avg	7d Avg	14d Avg
Myristate	c14:0	2.77%	1.69%	0.91%	0.83%	0.86%
Pentadecanoate	C15:0	0.38%	0.31%	0.12%	0.11%	0.10%
Palmitate	C16:0	43.94%	37.77%	24.62%	31.77%	30.93%
Palmitoleate	C16:1 n-9	0.30%	0.53%	0.24%	4.41%	4.71%
Heptadecanoate	C17:0	0.65%	0.59%	0.29%	0.25%	0.23%
Cis-10-heptadecenoate	C17:1 n-7	0.01%	0.01%	0.01%	0.08%	0.06%
Stearate	C18:0	39.66%	42.22%	48.84%	19.24%	20.02%
Oleate	C18:1 n-9	6.96%	8.84%	8.89%	24.64%	27.46%
Linoleate	C18:2 n-6	1.74%	2.62%	4.09%	12.75%	9.81%
y-Linolenate	C18:3 n-6	0.01%	0.02%	0.02%	0.17%	0.10%
Linolenate	C18:3 n-3	0.01%	0.02%	0.01%	0.81%	0.54%
Arachidate	C20:0	0.26%	0.23%	0.26%	0.41%	0.44%
Cis-11-eicosenoate	C20:1 n-9	0.06%	0.10%	0.11%	0.30%	0.31%
Cis-11-14-eicosadienoate	C20:2 n-6	0.02%	0.03%	0.05%	0.14%	0.12%
Cis-11-14-17-eicosatrienoate	C20:3 n-3	0.09%	0.14%	0.39%	0.29%	0.35%
Heneicosanoate	C21:0	0.05%	0.04%	0.04%	0.03%	0.03%
Cis-5-8-11-14-eicosatetraenoate	C20:4 n-6	2.10%	3.95%	9.18%	2.79%	3.00%
Cis-5-8-11-14-17-eicosapentaenoate (EPA)	C20:5 n-3	0.00%	0.01%	0.02%	0.14%	0.18%
Behenate	C22:0	0.25%	0.19%	0.26%	0.40%	0.35%
Erucate	C22:1 n-9	0.44%	0.12%	0.15%	0.05%	0.07%
Cis-13-16-Docosadienoate	C22:2 n-6	0.00%	0.00%	0.00%	0.01%	0.01%
Tricosanoate	C23:0	0.01%	0.01%	0.03%	0.06%	0.05%
Lignocerate	C24:0	0.02%	0.02%	0.03%	0.13%	0.08%
Cis-4-7-10-13-16-19-docosahexaenoate (DHA)	C22:6 n-3	0.23%	0.52%	1.41%	0.19%	0.21%
Nervonate	C24:1 n-9	0.02%	0.02%	0.04%	0.00%	0.00%

Works Cited

Chong, J., Wishart, D. S., & Xia, J. (2019). Using MetaboAnalyst 4.0 for Comprehensive and Integrative Metabolomics Data Analysis. *Current Protocols in Bioinformatics*, *68*(1). https://doi.org/10.1002/cpbi.86

Clasquin, M. F., Melamud, E., & Rabinowitz, J. D. (2012). LC-MS data processing with MAVEN: A metabolomic analysis and visualization engine. *Current Protocols in Bioinformatics*, *0 14*(SUPPL.37), Unit14.11. https://doi.org/10.1002/0471250953.bi1411s37

Ford, E., Voit, R., Liszt, G., Magin, C., Grummt, I., & Guarente, L. (2006). Mammalian Sir2 homolog SIRT7 is an activator of RNA polymerase I transcription. *Genes and Development*, *20*(9), 1075–1080. https://doi.org/10.1101/gad.1399706

Greiss, S., & Gartner, A. (2009, November). Sirtuin/Sir2 phylogeny, evolutionary considerations and structural conservation. *Molecules and Cells*. https://doi.org/10.1007/s10059-009-0169-x

Hiratsuka, M., Inoue, T., Toda, T., Kimura, N., Shirayoshi, Y., Kamitani, H., … Oshimura, M. (2003). Proteomics-based identification of differentially expressed genes in human gliomas: Down-regulation of SIRT2 gene. *Biochemical and Biophysical Research Communications*, *309*(3), 558–566. https://doi.org/10.1016/j.bbrc.2003.08.029

Jing, E., Gesta, S., & Kahn, C. R. (2008). *Sirt2 Regulates Adipocyte Differentiation Involving FoxO1 Acetylation/Deacetylation*.

Landry, J., Sutton, A., Tafrov, S. T., Heller, R. C., Stebbins, J., Pillus, L., & Sternglanz, R. (2000). The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(11), 5807–5811. https://doi.org/10.1073/pnas.110148297

Lombard, D. B., Alt, F. W., Cheng, H.-L., Bunkenborg, J., Streeper, R. S., Mostoslavsky, R., … Schwer, B. (2007). Mammalian Sir2 Homolog SIRT3 Regulates Global Mitochondrial Lysine Acetylation. *Molecular and Cellular Biology*, *27*(24), 8807–8814. https://doi.org/10.1128/mcb.01636-07

Mihelic, R., Winter, H., Powers, J. B., Das, S., Lamour, K., Campagna, S. R., Voy, B. H. (2019). Genes controlling polyunsaturated fatty acid synthesis are developmentally regulated in broiler chicks. Submitted to *British Poultry Science*. https://doi.org/10.1080/00071668.2020.1759788

Nakagawa, T., & Guarente, L. (2011, March 15). Sirtuins at a glance. *Journal of Cell Science*. https://doi.org/10.1242/jcs.081067

Nakagawa, T., Lomb, D. J., Haigis, M. C., & Guarente, L. (2009). SIRT5 Deacetylates Carbamoyl Phosphate Synthetase 1 and Regulates the Urea Cycle. *Cell*, *137*(3), 560–570. https://doi.org/10.1016/j.cell.2009.02.026

Nasrin, N., Wu, X., Fortier, E., Feng, Y., Baré, O. C., Chen, S., … Bordone, L. (2010). SIRT4 regulates fatty acid oxidation and mitochondrial gene expression in liver and muscle cells. *Journal of Biological Chemistry*, *285*(42), 31995–32002. https://doi.org/10.1074/jbc.M110.124164

North, B. J., & Verdin, E. (2004). Sirtuins: Sir2-related NAD-dependent protein deacetylases. *Genome Biology*. https://doi.org/10.1186/gb-2004-5-5-224

Ren, J., Xu, N., Ma, Z., Li, Y., Li, C., Wang, Y., … Kang, X. (2017). Characteristics of expression and regulation of sirtuins in chicken (*Gallus gallus*). *Genome*, *60*(5), 431–440. https://doi.org/10.1139/gen-2016-0125

Roberts, L. D., West, J. A., Vidal-Puig, A., & Griffin, J. L. (2014). *Methods for performing lipidomics in white adipose tissue*. *Methods in Enzymology* (1st ed., Vol. 538). Elsevier Inc. https://doi.org/10.1016/B978-0-12-800280-3.00012-8

Search - Gallus gallus - Ensembl genome browser 99. (n.d.). Retrieved April 9, 2020, from http://useast.ensembl.org/Gallus_gallus/Search/New?db=core

Singh, C. K., Chhabra, G., Ndiaye, M. A., Garcia-Peterson, L. M., MacK, N. J., & Ahmad, N. (2018, March 10). The Role of Sirtuins in Antioxidant and Redox Signaling. *Antioxidants and Redox Signaling*. Mary Ann Liebert Inc. https://doi.org/10.1089/ars.2017.7290