ABSTRACT

PHILLIPS, CHELSEA ADELE. Diet Affects Genomes for Generations. (Under the direction of Dr. Christopher Ashwell).

Several genomic modifications have been shown to be inherited from parent to offspring including methylation of DNA in the form of 5-methylcytosine. Changes in DNA methylation leads to changes in gene expression and ultimately phenotypes. These modifications can affect the health and growth of an organism. Environmental signals, including nutrition, are known to impact epigenetic modifications. The methylation of DNA requires enzymes and many dietary micronutrients referred to as methyl catalysts. Studies assessing parental methyl catalyst supplementation have indicated that parental nutrition plays a role in shaping the epigenetic pattern of the offspring. It was hypothesized that a diet with a greater concentration of methyl catalysts could alter DNA methylation in progeny compared to a control when fed to the parent generation. Methyl catalysts for our study included choline chloride, betaine, vitamin B12, folic acid, pyridoxine, and zinc sulfate in addition to nutrients endogenous to the dietary feedstuffs. In the following studies herein, quail parents were fed either the high methyl diet or control diet in the starter and layer diets. Two generations of progeny from both treatments, which were all fed a control diet, were evaluated. Genome wide CpG DNA methylation levels were evaluated for each generation (Chapter 2). Overall vast differences in global CpG DNA methylation were not detected between groups however differences were detected locally when evaluating methylation levels of genes involved in the DNA methylation cycle (Chapter 2 & 3). Transgenerational phenotypic changes occurred in egg size throughout the three generations (Chapter 2 & 4). RNA sequencing of liver was analyzed for two generations out from grandparents which were fed the high methyl treatment diet. Gene expression was evaluated using Ingenuity Pathway Analysis (IPA) to determine molecular

networks associated with each treatment. Metabolic pathways surrounding were found to have transgenerational impacts (Chapter 3). Gene expression data were compared with DNA methylation levels for specific genes where most of the genes which had high DNA methylation levels resulted in a decrease in expression (Chapter 3). Epigenetic changes in modifications to DNA associated with nutrient availability in the offspring leads to further questions regarding the mechanisms behind the alterations of the epigenetic landscape for the expression or suppression of specific genes. These questions include: "Do offspring remember an environmental condition to which they were never exposed," "How does this relate to an organism's ability to adapt to conditions to which the parents were exposed," and "What epigenetic modifications are made to the offspring genomic regions due to parental environmental exposures?" These data indicate that a change in DNA methylation can alter gene expression without altering the genome itself. Similar studies in mice have shown that feeding a diet high in methyl catalysts can lead to phenotypic changes along with improved health and life span of individuals. Further research is warranted on the manipulation of parental diets to impart positive epigenetic effects on progeny. The utilization of these ideas could be a useful tool to the poultry industry through manipulation of the parental environmental conditions to better prepare the offspring for future environmental exposures. In mammals, parental nutrition has been linked to adult onset disease through possible alterations in epigenetic marks. Research is needed into the many diseases affecting poultry production for the evaluation of susceptibility and associations with epigenetic modifications resulting in changes in gene regulation.

Diets Affect Genomes for Generations

by
Chelsea Adele Phillips

A dissertation submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Physiology

Raleigh, North Carolina
2020

APPROVED BY:

_____	_____
Dr. Christopher Ashwell Committee Chair	Dr. Jack Odle
_____	_____
Dr. Charlotte Farin	Dr. Benjamin Reading
_____	
Dr. Hsaio-Ching Liu	

BIOGRAPHY

Chelsea Adele Phillips was born in 1993 in the Appalachian Mountains of North Carolina. She was raised by her parents, Patrick Phillips and Tammy Bryant, and grandmother, Pat Phillips. Chelsea graduated from Mitchell High School in 2011 and moved to Raleigh, NC to major in Poultry Science at NC State University. This proudly made her a first-generation college student. As an undergraduate in poultry science and nutrition, she was successful in her course work and was able to work as a student researcher with Dr. Christopher Ashwell and Dr. Frank Edens. In this position Chelsea was given the opportunity to independently conduct research trials in poultry. Upon graduating early with a bachelor's degree in Poultry Science and a minor in nutrition, she was confident she wanted to further her education in poultry-related research. As such, she applied to become a graduate student within Christopher Ashwell's lab and was accepted as a doctoral student in the physiology program.

ACKNOWLEDGMENTS

To begin I would like to extend my appreciation to my advisor Chris Ashwell for giving me the tools and opportunities to succeed and grow not only as an academic but as a person. Your influence has forced me to become an independent researcher who can take initiative and answer questions. I am most thankful for how you have changed my perspective on the world and people around me.

Sincere gratitude is also due to Benjamin Reading, Charlotte Farin, Hsaio-Ching Liu, Jack Odle, and Kim Livingston for all their time and input during my preliminary exams and throughout the writing of my dissertation. There is no doubt their comments, suggestions, and criticisms have aided in my scholarly development.

My time at NC State would not have been possible without the help of our lab tech, Shelly Nolin, and undergraduates Heather Bundy and Mallori Hopper. Shelly was an essential person in helping me with everything from research, classes, to life problems. Her guidance and friendship have always been available and will be forever appreciated.

Lastly, I would like to acknowledge the continuous help, guidance and support I received from Coltin Caraway who entertained all my research thoughts and ideas and above all supported me on the good days and the bad. Thank you for helping me become the best version of myself.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Literature Review

Epigenetics

Naturally occurring epigenetic events link the prominent theory of nature versus nurture by explaining how the environment results in cellular memory for future generations. As our understanding of cellular memory as influenced by the environment expands, it has become clear that diet and other environmental factors can not only impact a single individual but future generations. This area of research has come to be known as a study of heritable traits that can be associated with changes in chemical modifications of DNA or of structural and regulatory proteins bound to it (Felsenfeld, 2014). This concept is dissimilar to genetics due to the fact that epigenetics is regulatory and does not include changes within the nucleotide sequence itself.

In 1942, Waddington devised the term epigenetics to explain unresolved aspects of development which included how a single fertilized egg can later give rise to a complex organism (Waddington, 1942). He was interested in how genes that interact with their environment could shape the organism. To explain his process of epigenetics he used the example of a ball rolling down a hill. This is known as the epigenetic landscape which is formed by the pull of genes on the surface (Waddington, 1957). The ball represents embryonic differentiation during development. An area with a lot of genetic pull would result in a valley where the zygote would be more likely to fall, referred to as developmental pathways. Once the ball has reached the bottom of the hill it is unable to simply go back up the hill on its own. Therefore, once a cell has differentiated into a specific cell type it is unable to reprogram on its own. Waddington was explaining the process cells use to form an identity during differentiation. Knowing every cell within an organism is made up of the same DNA sequence, epigenetic modification to the DNA sequence allow for a stem cell to differentiate into a target cell type, such as a liver cell, and to maintain this state.

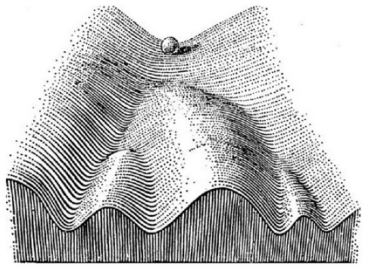

Figure 1.1. Waddington's Epigenetic Landscape.

The work of Laskey and Gurdon demonstrated the ball could be rolled back up the hill when the DNA of a somatic cell was added to a *Xenopus* egg with the nucleus removed but an embryo still developed (Laskey and Gurdon, 1970). It became clear the genetic material needed for cellular differentiation had not been removed from the differentiated cell but rather signals causing the expression of the genetic material had changed. Later, Yamanaka demonstrated that the four factors needed to reprogram somatic cells back to induced pluripotent stem cells were: Oct3/4, Sox2, c-Myc, and Klf4 (Takahashi et al., 2006).

DNA Methylation

Currently, the best understood epigenetic mechanisms are DNA methylation and histone modifications. Within an organism, the DNA in all somatic cells is the same, however transcript expression will determine the cell type it becomes. The differentiation in expression of these cell types is often controlled by epigenetic mechanisms. One such example is DNA methylation at cytosine, which was discovered as an epigenetic mechanism to repress gene expression (Razin and Riggs, 1980). This mechanism is accomplished by the covalent addition of a methyl group to the 5-carbon ring on cytosine (m^5C). Vertebrates have the greatest levels of m^5C, and it is dispersed over much of the genome, referred to as global methylation. The DNA methylation of cytosine can occur in three separate sequence frameworks including CpG, CHG, or CHH, where H stands for

the nucleotide adenine, thymine or cytosine. However, the most common sequence for DNA methylation in mammals is CpG (Pelizzola and Ecker, 2011).

Clusters of CpG dinucleotides, known as CpG islands, are regions of DNA greater than 200 base pairs and have a frequency of greater than 50% G+C content (Gardiner-Garden and Frommer, 1987). These islands are typically completely methylated or unmethylated and in the germline they tend to be unmethylated (Weber et al., 2007). CpG islands are typically associated with promoter regions and may affect the expression of the corresponding transcript. Throughout the genome, DNA methylation is unevenly distributed and DNA methylation occurring in vertebrates is found within repetitive and retroviral sequences as a means to keep these sequences silent (Felsenfeld, 2014). Unlike mammals where 40% of the genome is made up of retrotransposons, only approximately 15% of the chicken genome is made up of repetitive elements (Wicker et al., 2005). Much of this is thought to be due to their small genome size.

Gene expression is inhibited by m^5C through inhibition of DNA binding factors to their recognized DNA sequence or as a signal for the recognition of proteins involved in repression (Klose and Bird, 2006). Many methyl binding proteins (MBPs) have been identified in mammals. The MBPs specifically recognize and bind to methylated CpGs to mediate silencing of gene expression. Silencing can be achieved through targeting chromatin remodeling corepressor complexes to these regions (Jones et al., 1998). Cytosines, which are a part of a C-G dinucleotide (CpG), are typically highly methylated (~60-80%) in mammals. In differentiated tissue, the cytosines not a part of CpG dinucleotides are often unmethylated (~0.3-3%) (Pelizzola and Ecker, 2011).

When evaluating DNA methylation, genomic regions can be denoted as hypomethylated or hypermethylated, which refers to a gain or loss of methylation to a particular allele when

compared to a normal condition. This establishes an individual epigenetic pattern. Within a cell, the DNA methylation patterns can be passed on to the daughter cells and subsequently passed on to the next generation of cells.

DNA Methyltransferase Enzymes

DNA methyltransferase enzymes (DNMTs) are responsible for the direct methylation of DNA through the donation of a methyl group from the universal methyl donor S-adenosyl methionine (SAM). DNMT3 is a family of enzymes containing DNMT3A, DNMT3B, DNMT3C and DNMT3L. DNMT3A and DNMT3B are known as de novo methyltransferases that are needed to establish the epigenetic profile, or cell fate, for new cells being produced that do not yet contain methylation marks. In satellite cells, DNMT3A regulates the proliferation of satellite cells, and a loss in DNMT3A results in the inability of satellite cells to proliferate (Naito et al., 2016). DNMT3A and DNMT3L are both required for most of the genomic imprinting in the paternal and maternal germ line (Kaneda et al., 2004). DNMT3B is responsible for the methylation of CpG islands on autosomes and the X chromosome and the somatic methylation patterns early in development (Auclair et al., 2014). DNMT3L is a cofactor expressed during gametogenesis and is involved in maternal imprinting. It is not catalytic, therefore it is unable to methylate, although it is thought to be involved in transcriptional repression. DMT3C is a newly discovered de novo DNA methyltransferase that is required for fertility and responsible for specific methylation of young retrotransposon promoters in the male germ line (Barau et al., 2016). The process of detecting regions of the genome for *de novo* DNMT methylation activity has been detected through multiple target methods. The DNMT3 enzymes may recognize specific DNA or chromatin regions, the enzymes may be recruited through protein interactions or through the use of RNAi (Klose and Bird, 2006).

DNMT1 is the most abundant methyltransferase and is primarily responsible for maintenance. The unique feature to this DNMT is its capability to methylate hemi methylated regions in the genome, meaning DNMT1 can methylate double stranded DNA, which may already have methylated regions present. DNMT1 also plays a role in de novo methylation. This methyltransferase has an isoform found in the somatic nucleus of cells and a form that is found in the oocyte cytoplasm that will eventually be transported to the nucleus once development is underway. In mice, embryos which lack maternal DNMT1 exhibit genome wide demethylation and knockout of DNMT1 in the zygote, resulting in a complete loss of methylation at both paternal and maternal differentially methylated regions (DMRs) indicating that DNMT1 alone can maintain DNA methylation (Hirasawa et al., 2008). Lastly, DNMT2 is not believed to play a significant role in the methylation of DNA. When this DNMT2 is knocked out in embryonic stem cells, DNA methylation continues without any detectable effect (Okano et al., 1998).

Histone Modifications

For DNA to condense, it is wrapped around positively charged proteins known as histones. Two copies of histone H2A, H2B, H3 and H4 make up a nucleosome that is further packaged into 30-nm fibers (Kornberg and Thonmas, 1974). Histones have an extended tail of amino acids beyond the DNA that can be modified to alter histones, DNA interactions, and folding (Luger et al., 1997). Histone modifications are needed for establishing chromatin conformation, recruitment or removal of proteins, and regulating active or repressive gene expression. This can be accomplished by making DNA inaccessible to transcription factors through altered chromatin structure (Wolffe and Hayes, 1999). Activation or inactivation of transcription is based on the modifications associated with the DNA and histones. Histone modifications can be considered more plastic while DNA methylation is static. Loci expressed more often are usually associated

with histone proteins that promote expression, such as acetylation of lysine. In contrast, commonly repressed loci typically become associated with histone modifications that inhibit gene expression. There are at least eight modifications to histones with over 60 different sites known (Kouzarides, 2007). Similar to DNA methylation, histone modifications are not uniformly distributed throughout the genome. The modifications are dynamic and continuously changing.

An early misconception of histone modifications was that histone proteins were only required in the silencing of DNA sequences and in the absence of modifications, including chemical and the binding of proteins, the DNA sequence could actively be transcribed. However, acetylation of histones began to be correlated with the activation of genes showing that epigenetic modifications can both activate and silence gene expression (Allfrey, VG Faulkner, R Mirsky, 1964). If the chromatin domain has been activated, the DNA which is associated with the nucleosome region must be repositioned to allow for the accessibility to the DNA promoter region. This is accomplished by histone remodeling complexes such as SWI/SNF. Histones can be modified in many different ways.

Methylation modifications can typically be seen transgenerationally and result from one to three methyl groups covalently bonded to lysine or arginine residues on histone proteins by the enzyme histone methyltransferase (HMT). Some HMTs are lysine specific while others are arginine specific. Interestingly, some HMTs contain a possible methyl CpG binding domain that indicates the possibility of dual methylation of histones and DNA occurring simultaneously (Zhang and Reinberg, 2001). This indicates that DNA methylation and chromatin modifications can work in conjunction with each other to achieve silencing of a genomic region. Specifically, DNMTs have been shown to interact with histone methyltransferases and deacetylases (Geiman et al., 2004). Methyl CpG binding proteins may even recruit histone deacetylase complexes so that

the histone tails can become methylated instead of acetylated (Zhang and Reinberg, 2001). Histone methylation is thought to be static, however changes do occur during the cell cycle and other events such as heat shock (Zhang and Reinberg, 2001).

A common mechanism for the cell to maintain or alter a chromatin state is through the use of polycomb and trithorax proteins, which have been extensively studied as a way to permanently keep a cluster of genes in the active or inactive state throughout cell division. The polycomb group (PcG) stably silences chromatin domain through cell division (Grossniklaus and Paro, 2014). Once the chromatin has been altered this can lead to a cascade of downstream effects such as the recruitment of proteins to the modified region that can alter the structure and functional states of the chromatin. The histone modification will contain a binding site that will interact with a protein binder. This protein binder will have another site for an interaction with an enzyme writer, which can modify adjacent nucleosomes creating a domino effect until a boundary element is reached.

Often times the activation or repression of gene expression are specific to the histone, the lysine residue, and the genomic region they are in association with, meaning one modification can commonly be categorized as an active mark but in some situations, it could repress gene expression (Kouzarides, 2007). Trimethylation of lysine 4 on histone 3 (H3K4me3) and acetylation of lysine 27 on histone 3 (H3K27ac) is an active marker that can be found in the promoter of activated genes serving as a signal to be transcribed. This can result from the fact that acetylation of positively charges lysine residues can neutralize the charge resulting in less of an interaction between the histone tail and the nucleosome allowing for the chromatin to open. Histone acetylation modifications of lysine are accomplished by histone acetyltransferases (HATs) and histone deacetylases (HDACs).

Trimethylation favors a closed chromatin state and the repression of gene expression when the target is lysine 9, 36, and 27 on histone 3 (Hyun et al., 2017). In some cases, histone modifications can interfere with transcription factors attaching to their promoter region simply due to the bulk interference of modifications to the histone tail, especially in the case of trimethylation. Genome wide H3K4me3 and H3K27me3 histone modifications were evaluated for the spleen, thymus, erythrocytes and bursa of the chicken (Jahan et al., 2016; Luo et al., 2012; Mitra et al., 2012, 2015). Similar to mammals, H3K27me3 modifications were observed surrounding the transcriptional start site and resulted in repressed gene expression. H3K4me3 was observed to cause active gene expression.

Dosage Compensation

An early model of an epigenetic mechanism includes the inactivation of one X chromosome in female mammals, which contain two X chromosomes. This occurs as an epigenetic mechanism preventing transcription of twice as many genes often referred to as dosage compensation (Ohno et al., 1959). Inactivation is accomplished through the addition of epigenetic modifications by DNA methylation.

Genes on the stably inactivated X chromosome exhibit decreased amounts of histone H4 acetylation and an increase in methylation to the GC promoter region of housekeeping genes (Jeppesen and Turner, 1993; Wolf et al., 1984). With the discovery of the methylase inhibitor 5-azacytidine, it became clear that the expression of numerous genes are altered by DNA methylation (Jones, Taylor, Wilson, 1983). When inactivated X chromosomes were treated with 5-azacytidine the reactivation of the X chromosome consistently occurred, revealing the important role DNA methylation plays in the inactivation of gene expression (Gartler and Goldman, 1994).

In birds, the female is the heterogametic sex with chromosomes ZW and males are the homogametic sex with chromosomes ZZ. Therefore, male birds carry a double dose of sex-linked genes compared to the other sex. Originally, dosage compensation was not thought to occur in the avian but using RT-QPCR McQueen et al. (2001) found that genes which are Z linked in the bird are dosage compensated because the Z linked genes were not expressed twice as much in males (ZZ). The underling mechanism birds use for dosage compensation is unknown.

Genomic Imprinting

Genomic imprinting is an inheritance process which results in only one allele being expressed from one of the two parent alleles. Forms of imprinting have been detected in fungi, plants, insects, fish, and other animals (Tuiskula-Haavisto et al., 2004). It has long been known that each autosomal gene is represented by two alleles, one from the maternal genome and one from the paternal genome. In most cases expression occurs from both alleles; however, a small subset of genes are known to only be expressed from a single parent. The process of controlling this expression is accomplished epigenetically by DNA methylation. Imprinting is thought to influence the transfer of nutrients to the embryo, even in oviparous organisms, and imprinted genes are known to affect growth during development (Frésard et al., 2014).

Imprinted genes make up only a minority of the genes in the genome and are found in clusters 80% of the time with other imprinted genes (Reik and Walter, 2001). Imprinted regions tend to contain CpG islands. These regions are often found within or near the promoter regions of genes. Imprinted genes differ in DNA methylation of parental origin and properties. For example, some methylation patterns that are found in the parental genome are maintained throughout development and in the tissues, while others are open to changes during development and in specific tissues. It is generally believed that methylation to imprinted genes would result in inactivation of that

parental copy, although this can sometimes be the case it is not definite. Methylation to these regions can just as easily result in the active gene copy (Reik and Walter, 2001).

Forms of genomic imprinting have been demonstrated in plants, fungi, and mammals; however it has yet to be detected in reptiles, fish and avian species. Although it hasn't been discovered in these species does not mean it fails to exist. The mechanism may not be understood with our current understanding of maternal nutrient provisioning in oviparous organisms. Previous genome studies evaluating poultry quantitative trait loci (QTL) have been detected for traits including egg quality, egg production, feed consumption body weight and body fat (Ikeobi et al., 2002; Recoquillay et al., 2015; Schilling et al., 2015; Sewalem et al., 2002; Tuiskula-Haavisto et al., 2002, 2004; Van Kaam et al., 1998). In a study conducted by Tuiskula-Haavisto et al. (2004) parent of origin effects were analyzed for QTL production traits in the chicken. Significant phenotypic differences were found in age at first egg, number of eggs, egg weight, feed efficiency, feed intake (FI), and egg white quality. Using a parent of origin model, variation in the offspring was better explained than that of a Mendelian model. Evaluation of chromosome 1 in *Gallus gallus* (Gga1) displayed significant genome wide maternal expression for QTL affecting BW and FI, while significant paternal expression of QTL for age at first egg was evident. Maternally expressed QTL affecting feed intake was highly suggestive on linkage group 36. On Gga3, paternally expressed QTL for egg number was significant and on linkage group 30 QTL for egg weight were highly likely. More recently Harry et al., 2014 identified 20 QTL with a parent of origin expression effect including paternally expressed QTL of carcass and growth traits identified in the same region on Gga3 which Tuiskula-Haavisto et al., 2004 identified.

Imprinting was thought to primarily evolve due to a conflict in the maternal and paternal genes involved in the transfer of nutrients to the growing embryo (Reik et al., 2003). A balance

must occur in the nutrients removed from the mother and utilized by the embryo, because if too many nutrients were directed to the embryo this could harm the health of the mother; whereas, if not enough nutrients were available to the growing embryo this could result in an embryo that withstands less of chance in survival or even death preterm. In mammals, many of these genes are expressed in both the placenta and embryo. Maternally expressed genes typically regulate the transfer and supply of nutrients, whereas the paternally expressed genes typically control nutrient demand and growth (Tycko and Morison, 2002). Thus, maternally derived genes tend to be more conservative with resource allocation of nutrients than paternally derived genes. This theory is not clear for birds considering the egg has no physical connection to the mother once it has been laid.

Previous research comparing imprinted genes in mammals to that of birds has indicated an absence of imprinting and thus far all imprinted genes found in mammals are expressed bilaterally in the bird (Nolan et al., 2001; Yokomine et al., 2005). Imprinted genes in mammals cannot be compared to that in birds due to different placements of genes on the chromosomes and the vast physiological differences in reproduction. The majority of imprinted genes observed contain differentially methylated alleles meaning a different methylation status can be found across various cell types within an individual. When evaluating the differentially methylated regions (DMRs) in the chicken genome there was an absence of DMRs that would indicate the presence of imprinted genes (Li et al., 2011).

It is possible that the process for silencing genes could be different in the avian. It may be that imprinting in the avian is tissue specific or that maternal or paternal genes can be expressed differently depending on the tissue type. Considering most of the known imprinted genes in mammals are expressed in the placenta, it would be interesting to evaluate membranes of the egg during development such as the chorioallantoic membrane. This is a vascular membrane found in

the egg that is made up of the fusion between the allantois and chorion layers, which would be analogous to the placenta in mammals.

Developmental Epigenetics

Gametogenesis

During development, mechanisms occur allowing differentiated cells to preserve their epigenetic pattern from which they came. This makes it possible for every cell to have the same DNA sequence but allows for different expression patterns, resulting in an abundant array of cells that perform different roles. These epigenetic mechanisms also make it possible for a differentiated cell to revert back to pluripotency. The epigenetic pattern can be erased if a differentiated somatic cell nucleus is transplanted back into an oocyte and becomes totipotent once again (Kato et al., 1998; Wakayama et al., 1998). During gametogenesis, imprinted genes are marked epigenetically so that they express either the allele inherited from the maternal line or that from the paternal line. These epigenetic patterns are inherited by the embryo once fertilization has occurred and this pattern stays with the individual throughout their life.

Global DNA methylation reprogramming occurs during two stages in development (Jablonka and Raz, 2009; Li et al., 2018; Messerschmidt et al., 2014). The first being the process gametogenesis where primordial germ cells become gametes and the second is after fertilization has occurred, when the gametes from the male and female come together to form a zygote. This process is also a safety net to check for improper marks and to ensure the transfer of characteristics of a species between generations.

In the first stage, primordial germ cells during development are the source of male and female germ cells. As they relocate to their developing gonad they go through a reprogramming process throughout their entire genome. An organism's methylation pattern as well as their future

gametes are developed during their own development. Reprogramming makes possible the inheritance of epigenetic patterns as well as the expression of sex related genes needed to further develop the organism gametes that one day may result in another embryo. Demethylation of primordial germ cells leads to activation of genes that are essential for gametogenesis to occur (Sun et al., 2017).

Spermatogenesis

The inheritance of paternal factors on the offspring is an important area to provide information about epigenetic inheritance that has not been altered due to the exposure of offspring to maternal conditions. Stress, toxins, and nutrition have all been documented to alter the phenotype of the progeny (Rando, 2012). Studies in rodents evaluating the paternal diets, rather than the maternal diets, has shown a similar effect on offspring metabolism as that observed when the maternal diet is manipulated. The trend continued with males who were fed an undernourished diet resulted in offspring with altered glucose metabolism, while males who were fed a high fat diet resulted in daughters who were glucose intolerant (Anderson et al., 2006; Ng et al., 2010; Radford et al., 2012). Paternal diets low in protein (9% verses 18%) fed to mice resulted in impaired glucose tolerance in both male and female offspring (Watkins and Sinclair, 2014).

One area of research in spermatogenesis is the paternal role in embryo DNA methylation. It is known that for the sperm to fully mature, histones are replaced by protamines. This is essential in keeping the DNA compact and unharmed as it travels through the female reproductive tract. This process can occur because haploid DNA does not need to unwind for transcription unlike other diploid cells that need to be opened for the transcription machinery to reach the promoter region to transcribe. Once the sperm has been engulfed by the maternal oocyte the protamines keeping the male DNA compact are replaced with maternal histones (McLay and Clarke, 2003).

It was previously believed that this would result in the paternal line playing a minor role in the epigenetic pattern, however it is now understood that a small percentage of nucleosomes are retained on the paternal DNA that contribute to the organization of the embryos future epigenetic profile (Carrell and Hammoud, 2009). The location of the nucleosomes that were retained from the paternal line tend to occur in the hypomethylated promoter regions of transcription and signaling factors present during development. Later, in a study evaluating the nucleosome retention of males who were infertile, it was found that they exhibited alterations in the methylation pattern of promoter regions involved in development (Hammoud et al., 2011).

Oogenesis

Within the oocyte that is stalled at metaphase II, like somatic cells, the DNA is wrapped around histones. The main difference between oocyte histones and somatic histones is the absence of H1 linker histone (Clarke et al., 1998). H1 linker histone serves as another method to keep DNA compact. It is not a part of the nucleosome but instead sits on top keeping the coil together. In oocytes, H1 linker histone is replaced with a histone of a different H1 linker form used specifically in embryogenesis this alteration between somatic and oocyte packaging is still not completely understood. After birth, females experience genomic imprinting of their oocytes, which are then arrested in the diplotene stage of meiotic prophase 1. Later, once the oocyte has fully matured, it is completed by de novo methylation process.

Embryogenesis

In the second stage of epigenetic reprogramming, after fertilization and before the embryo has been implanted, the genome of the paternal and maternal line undergoes reprogramming (Messerschmidt et al., 2014). Epigenetic remodeling occurs differently in each parental genome

and is not evenly distributed throughout either genomes (Carrell and Hammoud, 2009; Curley et al., 2011).

The methylated cytosines of the parental genome are converted to hydroxymethyl cytosine by the enzyme Tet3. Tet3 erases all but a subset of the DNA cytosine methylation marks. Tet3 is a DNA hydroxylase enzyme that oxidizes 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) then to 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (Wu and Zhang, 2011). It is important to note that commonly used methods for DNA methylation detection are unable to discern 5mC from 5hmC (Huang et al., 2010). The regions responsible for imprinting are maintained epigenetic marks and do not become demethylated.

Once fertilized, the maternal genome is triggered to complete meiosis and experiences fewer epigenetic changes when compared to that of the paternal genome (Rando, 2012). The protein PGC7/Dppa3/Stella is a maternal factor expressed in embryonic stem cells which binds to DNA to prevent the demethylation by Tet3 of methylated cytosines associated with imprinted genes (Nakamura et al., 2007). This protein uses H3K9me2 as an indicator of regions to protect from conversion to 5-hydroxymethylcytosine. Similarly, imprinted H3K9me2 regions of sperm are protected from demethylation by PGC7 binding (Nakamura et al., 2012).

After methylation marks have been erased, *Oct4* (chicken analog *cPouV*) and *Nanog* are expressed, which are important in regulating pluripotency and to keep the embryo from differentiating. De novo methylation begins in the inner cell mass of the blastocyst where the levels of methylated DNA will increase (Rossant et al., 1986). It is important to note that the epigenetic pattern allows for cells to create their identity so the epigenetic patterns created will be tissue specific.

During embryogenesis cells go through a process of cell fate, deciding what cell lineages will survive and which cells to destroy. The cells that are destined for survival will be precursors to numerous other cells through multiple cell divisions. These cell lineages that are decided on for survival must be remembered through cell divisions. The cellular memory is accomplished through epigenetic marks (Hales et al., 2011). In the early stages of zygote formation, the maternal and paternal genomes remain asymmetrical until patterns are established by DNMT3a and DNMT3b. The entire genome is methylated except for the CpG islands. Once the embryo is implanted the maintenance of methylation by DNMT1 is just as crucial as before implantation. Reversible methylation is needed for many processes in genetic stability such as the expression of imprinted genes and X inactivation.

The requirement of epigenetic modifications, including DNA methylation and histone modifications, are needed for cellular differentiation and further development in the chick as well (Jiao et al., 2013). Tissue specific reprogramming of DNA methylation and H3K9 acetylation along with DNMT and HDAC activity has been discovered during chick embryogenesis (Li et al., 2015b). During development, global DNA methylation levels were highest in the gut when compared to the liver and muscle of chicks (Li et al., 2015b). Activity of the *de novo* methyltransferases DNMT3a/b, along with global DNA methylation, significantly decreased at E20 and hatch (Li et al., 2015b). Although levels began to rise in the continued weeks of evaluation.

Dietary Epigenetic Effects

Nutrition provided during different stages of life can alter epigenetic regulation and may influence how an organism utilizes certain nutrients (Choi and Friso, 2010; Lillycrop et al., 2014; Rando and Simmons, 2015). The methylation of DNA requires enzymes and many dietary

micronutrients referred to as methyl catalysts. Methyl catalysts typically consist of folic acid, riboflavin, vitamin B_6, vitamin B_{12}, betaine, choline, and lastly zinc, which is a cofactor that is required for amino acid metabolism and DNA modifications. Conditions where poultry are not given adequate methyl catalysts result in reduced BW, feed intake, FCR, perosis, and the failure of normal protein synthesis (Scott et al., 1982).

Methyl Catalyst Supplementation

Studies assessing maternal methyl catalyst supplementation *in utero* have clearly indicated that maternal nutrition plays a role in shaping the epigenetic pattern of the offspring (Waterland and Jirtle, 2003). This has been demonstrated by changed coat color phenotype of the offspring when maternal mice were supplemented with methyl catalysts before and during gestation (Wolff et al., 1998). The viable yellow (A^{vy}) mouse has a single gene mutation at the agouti gene and has a predisposition to obesity and tumor susceptibility (Yen et al., 1994). When the agouti gene was cloned it was revealed to encode a protein with an insertion of intracisternal A particle (IAP) retrotransposon which results in yellow pigmentation (Duhl et al., 1994). The nutritionally induced change in coat color phenotype of mice was later identified to be caused by an increase in methylation at the agouti locus (Waterland and Jirtle, 2003). The methyl-supplemented diet resulted in more brown offspring while the low methyl diet resulted in yellow offspring. The coat color change was due to hypo- or hypermethylation of the agouti gene IAP region (Waterland and Jirtle, 2003). As the percentage of methylated CpG regions of A^{vy} increased, the coat color was found to progressively change, from clear yellow , slightly mottled yellow, mottled yellow, heavily mottled yellow and pseudoagouti (Waterland and Jirtle, 2003).

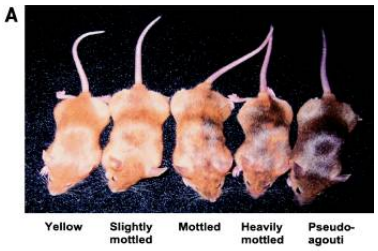

Figure 1.2. Dietary methyl catalyst supplementation of maternal mice results in phenotypic coat color alterations in A^{vy} offspring. Yellow mice are hypomethylated and hypermethylation results in agouti color (Waterland and Jirtle, 2003).

A similar example to the coat color agouti gene is the *Axin* fused ($Axin^{FU}$) mouse model where variations in methylation at the IAP retrotransposon of the *Axin* gene resulted in a kinky tail phenotype (Vasicek et al., 1997). Genetically identical $Axin^{FU}$ mice were supplemented with high methyl catalysts before and during gestation which resulted in hypermethylation of the IAP of the *Axin* gene and tail kinking in mice was reduced by half in offspring; whereas, if the region was hypomethylated the phenotype was very kinky tail (Waterland et al., 2006a).

Researchers examining paternal diet on offspring of male mice fed a low-protein or calorie restricted diet and discovered an increase in the expression of genes associated with lipid and cholesterol in the offspring when compared with those receiving a typical diet (Carone et al., 2010). When cytosine methylation patterns on the entire genome were analyzed in these rats, there was a similar methylation pattern present in all individuals including the control, suggesting that a minor modification in loci pattern can cause dramatic change (Carone et al., 2010). The effects of environmental exposures whether beneficial or not can be seen transgenerationally.

Although somewhat different, oviparous organisms are also influenced by their environment. A good example of this is the effect of incubation temperature. During development incubation temperatures can influence many physiological factors such as sex ratio in reptiles and in chicks, hatch wt and organ wt (Leksrisompong et al., 2007). When optimal nutrition was

provided to the laying hen, including greater levels of dietary vitamins, the result was higher levels of those vitamins found within the egg (Squires and Naber, 1993). This leads to the question of how this affects the offspring and their own requirements for the nutrition which was provided to the mother?

Studies on a variety of species have observed altered DNA methylation patterns resulting from environmental influences that the mother and/or father was exposed to, such as intrauterine diet, toxicants, or maternal disease states (Rando and Simmons, 2015; Zhang and Kutateladze, 2018). Many factors regarding how these environment influences affect an individual are still unclear and the changes regarding DNA methylation do not even affect the genome in the same way. Specific regions of the genome become hypomethylated, others are hypermethylated, while some areas are not affected at all. All of this can also depend on which tissue is being evaluated (Bird, 2002). Identifying the areas which can be influenced by extrinsic factors could broaden our understanding of how environmental factors not only influence that individual but also the offspring as well.

Folic Acid

Folate is an essential vitamin for birds and serves as a cofactor and cosubstrate for methylation reactions. Folic acid (FA), being the synthetic form of folate, is the oxidized form containing only a single conjugated glutamate residue (Shane, 2008). The predominant natural form of folate used by cells is a reduced form conjugated to a polyglutamate chain. In naturally occurring forms of folate, the polyglutamated tail is removed in the brush border of the mucosal cells by folate conjugates before it is absorbed. Folic acid is used as an additive because it is almost completely stable whereas the naturally occurring forms of folate often lose over half their activity over periods of days or weeks (Jaenicke, 1970).

Poultry are tolerant to high levels of FA and don't reach toxicity until 5,000 times the recommended value (Leeson et al., 2001). The bioavailability of FA in many products is generally found to exceed 70% (Pesti, 1995). In the presence of supplemental methionine and choline the folic acid requirement is often lowered (Pesti, 1991). By providing 2.64 mg per kg of FA to turkey breeders, the egg weight and hatch weight of the poults increased (Robel, 1993). The requirement for FA is higher for hatchability than egg production (Pesti, 1995). The normal concentration of folate found in an egg is 32 µg (Hoey et al., 2009). 80% of the total folate composition in eggs is 5-methyltetrahydrofolate (5-MTHF) (Seyoum and Selhub, 1998). When dietary FA is supplemented at 16mg/kg the concentration found in the egg can be increased to 75 µg of total folate content. Hyline W36 and W98 hens supplemented with increasing concentrations of FA for 21 days exhibited increased egg folate content and plasma folate with decreased homocysteine concentrations; however, bird performance was unaltered (Hebert et al., 2005). Egg folate content can be maximized with dietary supplementation of 2mg/kg or higher (Hebert et al., 2005). Knowing that FA must go through reduction and methylation reactions before being used, unlike the biologically active 5-MTHF, when dietary FA and 5-MTHF are fed and compared in laying hens they are equivalent in concentration of egg folates (Tactacan et al., 2010). The addition of dietary 5-MTHF in laying hens resulted in the production of heavier eggs and higher concentrations of folate in the egg and serum (Tactacan et al., 2010). Methionine synthase activity is decreased in the liver of birds supplemented with dietary FA or 5-MTHF (Tactacan et al., 2010).

Within the intestinal mucosal cells, folic acid is reduced to the di- and tetrahydro active forms in the body by the enzyme dihydrofolate reductase (DHFR). Tetrahydrofolate (THF) is converted to 5,10-methylene THF by the activity of serine hydroxymethyltransferase (SHMT) in the liver. Methylene tetrahydrofolate reductase (MTHFR) catalyzes the transformation of 5,10

methylene THF to 5-MTHF. After absorption, dietary folates move through plasma primarily as 5-methyl-THF mono-glutamate. 5-methyl THF is responsible for donating a methyl group to homocysteine. DNA methylation along with the intake of folate are positively correlated (Rampersaud et al., 2000; Pufulete et al., 2005). It is known that DNA methylation is both gene and tissue specific, meaning global DNA methylation can be increased when increasing folate but certain individual genes can be hypomethylated. The pathways determining what becomes hyper- and hypo-methylated is still unknown.

In humans, if folate is not provided adequately, DNA synthesis will be reduced resulting in a reduction in cell division, which will be most evident in cell types that divide rapidly such as red blood cells (WHO and FAO, 2001). The methylation cycle will be altered by increased homocysteine plasma levels because single carbon donors are unavailable for the remethylation of homocysteine.

Choline & Betaine

Unlike many vitamins, choline can be synthesized by poultry in adequate quantities; however, the one exception for this is quail which require 1500 mg per kg (Pesti, 1995). Dietary choline is necessary for the maintenance of egg size laid by quail (Pesti, 1995). While choline deficiency in chickens and turkey often results in perosis and slow growth, quail tend to have bowed legs and swollen hocks (Pesti, 1995). Choline chloride, which is soluble in water and alcohols, is the typical form added to poultry feed.

Choline is a source of methyl groups. Betaine is a metabolite of choline and serves as a methyl donor (Morris, 2001). Choline must be oxidized to betaine to serve as a methyl donor. Betaine like THF, plays a role in one carbon metabolism by the donation of a methyl group to homocysteine which can then be converted into methionine.

In the avian, betaine has been shown to improve the digestibility of methionine and reduce fat deposition (Scott et al., 1982). One possible mechanism for reduced fat alteration was a change in methylation status at the CpG promoter region of *lipoprotein lipase (LPL)*, ultimately decreasing gene expression in the presence of betaine supplementation (Xing et al., 2011). In rats from E11-17, a choline deficient parent diet resulted in global hypermethylation which could be due to hypomethylation of the CpGs associated with Dnmts resulting in an increase in the production of Dnmt1. However, when choline supplementation was given, there was an increase in S-adenosylmethionine found in both tissues (Kovacheva et al., 2007).

Considering adult birds can synthesize sufficient choline, laying hens that did not receive choline after 8 wks were able to synthesize enough choline during their laying period to sustain egg production while the hens that had received choline supplementation required continual supplementation for sustained egg production (Scott et al., 1982). This trend has also been observed with other nutrients such as phosphate requirements. Previous data have indicated that the birds fed a Ca and P-restricted diet during the starter phase utilize Ca and P more efficiently in the grower phase (Yan et al., 2005); however the effect on future generations has yet to be evaluated. It may be that nutrient requirements for offspring vary depending on what the parental generation received. In the future, it would be interesting to evaluate the effects of choline supplementation generationally to determine if the addition or absence of choline effected the requirements of the next generation.

Vitamin B_6

Vitamin B_6 is a compound that can be found in the forms: pyridoxine, pyridoxal, pyridoxamine all of which can be phosphorylated (Ueland et al., 2015). Absorption mainly occurs in the duodenum and jejunum by passive diffusion (Heard and Annison, 1986). Vitamin B_6 is found

in the liver and is mostly converted to the most active form pyridoxal 5'-phosphate (PLP). PLP is a coenzyme form needed for many catalytic functions (Ueland et al., 2015). One of which is the requirement as a cofactor for SHMT which as previously mentioned, converts THF to 5,10 methylene THF (Lamers et al., 2011). The three unphosphorylated forms are converted to a phosphorylated form with the help of pyridoxal kinase (PDXK) and pyridoxamine phosphate oxidase. Within the methylation cycle vitamin B_6 is a coenzyme to SHMT, which as previously mentioned, converts folate derived THF to 5,10 methylene THF.

Riboflavin

Riboflavin, also known as vitamin B_2, is found in three forms: free riboflavin and the coenzymes including flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) (Pinto and Zempleni, 2016). Free riboflavin is absorbed by the mucosal cells in the small intestine and most is converted to FMN by the enzyme flavokinase. Riboflavin then enters the blood as either free riboflavin or FMN. In the serum and eggs, riboflavin is bound by an essential, genetically controlled binding protein (Morris, 2001). A high correlation has been found between the amount of riboflavin in the diet and to the egg albumen indicating a potential for egg fortification. The average concentration of riboflavin within the egg is approximately 4.05 ug per g (Squires and Naber, 1993). Once in tissues most of the FMN is further converted to FAD by FAD-pyro phosphorylase. Although riboflavin is not stored in appreciable amounts, the liver contains the greatest concentration. The role of riboflavin within the methylation cycle occurs when 5,10 methylene THF is further reduced to 5-methyl THF by the enzyme MTHFR from the precursor FAD. A deficiency in riboflavin has been associated with a decreased activity of the flavoenzyme MTHFR and reduced levels of 5-methyl THF (Hustad et al., 2013).

Vitamin B_{12}

Poultry requirements for vitamin B_{12} are minimal varying from 3 to 10 ug per kg of feed (Pesti, 1995). It was determined that dietary levels of vitamin B_{12} could be several hundred times the requirement without the risk of toxicity (Leeson et al., 2001). Vitamin B_{12} in the egg yolk needed to support hatchability is between 1.3 and 2.6 µg/100 g (Squires and Naber, 1993). After 12 weeks, when laying hens were fed a diet with no vitamin B_{12}, one, two, or four times than the NRC requirement, the birds on low amounts of vitamin B_{12} had a decrease in egg production whereas an increase in B_{12} intake resulted in an increase in hen weight, hatchability, and egg weight (Squires and Naber, 1993). Vitamin B_{12} is necessary for one carbon metabolism in the production of methyl donors. Within the methylation cycle, vitamin B_{12} is the coenzyme of methionine synthetase, the enzyme responsible for the removal of the methyl group from 5-methyl THF to regenerate THF and donate a methyl group to homocysteine for the conversion to methionine (Anderson et al., 2012).

Zinc

Zinc (zn), unlike the other methyl catalysts, is a trace mineral and is recognized as an important cofactor in the methylation cycle (Vallee and Falchuk, 1993). When homocysteine is recycled back to methionine, the enzymes betaine homocysteine methyltransferase and 5-methy THF, are known to be zinc dependent. These enzymes catalyze the transfer of a methyl group to homocysteine.

Zn deficiency has been associated with abnormal development and poor progeny health in birds and mammals (Uriu-Adams and Keen, 2010). One possibility could be due the need of an increase in methylation by the developing oocyte (Tian and Diaz, 2013). Zn deficiency is known

to decrease histone and DNA methylation and as a result allow for an increase in expression of repetitive elements (Wallwork and Duerre, 1985).

Evaluation of the epigenetic role of Zn in birds has led to similar findings. Zn is involved in the regulation of inflammation in the gut through the anti-inflammatory zinc finger protein A20 which suppresses nuclear factor kappa beta (NFkB). When maternal birds were supplemented with organic zinc there was improved intestinal characteristics, including an increase in mucin 2 and secretory IgA production in the jejunum of progeny and maternal hens (Li et al., 2015a). NFkB was repressed in the offspring and maternal birds. With Zn supplementation, the A20 promoter region of maternal birds was hypomethylated and H3K9 was hyperacetylated, resulting in activation of A20 expression when compared to the control hens (Li et al., 2015a). Additionally, hyperacetylation and A20 gene expression was observed in offspring at 14d whose mother had received Zn supplementation (Li et al., 2015a).

Maternal stress in broiler breeders, as a result of high temperature, induced negative effects such as embryonic mortality and oxidative damage through epigenetic mechanisms (Zhu et al., 2017). Dietary Zn alleviated the embryonic mortality and resulted in improved antioxidant ability through the activity of metallothionein IV (Zhu et al., 2017). With the addition on Zn the promoter region of metallothionein IV was hypomethylated and there was increased H3K9 acetylation (Zhu et al., 2017).

Methionine

Methionine is not only an essential amino acid in poultry diets, but it serves as the precursor for cysteine and is a key intermediate in the methylation cycle. In typical poultry diets, methionine is one of the most limiting amino acids. In the liver, excess methionine is converted to an active form as s-adenosyl methionine (SAM) by an ATP-driven reaction of the methylation cycle

(Anderson et al., 2012). The end product of this cycle is homocysteine which is a nonprotein-forming amino acid. The enzyme responsible for the conversion of methionine to SAM is methionine adenosyl transferase. SAM is the active universal methyl donor for enzymatic methylation. DNMT enzymes covalently attach methyl groups from SAM to the 5-carbon position on cytosine bases resulting in 5-methylcytosine. SAM is generated through one carbon metabolism which is catalyzed in the presence of methyl donors (Anderson et al., 2012).

Each individual cell is responsible for the synthesis of SAM due to the inability of SAM to cross plasma membranes (Finkelstein, 1998). Once SAM has given up a methyl group it becomes s-adenosylhomocysteine (SAH). SAH is hydrolyzed to homocysteine which can then be remethylated for the production of methionine or trans-sulfuration for the formation of cysteine, taurine, and glutathione. In remethylation, homocysteine accepts a methyl group from 5-methyltetrahydrofolate in the folate and vitamin B_{12} dependent pathway or a methyl group can be accepted from betaine but this occurs predominately in the liver whereas the folate dependent pathway occurs in all tissues (Selhub, 1999). Homocysteine is converted into methionine by the enzymes homocysteine methyltransferase and 5-methyltetrahydrofolate is produced by methylenetetrahydrofolate reductase (MTHFR). Much of the DNA methylation reaction is dependent on the availability of SAM and removal of SAH, known to as the [SAM]:[SAH] ratio which has been referred to as the ‘methylation index’(Waterland, 2006).

Methionine status regulates homocysteine metabolism, resulting in remethylation to yield methionine or trans-sulfuration to yield cysteine. When adequate levels of dietary methionine are supplied, homocysteine recirculates approximately 1.5-2 times before going through the trans-sulfuration pathway but when methionine was halved the number of cycles increased two-fold. When methionine was in excess, homocysteine cycling levels fell below the basal level (Selhub,

1999). Knowing the methyl groups for DNA methylation are ultimately derived from methionine, it would seem logical that increasing the amount of dietary methionine would result in an increase in DNA methylation due to common recycling of methionine; however, in reality, excess methionine may result in the inability of DNA to become methylated by preventing the remethylation of homocysteine (Waterland, 2006). Not unlike studies feeding high methyl catalysts, when high levels (~1.5%) of methionine were fed to rats, tissue specific changes occur with DNA hypo- and hypermethylation to specific gene regions, however, the data for the methylation index [SAM]:[SAH] is inconsistent so overall it is still unclear whether MET diets result in hyper or hypomethylation (Finkelstein and Martin, 1984; Regina et al., 1993; Rowling et al., 2002).

The ability for the amount of homocysteine recycling to be influenced by dietary intake of methionine has indicated a regulation process between remethylation and trans-sulfuration. One part of the regulation process includes SAM acting as an inhibitor to the remethylation pathway by suppressing the synthesis of 5-methyltetrahydrofolate and therefore the transcriptional regulation of MTHFR synthesis, while promoting the activation of enzymes involved in the trans-sulfuration pathway (Selhub, 1999). Another process of regulation are the enzymes involved in the production of SAM. The enzymes responsible for the conversion of methionine to SAM have different affinities for methionine therefore, when methionine is in excess the enzyme with a higher affinity is rapidly active to convert the methionine to SAM for 5-methyltetrahydrofolate to be inhibited (Cabrero et al., 1988).

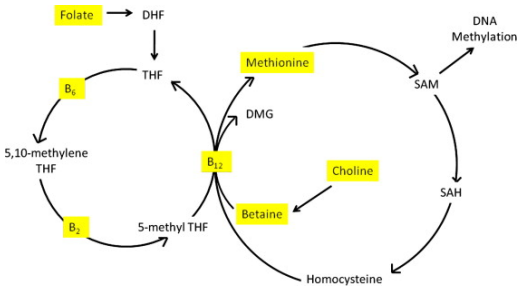

Figure 1.3. Dietary methyl catalysts highlighted in yellow that are involved in one carbon metabolism in the production of S-adenosylmethionine (Anderson et al., 2012).

Transgenerational Epigenetics

Speculation surrounds the parental dietary effects on offspring and questions regarding the transgenerational impact on offspring remain unanswered. Transgenerational epigenetics is the idea that epigenetic modifications can be inherited meiotically from generation to generation. In order for environmentally induced alterations to be considered transgenerationally epigenetic, the effect must span multiple generations. Parental nutrition has been identified as a factor that can influence offspring metabolism and alter phenotypes beyond the first generation (Radford et al., 2012, 2014). The idea that the adaptation, or programming, of parent stock to an environmental stimulus could be transferred to the progeny became known as “Lamarckian” inheritance after the early evolutionary biologist JB Lamarck.

The development of many diseases later in life have been linked to the intrauterine conditions of the mother (Hales and Barker, 2001; Ravelli et al., 1976, 1998; Valdez et al., 1994). The concept that poor nutrition early in life is correlated with poor fetal growth and later development of metabolic disorders is often denoted as the “thrifty phenotype hypothesis (Hales and Barker, 2001). There are many examples of epigenetic events in nature including the Dutch Hunger Winter where food supplies were blocked from going into the Netherlands and a period of famine was clearly defined. Individuals went from 2000 to 500 calories per day. Health records from this period still exist for this population. Women who were pregnant during this time period

gave birth to children whose metabolism, cardiovascular health and age-related cognitive function were affected by the famine throughout their lives (Schulz, 2010).

In certain areas of the human genome, a permanent epigenetic pattern is established during development on metastable epialleles, which is found in all the cell lines of an individual and the epigenetic variation is different from genetically identical individuals (Waterland et al., 2010). This has indicated that interferences in epigenetic patterns can result in a changed phenotype and the possibility that epigenetic variation at metastable epialleles may play a role in the probability of disease. An example of this has been observed in rural African Gambian villages where there is an annual season of hunger. Individuals born during the hungry season are 10 times more likely to die earlier in life due to the effect of malnutrition has on nutritional programming of the immune system in the developing embryo (Moore et al., 1997). It was found that DNA methylation at metastable epialleles was higher in individuals conceived during the hungry season. Thus, leading to evidences that methylation of DNA is systematic and occurs at specific loci in the human. It would be possible to observe similar patterns in other populations which have undergone a nutritional deficiency for a season.

The evaluation of a less complex organism, the *C. elegans,* has led to the accumulation of data regarding long term epigenetic effects. In one such example, the environmental impact of extreme temperature fluctuation resulted in gene expression alteration which was transmitted across more than ten generations (Klosin et al., 2017). A more complex example is a mutation ensuing the absence of the H3K4 demethylase LSD1 resulting in progressive sterility that is maintained for many generations (Katz et al., 2009). The sterility was thought to be due to the accumulation of H3K4me3 at loci involved in spermatogenesis, alluding to the essential role reprogramming plays during development. Many models of epigenetic modification have led to

the opinion that a single modification in the epigenomic landscape can result in an extreme phenotype. While this may be the case in some situations, many times it can occur over a time such as approximately 20 generations in this instance. These examples, although not always directly applicable to other species, have given insight into how environmental conditions during the time of development can influence the offspring for many generations.

Beyond providing DNA material for the next generation, parents provide bioactive molecules in the oocyte and sperm along with required nutrients for growth and development. The environmental conditions to which parents were exposed could result in epigenetic modifications of the parental genome thereby potentially preprogramming the offspring during development to withstand similar environmental conditions. In certain conditions this could be beneficial to offspring for a quick adaptation to an environmental stimulus that could potentially increase the chance of survival for the next generation. In contrast, poor nutrition during early stages of life could result in persistent negative alterations. Regardless of the environmental stimuli resulting in transgenerational effects, epigenetic modification involves chemical modifications of DNA or of structural and regulatory proteins bound to DNA that result in expression or repression of genes at various loci. Therefore, the changes in expression are not due to changes in the nucleotide sequence itself.

Many metabolic pathways can be altered by the availability of nutrients, although it appears reprogramming of mitochondrial activity is essential in allowing the developing embryo to survive a limited nutrient environment (Pejznochova et al., 2010). Following mitochondria activation to produce energy for the embryo, there is an increased production in reactive oxygen species (ROS) and oxidative stress, which can have detrimental effects on cells (Lattuada et al., 2008). If the embryo is restricted from essential nutrients, oxidative stress can be detected in multiple tissues

due to dysfunction of the mitochondria which may result in the later development of disease (Petersen et al., 2004).

Epigenetic changes associated with nutrient availability in the offspring has led to further questions regarding the mechanisms behind the alterations of the epigenetic landscape for the expression or suppression of specific genes due to the early embryonic environment that results in long term changes in phenotype. It could prove beneficial to understand the specific modifications which contribute to changes in phenotypes. As oviparous organisms, avian species could serve as interesting candidates to evaluate transgenerational inheritance because the embryo is developing in an incubator with many external environmental influences of the embryo's primordial germ cells. This is unlike mammals, wherein the primordial germ cells of the embryo are exposed to the same environmental conditions as the parent. These ideas could be a useful tool to the poultry industry by controlling the environmental conditions of the parents to better prepare the offspring for future environmental conditions in the hopes of producing a healthier bird with improved performance.

Avian Epigenetics

The chicken genome is less than half the size of mammalian genomes and is comprised of approximately 20,000-23,000 genes and approximately 1200 million base pairs (Hillier et al., 2004; Wicker et al., 2005). When compared to that of mammal's, the chicken genome has a 3-fold decrease in size due to the less interspersed repeat content, pseudogenes, and segmental duplications (Hillier et al., 2004). The chicken genome is made up of 38 paired autosomes and one pair of sex chromosomes (Z and W).

Many of the same mechanisms mentioned in the previous sections apply to vertebrates, not just mammals. However, less is known about epigenetic mechanisms in the avian. In general when

evaluating the DNA methylation profiles of the chicken it was found that the DNA methylation pattern follows the same rules as that of mammals and plants with hypermethylation found in repetitive regions and hypomethylation in most of the CpG islands (Asp et al., 2011). Approximately 38% of CpG islands are conserved between the human and chicken (Hillier et al., 2004). Decreased DNA methylation levels have been observed in promoter regions and transcriptional start sites and increased DNA methylation often occurs in the gene body regions (Hu et al., 2013). However, differences in methylation have been found between different tissues and strains of the avian, with hepatic DNA being less methylated when compared to the muscle (Xu et al., 2007).

Mandel and Chambon (1979) used CpG restriction enzymes first described by Bird and Southern (Bird, 1978) to evaluate the DNA methylation associated with three genes which are responsible for proteins found in egg albumen: ovalbumin, conalbumin, and ovomucoid. When these genes were evaluated in the laying hen, minimal methylation was detected in the oviduct where they are typically expressed and the highest amount of methylation was detected in the sperm where expression of these genes would not occur (Mandel and Chambon, 1979). This indicates the typical characteristic of an increase in DNA methylation leading to a decrease in gene expression due to the inability of transcription machinery to gain access to the promoter region to transcribe the DNA.

Maternal supplementation of either organic or inorganic Zn may epigenetically modify offspring by protecting them from damage caused by stressors. Zn sources Zn fed to broiler breeder hens resulted in an 11% increase in healthy chicks and eliminated the embryonic mortality induced by heat stress (Zhu et al., 2017). Both organic and inorganic Zn sources resulted in higher levels of global DNA methylation and lower global levels of histone 3 lysine 9 acetylation within the

liver (Zhu et al., 2017). However, it is important to note that some analyzed regions experienced the opposite effect, such as the promoter region of the MT4 gene which is involved in zinc metabolism. Previous reports in humans have indicated that lower levels of global DNA methylation caused by insufficient maintenance methylation can be associated with abnormal embryonic development (Yin et al., 2012).

Summary

Epigenetic modifications are essential for bacteria, plants, and mammals. In other organisms, such as drosophila, the importance of epigenetics is not yet clear.

The many examples discussed above have demonstrated that the intrauterine environment plays a role in the growth and development of the embryo along with the subsequent development of diseases. Considering birds are oviparous, it is possible that the environment the egg is exposed to including nutrients, temperature, humidity, UV lighting etc., could result in similar long-term modifications. In the maternal models, some of the earliest molecular events associated with nutrient restriction are mitochondrial dysfunction and oxidative stress and provide a link to adult onset disease through possible alterations in epigenetic marks.

It is important to note that any research study conducted to evaluate epigenetic alterations in the genome or phenotype could also be altered by many other variables. There are many unknowns when it comes to the bird. In the future, there is a need for the poultry science community to evaluate areas of the genome which are epigenetically altered by environmental conditions such as nutrition and stress. These areas may indicate some key regulators in environmental conditioning of the avian which could be beneficial to a broad area of disciplines such as how parental environmental stress can directly impact immune function, the effect of incubation protocols, or the transgenerational impact of parental nutrition status on progeny.

It is my belief that epigenetic programming of breeders in poultry presents a new field for realizing potential gains in health and performance of the offspring. For example, studies by Yan et al. (2005) indicate the possibility that feeding broiler breeders reduced Ca:P ratios may lead to a decreased need for these nutrients in the next generation. Studies such as these could be used within the poultry industry to help producers maximize the potential for the birds. However, there is a need for a basic understanding of the machinery involved in pathways resulting in epigenetic modifications and potential transgenerational phenotypic responses. To initiate progress towards fulfilling this need, the following research was conducted.

Chapter 2

Analysis of Genome-Wide DNA Methylation in *Coturnix japonica* in Response to Dietary Methyl Catalysts

Abstract

To provide a better understanding of DNA methylation in poultry, the genome-wide DNA methylation profile of female Japanese Quail (*Coturnix japonica*) in liver tissue was characterized using whole genome bisulfite sequencing. The methylation of DNA requires enzymes and many dietary micronutrients referred to as methyl catalysts. Studies assessing parental methyl catalyst supplementation have indicated that parental nutrition plays a role in shaping the epigenetic pattern of the offspring. It was hypothesized that a diet with a greater concentration of methyl catalysts (HiMet) could alter DNA methylation in progeny compared to a control (CON) when fed to the parent generation. The methyl catalysts included 7030 mg/kg choline chloride, 5 mg/kg betaine, 1.5 mg/kg vitamin B12, 7.5 mg/kg folic acid, 12 mg/kg pyridoxine, and 99 mg/kg zinc sulfate in addition to nutrients endogenous to the dietary feedstuffs. In the following study, quail parents were fed either the HiMet or CON diet in the starter and layer diets, yielding the following treatments: HiMet-HiMet, HiMet-CON, CON- HiMet, and CON-CON. For the parent generation of this multi-generational trial, 300 Japanese Quail were placed in a brooder at day of hatch. A total of 150 chicks received the CON diet while the remaining 150 received the Hi-Met diet. At 6 wk, half of each group continued consuming the same diet while the other half switched to the other dietary treatment. Two generations of progeny from both treatments, which were all fed a control diet with typical methyl catalyst levels, were evaluated. Liver gDNA was isolated, pooled and bisulfite converted for 12 samples from the three generations of female quail and four treatments. After conversion, single stranded DNA was used to create gDNA libraries using TruSeq DNA Methylation Kit. Libraries were sequenced using Illumina NextSeq with 75bp paired end runs with 800 million paired end reads total. DNA sequence reads were mapped using CLC Genomics workbench. Percent CpG hypermethylation and hypomethylation was determined for

each generation and treatment. Overall vast differences in global CpG DNA methylation were not detected between groups however differences were detected locally when evaluating methylation levels of genes involved in the DNA methylation cycle. Transgenerational phenotypic changes occurred in egg size throughout the three generations. Epigenetic changes in modifications to DNA associated with nutrient availability in the offspring leads to further questions regarding the mechanisms behind the alterations of the epigenetic landscape for the expression or suppression of specific genes. These questions include do offspring remember an environmental condition to which they were never exposed and how does this related to an organism's ability to adapt to conditions to which the parents were exposed, and what epigenetic modifications are made to the offspring genomic regions due to parental environmental exposures? Further research is warranted on the manipulation of breeder diets to impart positive epigenetic effects on progeny. This could be a useful set of tools to the poultry industry by manipulating the environmental conditions of the parents to better prepare the offspring for future environmental conditions.

Introduction

Healthy embryos and progeny are dependent on the environment in which development occurred. One way the organism responds to the environment is through epigenetic modifications of DNA such as DNA methylation. This is accomplished by the covalent addition of a methyl group to the 5-carbon ring on cytosine. This process can alter expression of genes without changing the DNA sequence. The maternal epigenome can contribute to optimal embryonic development and chick quality. The chick spends a significant portion of its life in the egg and many environmental conditions such as humidity, temperature, lighting, yolk hormones and nutrition provided during development can alter the quality of the chick (Ho et al., 2011). The avian egg contains all of the required nutrients for the embryo to develop during incubation. If nutrients are in abundance or provided adequately, this may result in more efficient deposition of nutrients that are transferred to the embryo by way of the egg (Hoey et al., 2009; Naber, 1979; Squires and Naber, 1993; Wilson, 1997) . This can result in a healthy chick at hatch and possibly long-lasting advantages to the offspring such as improved immune function (Wilson, 1997). A potential advantage to adequate maternal nutrition is offspring that could be better equipped for survival and productivity amongst the many stressors they may encounter.

Studies on a variety of species have observed changes in DNA methylation patterns resulting from environmental influences that the mother and/or father was exposed to, such as diet, toxicants, or stress (Hanson and Skinner, 2016). Many factors regarding how these environment influences affect an individual are still unclear. Furthermore, environmental inputs that alter DNA methylation do not affect the genome in the same way. Specific regions of the genome become hypomethylated, others are hypermethylated, while some areas are not affected at all by the same treatment (Bird, 2002). Specific methylation patterns also depend on the tissue being evaluated

(Xu et al., 2007). Identifying the areas influenced by extrinsic factors could broaden our understanding on how environmental factors not only influence that individual but their offspring, as well.

Nutrition provided during different stages of life can alter epigenetic regulation and may influence how an organism utilizes certain nutrients (Choi and Friso, 2010; Lillycrop et al., 2014; Rando and Simmons, 2015). The methylation of DNA requires enzymes and many dietary micronutrients referred to as methyl catalysts. Methyl catalysts involved in the methylation pathway consist of folic acid, riboflavin, vitamin B_6, vitamin B_{12}, betaine, choline and zinc. These nutrients and cofactors are required for the methionine conversion to the universal methyl donor S-Adenosylmethionine (SAM) (Finkelstein, 1998). Through interaction with DNA methyl transferases, SAM provides a methyl group for the direct methylation of DNA.

Parental nutrition has been identified as a factor that can influence offspring metabolism and alter phenotypes beyond the first generation (Radford et al., 2012, 2014). Studies assessing maternal methyl catalyst supplementation *in utero* have clearly indicated that maternal nutrition plays a role in shaping the epigenetic pattern of the offspring (Waterland and Jirtle, 2003). This has been demonstrated by changed coat color phenotype of the offspring when maternal mice were supplemented with methyl catalysts before and during gestation (Wolff et al., 1998). The viable yellow (A^{vy}) mouse has a single gene mutation at the agouti gene and has a predisposition to obesity and tumor susceptibility (Yen et al., 1994). When the agouti gene was cloned it was revealed to encode a protein with an insertion of intracisternal A particle (IAP) retrotransposon which results in yellow pigmentation (Duhl et al., 1994). The nutritionally induced change in coat color phenotype of mice was later identified to be caused by an increase in methylation at the agouti locus (Waterland and Jirtle, 2003). The methyl-supplemented diet resulted in more brown

offspring while the low methyl diet resulted in yellow offspring. The coat color change was due to hypo- or hypermethylation of the agouti gene IAP region (Waterland and Jirtle, 2003). As the percentage of methylated CpG regions of A^{vy} increased, the coat color was found to progressively change, from clear yellow (CY), slightly mottled yellow (SMY), mottled yellow (MY), heavily mottled yellow (HMY) and pseudoagouti (PA) (Waterland and Jirtle, 2003).

DNA methylation alterations can occur in diets deficient in methyl catalysts as well. In post weaning mice this deficiency caused hypomethylation of IGF2 resulting in permanent loss of imprinting and dysregulation of transcription (Waterland et al., 2006b). In rats, a methyl catalyst deficient diet for only 4 wks resulted in hypomethylation of genes that play a role in growth regulation which persisted 3 wks after the diet had been supplemented (Christman et al., 1993). Conditions where poultry are not given adequate methyl catalysts result in reduced BW, feed intake, FCR, perosis and the failure of normal protein synthesis (Scott et al., 1982). The question in poultry remains, however, whether the only consequence of these nutrient inadequacies is simply reduced productivity or a more complicated cascade of pathways resulting in epigenetic modifications that could impact generations – for better or worse.

Speculation surrounds the parental dietary effects on offspring and questions regarding the transgenerational impact on the offspring remain unanswered. Transgenerational epigenetics is the idea that epigenetic modifications can be inherited meiotically from generation to generation. Japanese quail serve as a useful transgenerational model for chickens due to their small generation time as well as their similar genome size (1.2×10^9 base pairs) and the same karyotype of $2n = 78$ chromosomes, consisting of macrochromosomes and microchromosomes (>20Mb) (Sasazaki et al., 2006). Homology of chromosomes and gene order has been observed as being similar between the two species (Schmid et al., 2000; Shibusawa et al., 2001). The chicken genome is less than half

the size of mammalian genomes and is comprised of approximately 20,000-23,000 genes and approximately 1200 million base pairs (Hillier et al., 2004; Wicker et al., 2005). When compared to that of mammals the chicken genome has a 3-fold decrease in size due to the less interspersed repeat content, pseudogenes and segmental duplications (Hillier et al., 2004). A total of 20,244 CpG islands have been detected in the chicken using MeDip-Seq (Li et al., 2011).

By using two populations of Japanese Quail, one fed a standard control diet and the other fed a methyl catalyst supplemented diet, this allowed for the evaluation of the transgenerational epigenetic effect that an increase in DNA methylation has on phenotypic parameters such as egg weight and hatch weight along with molecular parameters including global CpG DNA methylation and the methylation level of genes involved in the methylation pathway. We show that diet can affect genotypes and phenotypes for generations.

Materials & Methods

Experimental Birds and Housing

The study was conducted at the Prestage Department of Poultry Science's Animal Facilities in Scott Hall at North Carolina State University. For all three generations, quail chicks were hatched from eggs obtained from North Carolina State University's residential meat-type Japanese Quail lines in Scott Hall. At placement, each brooder level was equipped with two side feeders, two plastic jar drinkers, and one supplemental feed tray. The supplemental feed tray was removed at 14 d. Birds were raised in thermostatically controlled battery-style brooders from 0-21 d and battery-style layer cages with raised wire flooring after 21 d. Room temperature at chick placement was 32.2°C (90°F). Air temperature was reduced daily until 7 d of age and was then maintained at approximately 23.9°C (70°F). Lighting was provided for 23 h from 0 – 3 d, 20 h from 4 – 10 d and

18 h from 11 d until the end of that generation. All birds were cared for according to North Carolina State University Institutional Animal Care and Use Committee (IUCAC) guidelines.

Generation 0

For the first generation (G0) of this multi-generational trial, 324 newly hatched Japanese Quail were weighed and randomly divided into four equal groups and placed in four separate brooder cages. Two brooder levels were given a starter feed that contained high levels of methyl catalysts while the other two levels were given a control feed that contained standard levels of methyl catalysts (Figure 2.1). The methyl catalyst package included an additional 7030 mg/kg choline chloride, 5 mg/kg betaine, 1.5 mg/kg vitamin B12, 7.5 mg/kg folic acid, 12 mg/kg pyridoxine, and 99 mg/kg zinc sulfate (Table 2.1). This provided 10 times the amount found in the control diet. The control diet was formulated to meet NRC quail requirements for typical growth and reproduction. Chicks were given feed and water *ad libitum*. Individual chicks were weighed weekly for a group body weight while in the brooder from hatch until 3 wks.

At three weeks of age birds were moved to 32 cages containing 4 females and 1 male per cage. From the two groups of either high or low methyl starter, 40 birds (32 females and 8 males) from each group were fed high or low methyl layer diets (Table 2.1). This resulted in four total groups of high methyl starter to low methyl layer, high methyl starter to high methyl layer, low methyl starter to high methyl layer and low methyl starter to low methyl layer. A total of 160 birds were placed in cages. Each generation consisted of four treatment groups indicated by the diet G0 received in each dietary phase: High Starter High Layer (HiMet-HiMet), High Starter Control Layer (HiMet-Con), Control Starter High Layer (Con-HiMet) and Control Starter and Control Layer (Con-Con) (Figure 2.1). Individual birds were weighed in layer cages for a group BW at 4 wks, 5 wks and then again at 16 wks. For all generations, bird sex was not separated for the BW

measured in the brooder because they were too young to differentiate. However, once birds were moved to layer cages, 4 females and a single male were weighed for each cage.

Table 2.1. Composition of basal starter and layer diets (HiMet-HiMet, HiMet-Con, Con-HiMet, and Con-Con).

Ingredients (%)	Starter	Layer
Corn	49.18	48.56
Soybean meal (48% CP)	32.29	37.83
Poultry By-Product Meal	14.03	----
Soybean Oil	1.00	3.82
Mono-Dicalcium phosphate	0.04	1.60
Limestone	1.02	5.85
Salt	0.50	0.48
DL-Methionine	0.33	0.30
Choline chloride (60%)	0.10	0.10
L-Threonine	0.01	0.06
Vitamin premix[2]	0.10	0.10
Mineral premix[3]	0.20	0.20
PrimaLac	0.05	----
Lasolocid	0.05	----
Celite/Methyl Package	1.10	1.10
Calculated nutrient content		
Crude protein	27.90	22.60
Calcium	1.00	2.60
Available phosphorus	0.50	0.45
Total methionine	0.74	0.64
Metabolizable energy (kcal/kg)	2,850	2,800

[1]Starter diet was fed to approximately 6 wk of age
[2]Vitamin premix supplied the following per kg of diet: 13,200 IU vitamin A, 4,000 IU vitamin D_3, 33 IU vitamin E, 0.02 mg vitamin B_{12}, 0.13 mg biotin, 2 mg menadione (K_3), 2 mg thiamine, 6.6 mg riboflavin, 11 mg d-pantothenic acid, 4 mg vitamin B_6, 55 mg niacin, and 1.1 mg folic acid.
[3]Mineral premix supplied the following per kg of diet: manganese, 120 mg; zinc, 120 mg; iron, 80 mg; copper, 10 mg; iodine, 2.5 mg; and cobalt, 1 mg.
[4] PrimaLac is a probiotic manufactured by Star-Labs/Forage Research, Inc.
[5]Lasolocid is an anticoccidal with the trade name Avatec

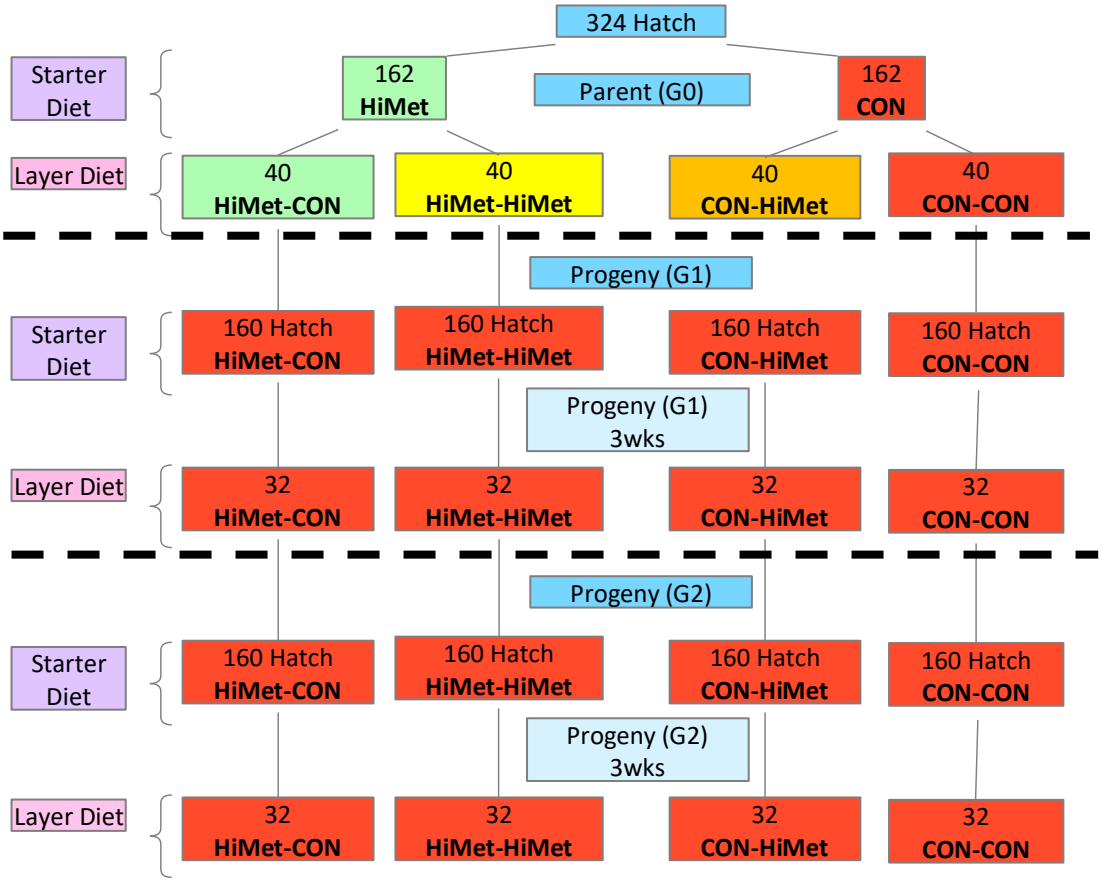

Figure 2.1. Outline of Transgenerational Trial Design.

At 3 wks when birds were moved to layer cages and at 16 wks when they were removed to make room for the next generation, liver samples were collected randomly from females for each of the 32 cages and treatment groups. At 3 wks of age male gonads were also sampled for starter dietary treatment. Genomic DNA (gDNA) was isolated from six liver samples from female quail representing each of the four treatment groups using Qiagen DNeasy Blood and tissue kit according to the manufacturer's protocol (Qiagen, Valencia, CA). Genomic DNA was then pooled by treatment for bisulfite conversion and sequencing with a total of one biological replicate for each generation and treatment. The DNA quality was assessed by Nanodrop 2000 spectrophotometer (Thermo, USA).

Eggs were counted and collected daily from the birds in grow-out cages. Starting at 9 weeks of age, eggs were weighed daily for 7 wks and average egg weight was calculated. Eggs from the four parental treatment groups (HiMet-HiMet, HiMet-CON, CON-HiMet, CON-CON) were collected at 12 wks of age for 7 d and approximately 20 eggs per cage were incubated for 18 d to hatch G1 progeny.

Generation 1 & 2

Generation 1 (G1) consisted of 640 chicks from the four G0 treatment groups (HiMet-HiMet, HiMet-CON, CON-HiMet, CON-CON). All chicks were individually weighed and grouped based on G0 treatment. For placement, 10 chicks from each parent pen placed in the 5 brooders were selected based on BWs that represented average hatch weight from the previous generation's hatch weight (10 g). These 10 chicks from each of the 32 parental groups were tagged and identified by parental cage number. Two chicks from each parent cage were randomly placed in 5 separate brooder levels. Thus, each of the 5 brooder levels housed an equal mixture of properly identified chicks from each of the 4 parental treatments. All chicks were fed the same control diet fed to the G0 CON-CON treatment. The same methods were followed for generation 2 (G2).

At 3 wks of age four females from each parental cage were randomly selected and placed in the same cage group as their mother. One male from the same G0 dietary treatment but from a different cage group to avoid inbreeding was assigned to the three females. This marked the beginning of the layer phase. At this time, sex-separate BW data were collected for G1 and G2. Liver samples from birds not placed in layer cages were collected randomly from both males and females from each of the G1 and G2 groups. Liver gDNA was isolated using Qiagen DNeasy Blood and tissue kit from female birds and later pooled by treatment for bisulfite conversion and sequencing. DNA quality was assessed by Nanodrop 2000 spectrophotometer (Thermo, USA).

Similar to G0, eggs were counted and collected daily from each group of birds in layer cages beginning at the start of lay for G1 and for G2 for approximately 8 wks for G1 and 5 wks for G0. Eggs from G1 & G2 were collected and set for incubation following the same procedures used for G0.

Bisulfite Conversion & Sequencing

Currently, the best-known method to determine DNA methylation is through genome wide bisulfite-conversion and sequencing. During bisulfite conversion, all unmethylated cytosines are converted into uracil while methylated cytosines are protected from the conversion process. Uracils are read as thymines by DNA polymerase and when bisulfite treated DNA is amplified using PCR, the products of unmethylated cytosine appear as thymines. Methylated and unmethylated cytosines can therefore be distinguished after sequencing. A total of 12 samples representing each of the four treatments from the three generations of female Japanese Quail were subject to bisulfite conversion. 100 ng liver gDNA from each sample was prepared for bisulfite conversion. Liver samples for G0 were taken at 16 wk and for G1 and G2 at 3wks.

Bisulfite conversion was accomplished using EZ DNA Methylation-GoldTM Kit by Zymo following the manufacture's protocol. After conversion, 50 ng of single stranded DNA was used to create gDNA libraries using TruSeq DNA Methylation Kit following the manufacture's protocol (Illumina, San Diego, Ca). Libraries were sequenced using Illumina NextSeq with 75bp paired end runs with 800 million paired end reads total. A total of 12 samples were sequenced with two runs of NextSeq-75 PE run with 800 PE reads total.

Genomic DNA from female liver and male gonad were taken at 3 wks for the starter phase of G0 and sent to Zymo Research for bisulfite conversion, sequencing and data analysis.

Reference Genome and Gene Annotation

The *Coturnix japonica* 2.0 reference genome along with annotations was downloaded from NCBI (https://www.ncbi.nlm.nih.gov/assembly/GCF_001577835.1).

Sequence Alignment and Data Analysis

DNA Sequence reads were mapped using CLC Genomics Workbench Bisulfite Sequencing Plugin (Version 11; Qiagen, Valencia, CA, USA; licensed to NCSU) following the software manual (http://resources.qiagenbioinformatics.com/manuals/clcgenomicsworkbench/current/User_Manual.pdf). 800 bp sequencing reads and resulting FASTQ files were aligned to the *Coturnix japonica* reference genome. Due to the complexity of BS-seq alignments, standard sequencing alignment software was not used. Both cytosine and thymine must be considered as potential matches to a genomic cytosine. This process allows for high sensitivity because what is present is typically utilized. However, this can cause a bias towards methylated sequences because these carry more information than unmethylated sequences and they are aligned with higher efficiency, resulting in an overestimation in methylation levels. To alleviate this issue, any residual cytosines in the BS treated sequences and all cytosines in the reference genome were converted to thymines before alignment, making the sequence that is aligned an exact match and unaffected by methylation status. The two strands of DNA in the reference genome must be considered separately. The reference genome is converted to a CT and GA version and the read pairs are individually mapped to both. The better of the two mappings is reported as final mapping result.

Statistical Analysis

Simple means of relevant measurements for all generations of quail from HiMet-HiMet, HiMet-CON, CON-HiMet, CON-CON treatments were analyzed using the One-way ANOVA function of the Basic Analysis platform of JMP 13.2.1 (SAS Institute, Cary, NC). Differences were

considered statistically significant at $P \leq 0.05$ and significantly different means were separated using Tukey's test. A total of 32 pens were utilized in this study and each was considered an experimental unit resulting in *n=8* for each treatment for phenotypic parameters measured.

Statistical analysis for CpG DNA methylation sites for the three generations of quail from HiMet-HiMet, HiMet-Con, Con-HiMet, Con-Con treatments in this study were analyzed as a 3 x 4 factorial arrangement with the Standard Least Squares ANOVA within the Fit Model platform of JMP 14 (SAS Institute, Cary, NC). Differences among means were considered statistically significant when $P \leq 0.05$ and means were separated using Tukey HSD-test in JMP 14. A total of 12 samples were utilized in this study, therefore *n=3* for each treatment main effect, *n=4* for each generation main effect, and *n=1* for each interaction.

Results

Parent Flock – Body Weight

Following 18d of incubation, 324 quail chicks were weighed at hatch and no significant difference in BW was observed (Figure 2.2). The birds were randomly placed in the brooder and by 7 d the birds fed high methyl starter diet weighed 30g and were significantly heavier than those fed the control starter diet that weighed 29g ($p<0.058$; Figure 2.2). At 14 d, no statistical difference in BW was observed. However, when birds reached 21 d, those consuming the high methyl starter diet weighed 94g and were significantly heavier than those on the low methyl starter diet that weighed 86g. Following the transfer to layer cages, individual birds were weighed to calculate an average group weight for each pen at 4, 5, and 16 wks. No significant differences in BW were observed at any time point in the layer phase (Figure 2.3). No differences in mortality were detected.

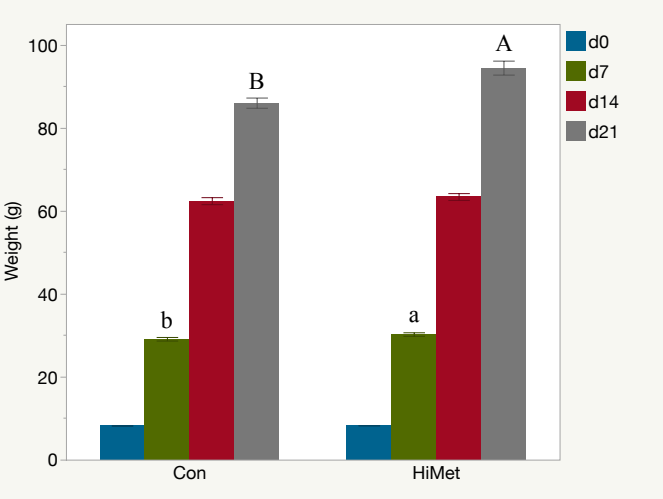

Figure 2.2. BW of G0 as affected by the high methyl starter or the low methyl starter during starter phase; P<(0.0001) and P<(0.0500). Each error bar is constructed using 1 standard error from the mean.

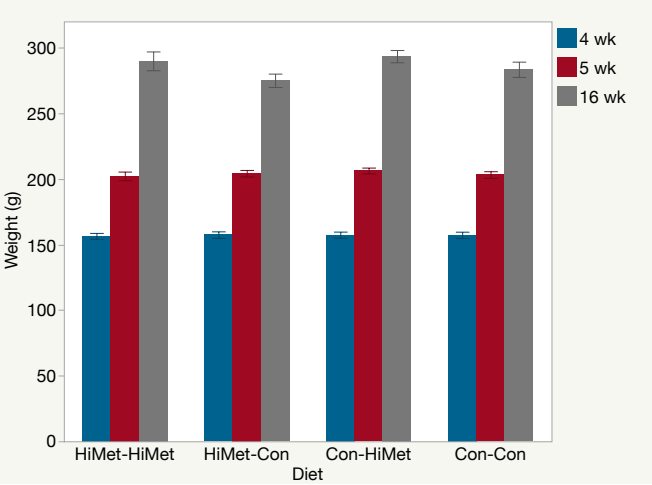

Figure 2.3. BW of G0 as affected by dietary treatment during the layer phase. Each error bar is constructed using 1 standard error from the mean.

Parent Flock – Egg Weight

Eggs laid by quail from the HiMet-HiMet treatment and those from the Con-HiMet were significantly heavier (12.61 g and 12.61 g, respectively; p<0.0001) than those laid by birds from both the HiMet-Con and Con-Con treatments (12.34 g and 12.31 g, respectively; Figure 2.4). Although there were differences in the weight of eggs there were no significant differences in the number of eggs laid between treatment groups (Table 2.2). Weekly eggs laid per hen for the HiMet-

HiMet, HiMet-Con, Con-HiMet and Con-Con group were 5.43 eggs/hen/week, 5.73 eggs/hen/week 5.75 eggs/hen/week, and 5.32 eggs/hen, respectively (Table 2.2).

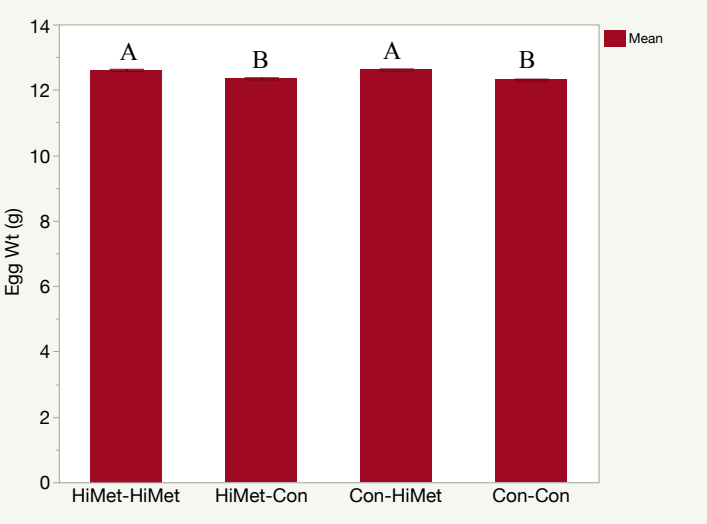

Figure 2.4. G0 Average egg weight between 9-16 wks of age as affected by dietary treatment in the brooder and layer phase; P<0.0001. Each error bar is constructed using 1 standard error from the mean.

Table 2.2. Number of Eggs for G0 by treatment measured for 7 consecutive wks beginning at 9 wks of age.

	Eggs/Hen/Week	Std Error
HiMet-HiMet	5.43	7.40
HiMet-Con	5.73	7.40
Con-HiMet	5.75	7.40
Con-Con	5.32	7.40
p-value	**0.5962**	

Generation 1 – Body Weight

All progeny was fed the same standard control diet given to the G0 Con-Con group through the entirety of the brooder and layer phases. Generation 1 (G1) consisted of 640 chicks from the four G0 treatment groups (HiMet-HiMet, HiMet-Con, Con-HiMet, Con-Con). At hatch the HiMet-HiMet group weighed 8.8645 g and the Con-HiMet group weighed 8.8442 which was significantly heavier (p<0.0001) than the HiMet-Con group weighing 8.5695 g and the Con-Con group weighing 8.4556 g (Figure 2.5). No significant differences were observed at 1, 2 or 3 wks in the

brooder (Figure 2.5). At 3 wks of age, birds were moved to layer cages and BW was measured at 4 and 5 wks. Significant differences were not observed (Figure 2.6). No differences in mortality were detected.

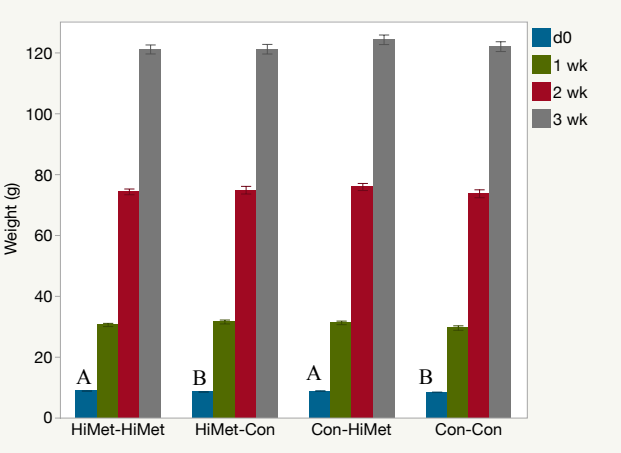

Figure 2.5. G1 BW during starter phase; P<(0.0001). Each error bar is constructed using 1 standard error from the mean.

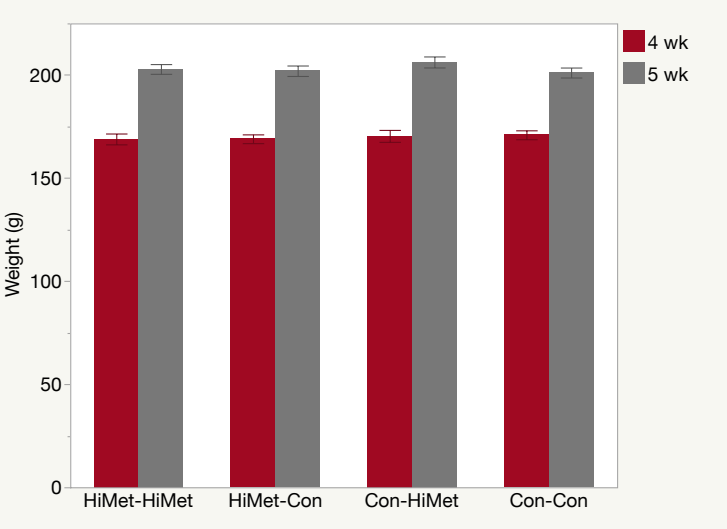

Figure 2.6. G1 BW during layer phase. Each error bar is constructed using 1 standard error from the mean.

Generation 1 – Egg Weight

Eggs were counted and collected daily from each group of birds in layer cages beginning at the start of lay for G1 (Figure 2.7 & Table 2.3) for 8 wks. Eggs from birds whose parents from the HiMet-HiMet and HiMet-Con treatments laid significantly heavier eggs (11.98 g and 12.08 g,

respectively) than the progeny from the HiMet-Con and Con-Con parent generation (11.83 g and 11.82 g, respectively) where p<0.0001 (Figure 2.7). Although differences in egg weight were observed, there were no significant differences in the number of eggs laid between treatment groups (Table 2.4). Weekly eggs laid per hen each week for the HiMet-HiMet, HiMet-Con, Con-HiMet and Con-Con group were 4.52 eggs/hen/week, 4.70 eggs/hen/week, 3.91 eggs/hen/week, and 4.41 eggs/hen/week respectively (Table 2.3).

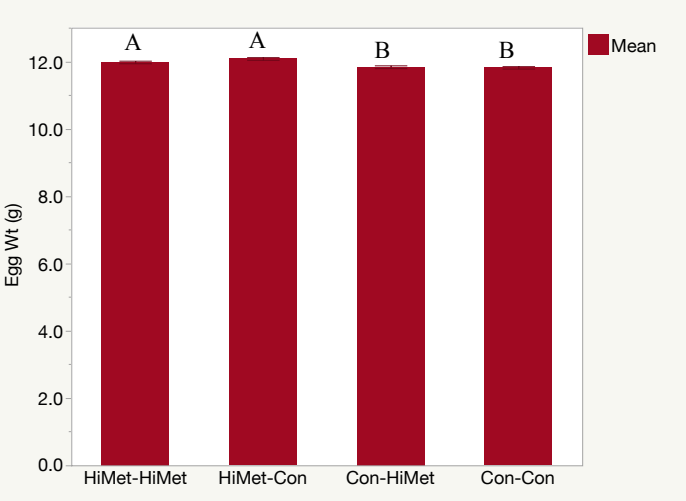

Figure 2.7. G1 Average egg weight measured from the start of lay (6wks-14wks); P<0.0001. Each error bar is constructed using 1 standard error from the mean.

Table 2.3. Number of Eggs for G1 by treatment measured for approximately 8 consecutive wks beginning at 6 wks of age.

	Eggs/Hen/Week	Std Error
HiMet-HiMet	4.52	10.21
HiMet-Con	4.70	10.21
Con-HiMet	3.91	10.21
Con-Con	4.41	10.21
p-value	**0.7757**	

Generation 2 – Body Weight

Generation 2 (G2) was fed the same standard control diet consumed by the G0 Con-Con group treatment. G2 consisted of 640 chicks hatched from G1and continued to be differentiated

by the four G0 treatment groups: HiMet-HiMet, HiMet-Con, Con-HiMet, Con-Con. Evaluation of BW from hatch through 8wks indicated no significant differences in the brooder or layer phases (Figure 2.8 & Figure 2.9). No differences in mortality were detected.

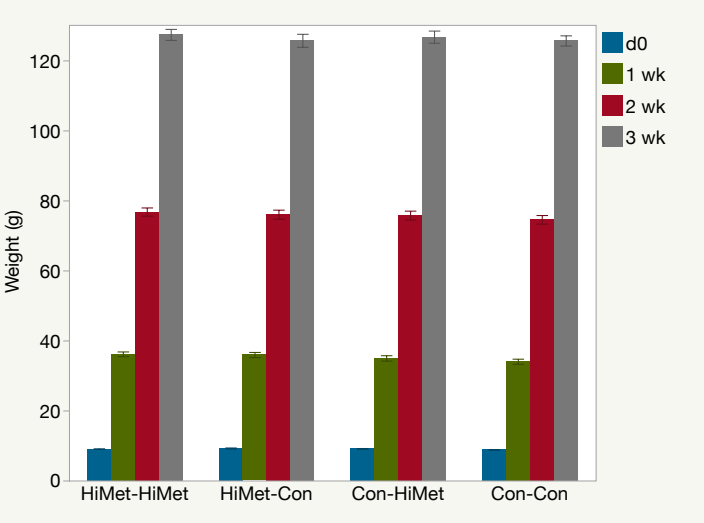

Figure 2.8. G2 BW during starter phase. Each error bar is constructed using 1 standard error from the mean.

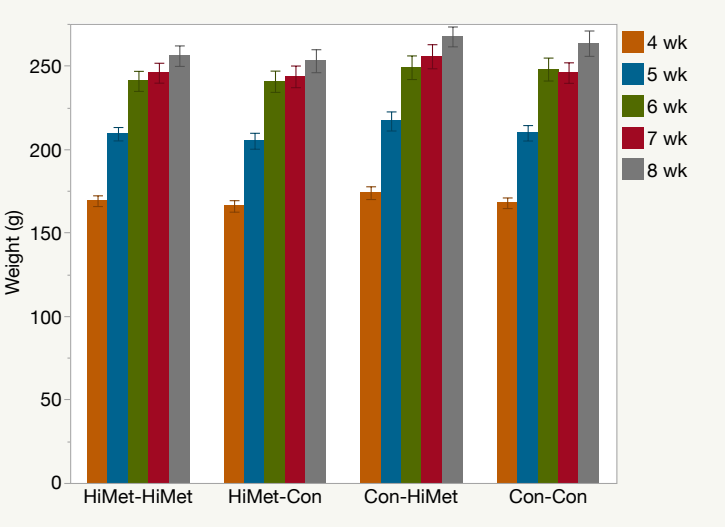

Figure 2.9. G2 BW during the layer phase. Each error bar is constructed using 1 standard error from the mean.

Generation 2 – Egg Weight

Similar to previous generations, eggs were counted and collected daily from each group of birds in layer cages beginning at the start of lay for G2 (Figure 2.10 & Table 2.4) for approximately

5 wks. Somewhat opposite effects on egg weight were observed for G2. Eggs from birds whose grandparents from the Con-Con treatment laid significantly heavier eggs that weighed 12.01 g compared to eggs laid by birds from HiMet-HiMet and Hi-Met Con grandparents which weighed 11.48 g and 11.39 g, respectively, where $p<0.0001$ (Figure 2.10). Although there were differences in the weight of eggs there were no significant differences in the number of eggs laid between treatment groups (Table 2.4). Weekly eggs laid per hen for the HiMet-HiMet, HiMet-Con, Con-HiMet and Con-Con group were 4.14 eggs/hen/week, 4.42 eggs/hen/week, 4.29 eggs/hen/week, and 4.33 eggs/hen/week, respectively (Table 2.4).

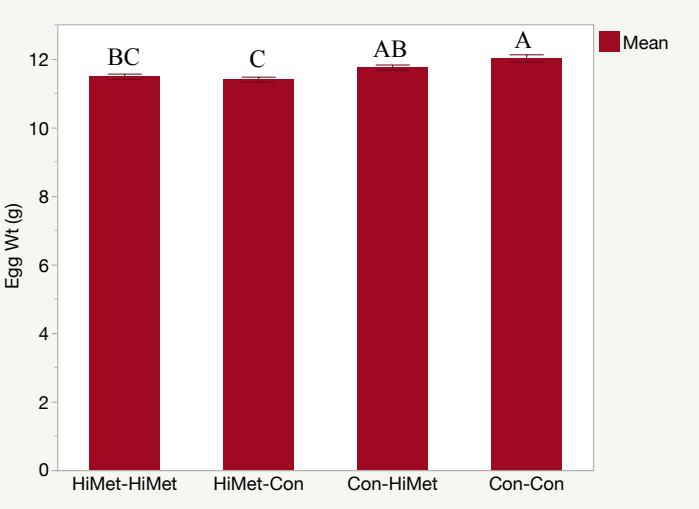

Figure 2.10. G2 egg weight measured from the start of lay (6wks-11wks); ($P<0.001$). Each error bar is constructed using 1 standard error from the mean.

Table 2.4. Number of Eggs for G2 by treatment measured for approximately 5 consecutive wks beginning at 6 wks of age.

	Eggs/Hen/Week	Std Error
HiMet-HiMet	4.14	3.28
HiMet-Con	4.42	3.28
Con-HiMet	4.29	3.28
Con-Con	4.33	3.28
p-value	**0.9531**	

Analysis of CpG DNA Methylation Level

Genome wide CpG DNA methylation was evaluated in the female liver tissue and male gonads from G0 starter phase. In the liver CpG promoter methylation level was highest for the treatment group receiving the high methyl starter diet compared to the control diet (Figure 2.11). The CpG DNA methylation overview for each chromosome was similar for both groups (Figure 2.12). Evaluation of the male gonads revealed no differences in CpG promoter methylation level (Figure 2.13), and no differences were observed across chromosomes (Figure 2.14).

Total read number for the liver was over 15 and 17 million with 30% mapping efficiency (Table 2.5). Total read number for the teste was over 28 and 30 million for the gonads with 30% mapping efficiency (Table 2.5). Approximately 2 million unique CpGs were detected. The liver tissue had approximately 16 times coverage and the teste had 27 times coverage (Table 2.5). The bisulfite conversion rate for both tissue types was 98-99% (Table 2.5).

At a later timepoint, genome wide CpG methylation of female liver tissue was evaluated from each generation and treatment. More than 7 million individual CpG sites were identified from each group and generation with varying methylation levels. Our results demonstrate no vast difference in percent methylation globally between generation and group (Table 2.6).

Approximately 50-60 million total reads were detected and roughly 30-40 million were used in the analysis (Table 2.7). The mapping efficiency was measured to be approximately 50 percent (Table 2.7). At least half of the alignment was required to match the reference sequence before being included in the analysis and the minimum percentage of identity between the aligned region of the read and the reference was set to 80 percent.

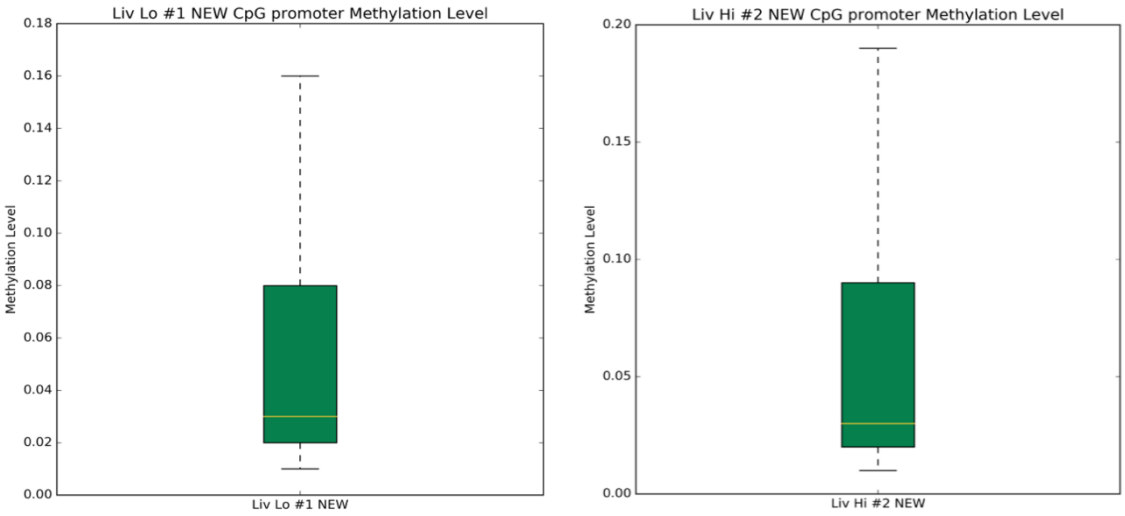

Figure 2.11. G0 Starter Phase Global CpG Promoter DNA Methylation Level of Female Liver for HiMet and Control (Lo).

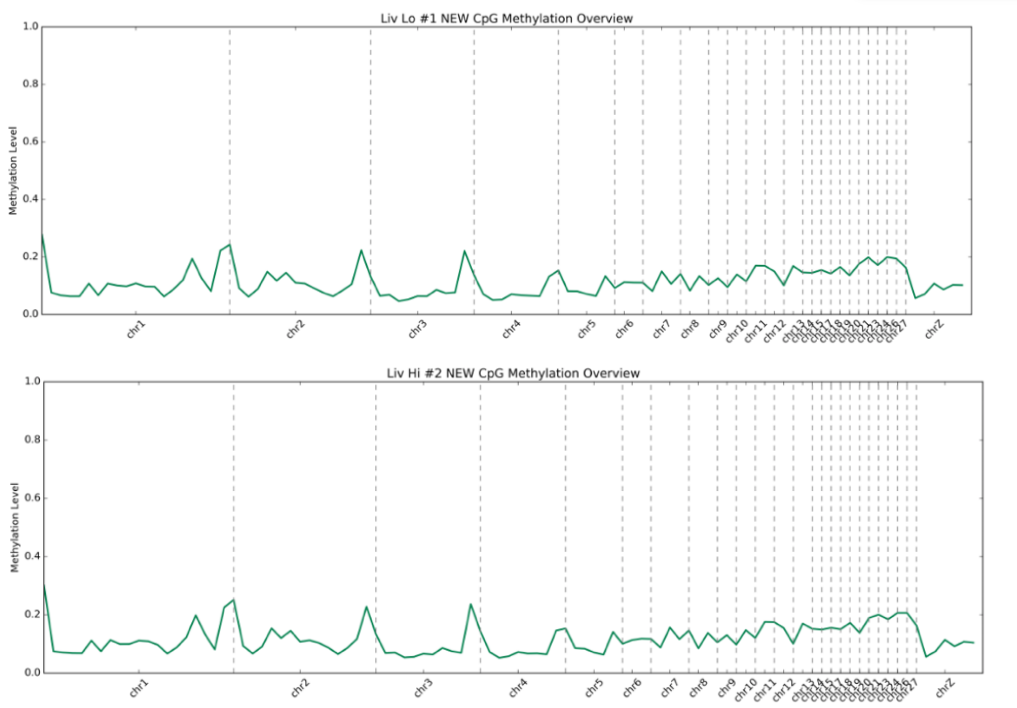

Figure 2.12. G0 Starter Phase Global CpG Chromosome DNA Methylation Level of Female Liver for HiMet and Control (Lo).

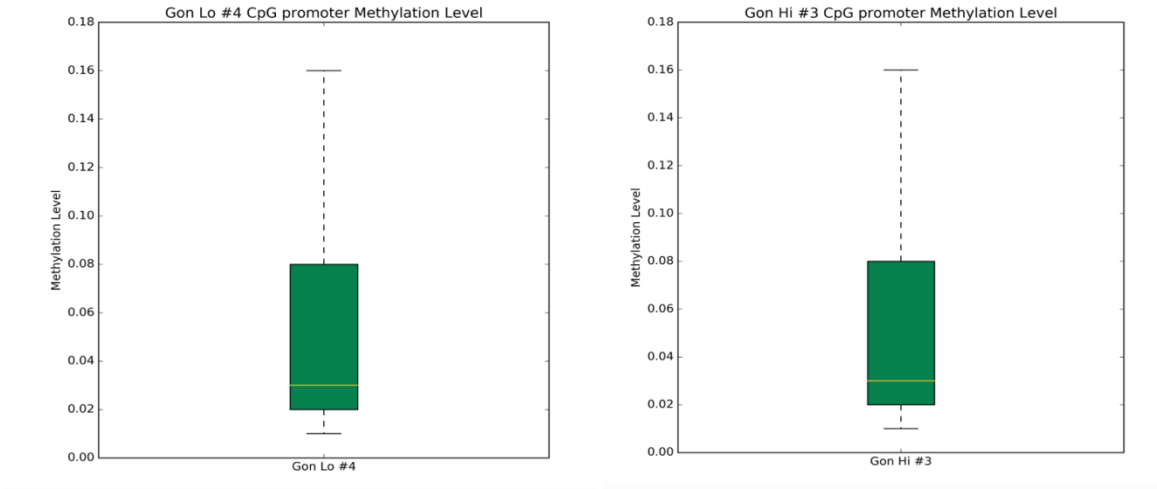

Figure 2.13. G0 Starter Phase Global CpG Promoter DNA Methylation Level Male Gonads for HiMet and Control (Lo).

Figure 2.14. G0 Starter Phase Global CpG Chromosome DNA Methylation Level of Male Gonad for HiMet and Control (Lo).

Table 2.5. Sequence Readings and Mapping Efficiency for G0 Starter Phase.

G0	Total Read Number (Read Pairs)	Mapping Efficiency	Unique CpGs	Avg. CpG Coverage	Bisulfite Conversion Rate
HiMet Liver	17,879,614	29%	1,860,124	16x	99%
Con Liver	15,785,476	30%	1,788,752	16x	99%
HiMet Teste	28,263,460	30%	2,061,648	27x	98%
Con Teste	30,058,472	27%	1,998,739	27x	98%

Table 2.6. CpG DNA Methylation and Unmethylated Regions and Percent Methylated for Generations and Treatments.

	Unmethylated	Methylated	Percent Methylated
G0 HiMet-HiMet	20,090,710	24,103,784	54.54
G0 HiMet-Con	21,615,764	25,153,006	53.78
G0 Con-HiMet	18,135,680	21,601,599	54.36
G0 Con-Con	19,458,373	22,686,004	53.83
G1 HiMet-HiMet	20,627,022	22,665,757	52.35
G1 HiMet-Con	22,438,351	24,515,690	52.21
G1 Con-HiMet	18,064,412	20,174,520	52.76
G1 Con-Con	20,908,373	23,757,569	53.19
G2 HiMet-HiMet	19,219,811	21,703,758	53.03
G2 HiMet-Con	22,479,672	24,516,962	52.17
G2 Con-HiMet	21,384,824	21,685,581	50.35
G2 Con-Con	20,631,693	22,825,258	52.52

Table 2.7. Sequence Readings and Mapping Efficiency for Generations and Treatments.

	Total Read Number (Read Pairs)	Included in Analysis	Mapped to GA Reference	Mapping Efficiency
G0 HiMet-HiMet	59,629,831	40,916,279	20,779,996	50.79
G0 HiMet-Con	64,047,363	43,238,239	22,003,553	50.89
G0 Con-HiMet	53,116,991	35,956,671	18,328,113	50.97
G0 Con-Con	56,524,405	38,837,208	19,770,818	50.91
G1 HiMet-HiMet	59,231,466	40,369,797	20,576,963	50.97
G1 HiMet-Con	64,715,704	43,826,629	22,316,677	50.92
G1 Con-HiMet	52,057,553	35,725,566	18,217,927	50.99
G1 Con-Con	60,897,982	40,992,807	20,905,514	51.00
G2 HiMet-HiMet	58,539,969	40,106,625	20,259,245	50.51
G2 HiMet-Con	66,013,511	44,691,643	22,614,369	50.60
G2 Con-HiMet	54,249,610	32,899,546	16,846,300	51.21
G2 Con-Con	60,232,605	40,649,620	20,545,918	50.54

Methylation Level of Methylation Cycle Genes

CpGs in regions associated with genes involved in the methylation cycle were analyzed comparing methylation levels between generation and treatment. The genes analyzed are responsible for the production of the enzymes illustrated in Figure 2.15 below. These enzymes are ultimately involved in the production of S-adenosyl methionine (SAM), the universal methyl donor needed for the direct methylation of DNA. A key of the genes involved in the DNA Methylation Cycle is available in Table 2.9.

CpG DNA methylation level was decreased in G0 (the only generation which received the dietary treatment) and increased in G1 and G2 for genes *SHMT1, MAT1A* and *AHCY* (Table 2.8). The opposite was detected for *PDXK* where G0 resulted in higher CpG DNA methylation levels when compared to G1 and G2 (Table 2.8). Differences were detected due to dietary treatment for the genes *DNMT3A, MAT2B, MTR*, and *SHMT2*. The HiMet-HiMet treatment group had increased CpG DNA methylation levels and the HiMet-Con treatment group had decreased CpG DNA methylation levels for the *DNMT3A* gene (Table 2.8). The *MAT2B* gene had increased CpG DNA methylation levels in the Con-HiMet treatment group and again decreased CpG DNA methylation levels were detected for the HiMet-Con group (Table 2.8). Both the HiMet-Con and Con-HiMet group were observed to have decreased CpG DNA methylation levels for *SHMT2* and increased CpG DNA methylation for the Con-Con treatment group (Table 2.8). Differences in the interaction between treatment and generation were identified for *DNMT3A* and *MAT2B* (Table 2.8). Evaluation of CpG DNA methylation of *DNMT3A* resulted in G0 and G1 HiMet-HiMet treatment group being significantly different from the G2 HiMet-HiMet treatment group (Table 2.8). The G0 and G1 Con-HiMet group was also significantly different than the G2 Con-HiMet group (Table 2.8). The interaction between generation and treatment groups for *MAT2B* resulted in the HiMet-

HiMet group having increased CpG DNA methylation in G0 and G1 and decreased methylation in G2. Differences were not detected for HiMet-Con between the three generations and the Con-HiMet group resulted in G1 being different from G0 and G2. CpG DNA methylation levels were not different between generations for the Con-Con group (Table 2.8). The number of CpG sites associated with each gene varied (Table 2.8).

Table 2.8. CpG DNA Methylation Level of Genes involved in Methylation Cycle.

		Locus															
Generation[1]	Treatment[2]	DHFR	SHMT1	SHMT2	MTHFR	MTRR	MTR	MAT1A	MAT2A	MAT2B	CHDH	AHCY	RFK	DNMT3A	DNMT3B	FLAD1	PDXK
		CpG Methylation Level															
G0	-	0.9210	0.6244^{B}	0.7754	0.7511	0.8911	0.8847	0.7883^{B}	0.8081	0.8356	0.8264	0.7776^{B}	0.8837	0.7372	0.7450	0.6804	0.8893^{A}
G1	-	0.9424	0.6913^{A}	0.7878	0.7940	0.8922	0.8639	0.8529^{A}	0.7941	0.8365	0.8235	0.8175^{A}	0.7937	0.7341	0.7602	0.6932	0.8617^{B}
G2	-	0.9162	0.7041^{A}	0.7744	0.7833	0.8852	0.8615	0.8609^{A}	0.7857	0.8037	0.8590	0.7952^{AB}	0.8080	0.7379	0.7440	0.6818	0.8416^{B}
P-value		0.5244	**0.0001**	0.6467	0.0636	0.8547	0.0610	**0.0001**	0.3987	0.2963	0.4758	**0.0303**	0.2537	0.7727	0.1332	0.6433	**0.0001**
SEM3		0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.03	0.004	0.01	0.01	0.01
-	HiMet-HiMet	0.9159	0.6639	0.7813^{AB}	0.7914	0.8785	0.8649^{AB}	0.8437	0.8148	0.8520^{A}	0.8255	0.7974	0.8253	0.7495^{A}	0.7559	0.6819	0.8674
-	HiMet-Con	0.9498	0.6817	0.7548^{B}	0.7620	0.8880	0.8504^{B}	0.8242	0.7789	0.7949^{B}	0.8074	0.7779	0.8242	0.7266^{B}	0.7431	0.6840	0.8739
-	Con-HiMet	0.9041	0.6922	0.7653^{B}	0.7733	0.8936	0.8946^{A}	0.8357	0.7828	0.7951^{B}	0.8835	0.8176	0.7810	0.7340^{AB}	0.7565	0.6977	0.8493
-	Con-Con	0.9364	0.6553	0.8155^{A}	0.7778	0.8978	0.8702^{AB}	0.8324	0.8073	0.8591^{A}	0.8288	0.7941	0.8833	0.7355^{AB}	0.7435	0.6770	0.8661
	P-value	0.3988	0.2547	**0.0072**	0.5813	0.5876	**0.0056**	0.7454	0.1575	**0.0217**	0.2579	0.1596	0.4521	**0.0058**	0.3741	0.6659	0.2279
	SEM[3]	0.02	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	0.04	0.004	0.001	0.01	0.01
G0	HiMet-HiMet	0.8925	0.6169	0.7840	0.7598	0.8781	0.8627	0.8015	0.8242	0.9017^{A}	0.8452	0.7753	0.8333	0.7284^{BC}	0.7629	0.6688	0.8760
G0	HiMet-Con	0.9166	0.6239	0.7174	0.7455	0.8880	0.8821	0.8088	0.8043	0.7652^{CD}	0.8177	0.7501	0.9777	0.7261^{BC}	0.7342	0.6837	0.9055
G0	Con-HiMet	0.9583	0.6450	0.7707	0.7295	0.8887	0.9010	0.7644	0.7947	0.8025^{BCD}	0.8500	0.8233	0.8571	0.7453^{B}	0.7411	0.6821	0.8814
G0	Con-Con	0.9166	0.6119	0.8295	0.7694	0.9095	0.8933	0.7783	0.8092	0.8732^{AB}	0.7926	0.7618	0.8666	0.7491^{AB}	0.7419	0.6871	0.8942
G1	HiMet-HiMet	0.9266	0.6626	0.8036	0.8235	0.8911	0.8552	0.8583	0.7921	0.8681^{AB}	0.7932	0.7897	0.8928	0.7333^{BC}	0.7520	0.6956	0.8729
G1	HiMet-Con	0.9814	0.6883	0.7708	0.7644	0.8938	0.8309	0.8372	0.7748	0.7790^{BCD}	0.8765	0.8360	0.7489	0.7313^{BC}	0.7519	0.6985	0.8658
G1	Con-HiMet	0.8928	0.7235	0.7660	0.8317	0.9085	0.9120	0.8441	0.7902	0.8530^{ABC}	0.8300	0.8148	0.7500	0.7524^{AB}	0.7949	0.7297	0.8477
G1	Con-Con	0.9687	0.6906	0.8108	0.7565	0.8752	0.8575	0.8719	0.8193	0.8459^{ABC}	0.7944	0.8296	0.7833	0.7192^{BC}	0.7421	0.6490	0.8606
G2	HiMet-HiMet	0.9285	0.7121	0.7565	0.7909	0.8664	0.8770	0.8714	0.8281	0.7862^{BCD}	0.8367	0.7874	0.7500	0.7867^{A}	0.7528	0.6813	0.8534
G2	HiMet-Con	0.9513	0.7329	0.7761	0.7761	0.8822	0.8383	0.8266	0.7576	0.8405^{ABC}	0.8112	0.7940	0.7460	0.7224^{BC}	0.7433	0.6698	0.8505
G2	Con-HiMet	0.8613	0.7082	0.7591	0.7588	0.8835	0.8709	0.8985	0.7634	0.7299^{D}	0.9242	0.7936	0.7361	0.7044^{C}	0.7336	0.6812	0.8189
G2	Con-Con	0.9238	0.6634	0.8061	0.8074	0.9086	0.8600	0.8471	0.7936	0.8584^{AB}	0.8638	0.8057	1.0000	0.7382^{BC}	0.7464	0.6950	0.8435
p-value		0.3763	0.6665	0.4106	0.2374	0.6275	0.1663	0.2602	0.2602	**0.0299**	0.9080	0.6206	0.1176	**0.0001**	0.0943	0.3068	0.8215
n		359	1612	1727	1317	1520	2843	1343	1426	757	477	1924	157	15014	5204	2113	2958
SEM[3]		0.04	0.02	0.02	0.02	0.01	0.01	0.02	0.02	0.03	0.03	0.02	0.07	0.01	0.01	0.02	0.01

[A,B] Connecting letters report using Tukey HSD

[1] Three total generations (G0, G1 and G2), where only G0 was provided the high methyl catalyst diet

[2] Treatment: Four total treatment groups of high methyl catalysts during the starter phase and/or layer phase (HiMet-HiMet, HiMet-Con, Con-HiMet, Con-Con)

[3] Standard error of mean (SEM) for n=12 samples

Table 2.9. Key of Genes involved in DNA Methylation Cycle.

Gene ID	Name	Activity
DHFR	Dihydrofolate reductase	converts DHF into THF
SHMT1	Serine hydroxymethyltransferase (Cystolic)	a pyridoxal phosphate-containing enzyme that catalyzes the reversible conversion of serine and THF to glycine and 5,10-methylene THF
SHMT2	Serine hydroxymethyltransferase (Mitochondrial)	a pyridoxal phosphate-dependent enzyme that catalyzes the reversible reaction of serine and THF to glycine and 5,10-methylene THF
MTHFR	Methylenetetrahydrofolate reductase	catalyzes the conversion of 5,10-methylene THF to 5-methyl THF
MTRR	Methionine Synthase Reductase	regenerating methionine synthase to a functional state
MTR	Methionine Synthase	encodes the 5-methyl THF-homocysteine methyltransferase
MAT1A	Methionine Adenosyltransferase 1A	catalyzes a two-step reaction that involves the transfer of the adenosyl of ATP to methionine to form SAM and tripolyphosphate
MAT2A	Methionine Adenosyltransferase 2A	catalyzes the production of SAM from methionine and ATP
MAT2B	Methionine Adenosyltransferase 2B	the regulatory beta subunit of MAT which catalyzes the biosynthesis of SAM from methionine and ATP
CHDH	Choline Dehydrogenase	a choline dehydrogenase
BHMT1	Betaine--Homocysteine S-Methyltransferase	a cytosolic enzyme that catalyzes the conversion of betaine and homocysteine to dimethylglycine and methionine
BHMT2	Betaine--Homocysteine S-Methyltransferase 2	one of two methyl transferases that can catalyze the transfer of the methyl group from betaine to homocysteine
AHCY	S-adenosylhomocysteine hydrolase	catalyzes the reversible hydrolysis of SAH to adenosine and L-homocysteine
RFK	Riboflavin kinase	enzyme that catalyzes the phosphorylation of riboflavin to form FMN, an obligatory step in vitamin B2 utilization and flavin cofactor synthesis
DNMT3A	DNA methyltransferase	de novo methylation
DNMT3B	DNA methyltransferase	de novo methylation
FLAD1	Flavin Adenine Dinucleotide Synthetase 1	enzyme that catalyzes adenylation of flavin mononucleotide (FMN) to form flavin adenine dinucleotide (FAD) coenzyme
PDXK	Pyridoxal Kinase	phosphorylates vitamin B6, a step required for the conversion of vitamin B6 to pyridoxal-5-phosphate

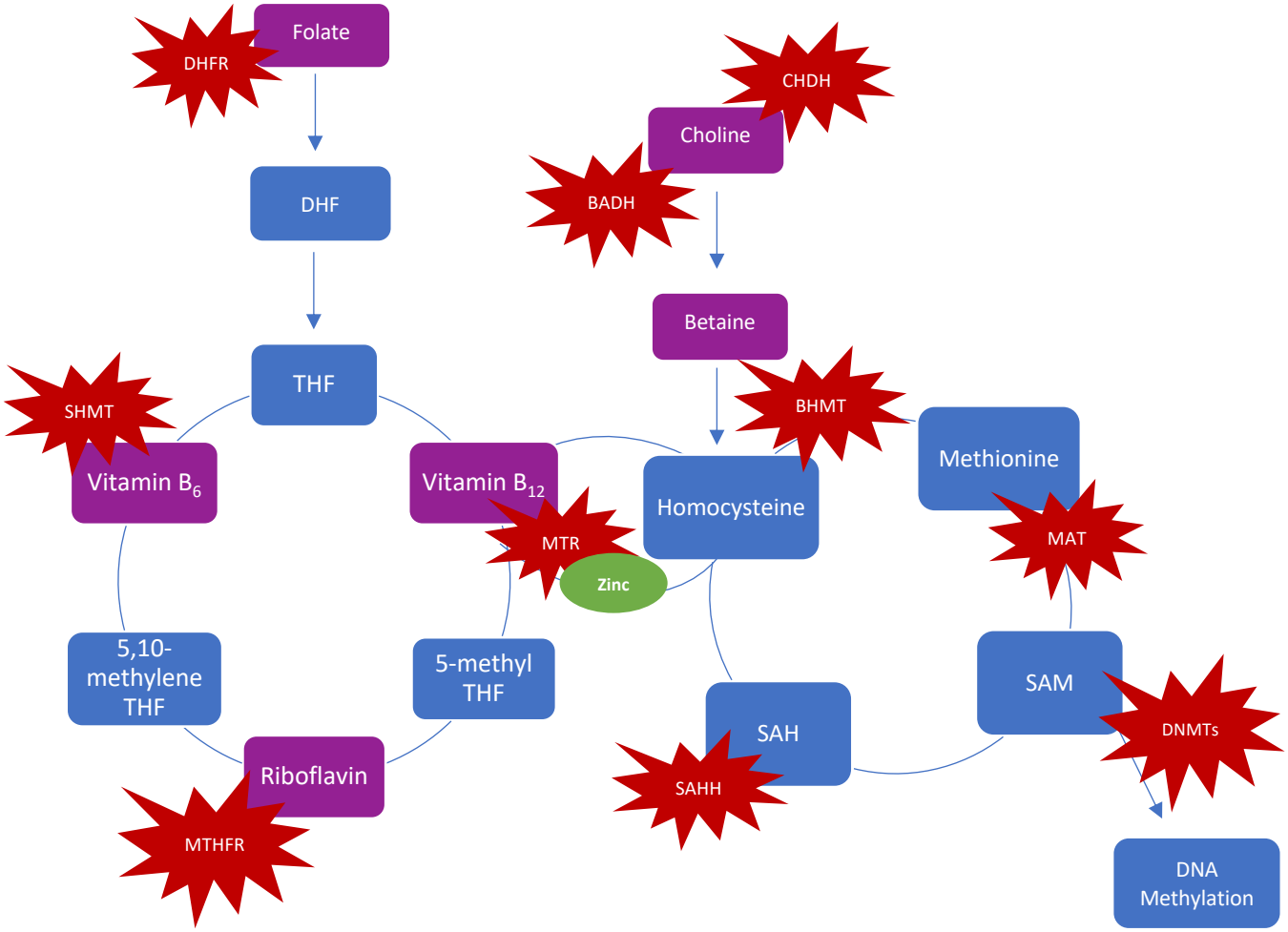

Figure 2.15. Methylation Cycle with Methyl Catalysts and Enzymes.

Discussion

In general, when evaluating the DNA methylation profiles of the chicken it was found that the DNA methylation pattern follows the same rules as that of mammals and plants with hypermethylation found in repetitive regions and hypomethylation in most of the CpG islands (Asp et al., 2011). Approximately 38% of CpG islands are conserved between the human and chicken (Hillier et al., 2004). Birds and mammals have lower cytosine methylation levels of 5.2% when compared to fish, amphibians and reptiles with 9.08% (Colwell et al., 2018). Decreased DNA methylation levels have been observed in promoter regions and transcriptional start sites and increased DNA methylation often occurs in the gene body regions (Hu et al., 2013). However, differences in methylation have been found between different tissues and strains of the avian, with hepatic DNA being less methylated compared to muscle DNA (Xu et al., 2007).

Mandel and Chambon (1979) used CpG restriction enzymes first described by Bird and Southern (Bird, 1978) to evaluate the DNA methylation associated with three genes which are responsible for proteins found in egg whites: ovalbumin, conalbumin and ovomucoid. When these genes were evaluated in the laying hen, minimal methylation was detected in the oviduct where they are typically expressed and the highest amount of methylation was detected in the sperm where expression of these genes would not occur (Mandel and Chambon, 1979). This indicates the typical characteristic of increased DNA methylation leading to decreased gene expression due to the inability of transcription machinery to gain access to the promoter region to transcribe the DNA. In our study analysis of global CpG DNA methylation levels of the male gonad, the addition of high methyl catalysts during the starter phase resulted in minimal differences in methylation levels when compared to the control (Figure 2.13). Evaluation of CpG methylation across the chromosomes for the male gonad appeared similar for both groups, with variable high points and

low points across each chromosome (Figure 2.14). Similarly, evaluation of CpG methylation across the chromosomes for the female liver appeared similar for both groups, with variable high points and low points across each chromosome (Figure 2.12); however, global CpG DNA methylation levels of the female liver resulted in a slight increase in methylation levels due to the addition of high methyl catalysts during the starter phase when compared to the control (Figure 2.11). Variation in global CpG DNA methylation level was detected between gonad and liver treatment tissue types (Figure 2.11 and Figure 2.13), which has previously been observed in the chicken when comparing the broiler chicken muscle and liver; however methylation percentage also varied between the tissue types of the broiler chicken and red jungle fowl where the red jungle fowl liver was 5.7% methylated and that of the broiler was 9.1% methylated using MeDip-seq methods (Li et al., 2011). The highest density of genes in the chicken are found on the microchromosomes which in turn also contain the highest percentage of CpG islands (David et al., 2017; Warren et al., 2017).

Within the liver, excess methionine is converted to the active form, s-adenosyl methionine (SAM), by an ATP- driven reaction of the methylation cycle (Anderson et al., 2012). The product of this cycle is homocysteine which is a nonprotein-forming amino acid. The enzyme responsible for the conversion of methionine to SAM is methionine adenosyl transferase (MAT) (Figure 2.15 & Table 2.9). CpG Methylation level of genes for *MAT1A, MAT2A, and MAT2B* were analyzed. Increased CpG DNA methylation was detected for *MAT1A* ($p<0.0001$) in G1 and G2 (Table 2.8) with the lowest levels detected in G0, which was provided with the methyl catalyst diet. The interaction between generation and treatment groups for *MAT2B* resulted in the HiMet-HiMet group having increased CpG DNA methylation in G0 and G1 and decreased methylation in G2 ($p<0.0299$). Differences were not detected for HiMet-Con between the three generations and the

Con-HiMet group resulted in G1 being different from G0 and G2. CpG DNA methylation levels were not different between generations for the Con-Con group (Table 2.8). Changes within G2 could potentially be explained by G2 being the first true generation that could not have been directly influenced by dietary treatment. G1 was not fed the treatment diet, however the oocytes for this generation were formed inside the maternal line which were fed the treatment diets. It is known that nutrition provided during different time points can result in different transgenerational epigenetic effects which is the reason the methyl catalyst diet was evaluated separately for the growth and layer phase (Zambrano et al., 2005).

SAM is the active universal methyl donor for enzymatic methylation (Friedel et al., 1989). DNMT enzymes covalently attach methyl groups from SAM to the 5-carbon position on cytosine bases resulting in 5-methylcytosine (Anderson et al., 2012). The only DNA methyltransferases detected were *DNMT3A* and *DNMT3B*, which are de novo methyltransferases that are needed to establish the epigenetic profile or cell fate for new cells being produced that do not yet contain methylation marks (Table 2.9) (Lyko, 2018; Wu et al., 2012). Differences in *DNMT3A* CpG DNA methylation were detected in the treatment main effect and the interaction between generation and treatment (Table 2.8). Evaluation of treatment effects resulted in the HiMet-HiMet group and the HiMet-Con group being significantly different ($p<0.0058$). Similar to *MAT2B*, the HiMet-HiMet G0 and G1 group had different methylation levels compared to G2. This trend was true for the Con-HiMet group as well, with G0 and G1 being different from G2 (Table 2.8)

Changes in CpG DNA methylation to G2 could also explain the reversal of the egg size trend observed in G2 compared to G0 and G1. Increased egg weight was observed as a result of the HiMet-HiMet treatment in G0 and G1 but this trend was not observed in G2 (Figures 2.4, Figure 2.7, Figure 2.10). Our study is not the first to detect changes in egg modification due to the

environmental conditions of the previous generation. This has been observed in Japanese quail exposed to stressors resulting in increased egg size and egg testosterone levels (Guibert et al., 2011). In the liver of Japanese quail that are laying eggs, hypomethylation along with a relaxed chromatin state were detected for vitellogenin II which is responsible for the deposition of yolk into the egg (Gupta et al., 2006). In the same study, older Japanese quail which had ceased egg production, vitellogenin II was hypermethylated and chromatin was in a compact state.

SAM is generated through one carbon metabolism and this is catalyzed by the presence of methyl catalysts (Figure 2.15) (Anderson et al., 2012). Each individual cell is responsible for the synthesis of SAM due to the inability of SAM to cross plasma membranes (Finkelstein, 1998). Once SAM has given up a methyl group it becomes s-adenosylhomocysteine (SAH). SAH is hydrolyzed to homocysteine by the enzyme s-adenosylhomocysteine hydrolase (SAHH) (Figure 2.15). The gene responsible for SAHH production is adenosylhomocysteinase (*AHCY*; Table 2.9). Increased CpG DNA methylation levels were detected in G1 when compared to G0 (Table 2.8). Methylation levels were not different in G1 and G2 which could be a result of the dietary treatment given to G0; however, G2 and G0 were also not different from one another. In this case, it seems the dietary treatment had an effect on the next generation, but the effect was not observed in the subsequent generation.

Homocysteine can be remethylated by methionine synthesis or undergo trans-sulfuration for the formation of cysteine, taurine, and glutathione. For remethylation, homocysteine accepts a methyl group from 5-methyltetrahydrofolate in the folate and vitamin B_{12} dependent pathway. Alternatively, a methyl group can be accepted from betaine but this occurs predominately in the liver whereas the folate dependent pathway occurs in all tissues (Selhub, 1999). Homocysteine is converted into methionine by the enzymes homocysteine methyltransferase and 5-

methyltetrahydrofolate is produced by methylenetetrahydrofolate reductase (MTHFR) (Figure 2.15 & Table 2.9). No significant differences were detected between generation and treatment in CpG methylation level for this gene (Table 2.8). *MTHFR* CpG methylation levels have not been evaluated in birds; however when broiler breeders were fed a diet deficient in folate their offspring had higher serum levels of MTHFR and dihydrofolate reductase (DHFR) when compared to the control offspring. Gene expression was analyzed from these offspring and MTHFD2L and SHMT1 was increased from the progeny from folate deficient hens (Xing et al., 2018).

Folic Acid

Folate is an essential vitamin for birds and serves as a cofactor and cosubstrate for methylation reactions. Folic acid, being the synthetic form of folate, is the oxidized form containing only a single conjugated glutamate residue (Shane, 2008). Folic acid is used as an additive because it is almost completely stable whereas the naturally occurring forms of folate often lose over half their activity (Jaenicke, 1970).

Within the intestinal mucosal cells, folic acid is reduced to the di- and tetrahydro- active forms in the body by the enzyme dihydrofolate reductase (DHFR;Table 2.9) (Shane, 2008). When evaluating the CpG DNA methylation level for this enzyme, changes in CpG methylation level for *DHFR* was not detected (Table 2.8). Tetrahydrofolate (THF) is converted to 5,10 methylene THF by the activity of serine hydroxymethyltransferase (SHMT) in the liver (Figure 2.15). *SHMT1* and *SHMT2* were genes evaluated for CpG DNA methylation levels. The cytosolic serine hydroxymethyltransferase (*SHMT1*) was significantly impacted ($p<0.0001$) with the trend of G0, the only generation given the dietary treatment, being different from G1 and G2 (Table 2.8). *SHMT2,* the mitochondrial serine hydroxymethyltransferase, resulted in HiMet-Con and Con-HiMet treatment group being different from the Con-Con treatment (*SHMT2*; Table 2.8). The two

treatment groups which are not consistently given the same treatment (HiMet-Con and Con-HiMet) like the HiMet-HiMet and Con-Con group result in differences in methylation levels across the genes analyzed. This is of interest because this is the evaluation of a change in dietary resources provided at one period either growth or egg production. It appears that either taking the supplementation away during the lay period or adding the supplementation only to the lay period has an effect on CpG DNA methylation which is not always generation dependent (Table 2.8)

DNA methylation, along with the intake of folate, have been shown to be positively correlated (Pufulete et al., 2005; Rampersaud et al., 2000). It is known that DNA methylation is both gene and tissue specific, meaning global DNA methylation can be increased when increasing folate but certain individual genes can be hypomethylated. The pathways dictating which genes becomes hyper- or hypo- methylated have not been identified. This could explain why large changes in global CpG DNA methylation were not detected (Table 2.6), however when evaluating individual genes significant changes were detected (Table 2.8). If folate is not provided adequately, the methylation cycle will be altered by increased homocysteine plasma levels because single carbon donors are unavailable for the remethylation of homocysteine.

Choline & Betaine

Unlike many vitamins, choline can be synthesized by poultry in adequate quantities; however, the one exception for this is quail which require the addition of choline in the diet (Latshaw and Jensen, 1971). Dietary choline is necessary for the maintenance in egg size within quail (Latshaw and Jensen, 1971). Evaluation of the eggs from G0, which was the only generation fed an increased amount of choline and betaine, revealed that eggs from the HiMet-HiMet group and the Con-HiMet group were the heaviest (Figure 2.4). In agreement with previous research, groups supplemented with additional choline and betaine during the layer phase resulted in

increased egg weight, however this would explain why changes in egg size persisted without dietary treatment in the next generations without an epigenetic factor (Figure 2.4, Figure 2.7, Figure 2.10). It is important to note that even though changes in egg size were detected, there were no significant changes in the number of eggs laid (Table 2.3, Table 2.4, Table 2.5). This is stated because increased egg weight has been shown to be negatively correlated with total egg production (Ekmay et al., 2013). This research revealed the potential for a transgenerational increase in weight of eggs laid by progeny (G1 & G2) in response to increased methyl catalysts in G0. It is not known whether the observed response in G1 was caused by an epigenetic factor or direct manipulation during oocyte formation since the diet fed to G0 could have exerted influence. However, the observed response in G2 egg weight could likely be attributed to epigenetic influence because the oocytes from which these progenies developed would not have been exposed to the different levels of dietary methyl catalysts fed to G0.

Choline is a source of methyl groups. Betaine is a metabolite of choline and serves as a methyl donor (Morris, 2001). Choline must be oxidized to betaine to serve as a methyl donor by the enzymes choline dehydrogenase (CHDH) and betaine aldehyde dehydrogenase (BADH) (Figure 2.15) (Obeid, 2013). Betaine, like THF, plays a role in one carbon metabolism by the donation of a methyl group to homocysteine which can then be converted into methionine. *BADH* was not detected when evaluating CpG DNA methylation. *CHDH* CpG DNA methylation levels were detected however they were not significant (Table 2.8). Betaine homocysteine S-methyltransferase (BHMT) is an enzyme which catalyzes the conversion of betaine and homocysteine to methionine (Figure 2.15) (Shane, 2008). CpG methylation levels were not detected for this gene.

Vitamin B_6

Vitamin B_6 is a compound that can be found in the forms: pyridoxine, pyridoxal, and pyridoxamine all of which can be phosphorylated (Ueland et al., 2015). Absorption mainly occurs in the duodenum and jejunum by passive diffusion (Heard and Annison, 1986). Vitamin B_6 is found in the liver and is mostly converted to the most active form pyridoxal 5'-phosphate (PLP). PLP is a coenzyme form needed for many catalytic functions (Ueland et al., 2015). One of which is the requirement as a cofactor for SHMT which as previously mentioned, converts THF to 5,10 methylene THF (Lamers et al., 2011). The three unphosphorylated forms are converted to a phosphorylated form with the help of pyridoxal kinase (PDXK) and pyridoxamine phosphate oxidase (Table 2.9). CpG DNA methylation of *PDXK* was significantly different ($p<0.0001$) where again G0 was different from G1 and G2 (Table 2.8). Within the methylation cycle vitamin B_6 is a coenzyme to SHMT, which as previously mentioned, converts folate derived THF to 5,10 methylene THF (Figure 2.15).

Riboflavin

Riboflavin, also known as vitamin B_2, is found in three forms: free riboflavin and the coenzymes including flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) (Pinto and Zempleni, 2016). Free riboflavin is absorbed by the mucosal cells in the small intestine and most is converted to FMN by riboflavin kinase (RFK) (Barile et al., 2016). Riboflavin then enters the blood as either free riboflavin or FMN (Powers, 2003). Once in tissues, most of the FMN is further converted to FAD by flavin adenine dinucleotide synthetase 1 (FLAD1) (Barile et al., 2016). Although riboflavin is not stored in appreciable amounts, the liver contains the greatest concentration. The role of riboflavin within the methylation cycle occurs when 5,10 methylene THF is further reduced to 5-methyl THF by the enzyme MTHFR from the precursor FAD (Figure

2.15) (Anderson et al., 2012). A deficiency in riboflavin has been associated with a decreased activity of the flavoenzyme MTHFR and reduced levels of 5-methyl THF (Hustad et al., 2013). Significant differences in CpG DNA methylation were not detected for *RFK* or *FLAD* (Table 2.8).

Vitamin B_{12}

Vitamin B_{12} is necessary for one carbon metabolism in the production of methyl donors. It is mostly absorbed in the small intestine by interaction with specific receptors and within the organism the highest concentration is found within the liver (Shane, 2008). Within the methylation cycle, vitamin B_{12} is the coenzyme of methionine synthetase (MTR), the enzyme responsible for the removal of the methyl group from 5-methyl THF to regenerate THF and donate a methyl group to homocysteine for the conversion to methionine (Figure 2.15) (Anderson et al., 2012). MTR is a zinc metalloprotein which contains a specific domain for the binding of vitamin B12 as a cofactor and a catalytic domain for the binding of homocysteine (Shane, 2008). The MTR enzyme is reactivated by methionine synthase reductase (MTRR). This enzyme contains binding sites for NADPH, FAD and FMN, with the latter two being forms of the riboflavin compound (Shane, 2008). CpG DNA methylation for methionine synthase was significantly different within treatment groups (Table 2.8), with the Con-HiMet group being different from the HiMet-Con group. This could indicate that the effect of increased methyl catalyst intake on methionine synthase methylation level could be dependent upon the phase in which the dietary treatment was applied. Differences were not detected for *MTRR*, which is involved in regenerating *MTR* to a functional state (Table 2.8).

Zinc

Zinc (Zn), unlike the other methyl catalysts, is a trace mineral and is recognized as an important cofactor in the methylation cycle (Vallee and Falchuk, 1993). When homocysteine is

recycled back to methionine, the enzymes betaine homocysteine methyltransferase and 5-methy THF, are known to be zinc dependent. These enzymes catalyze the transfer of a methyl group to homocysteine. Zn deficiency is known to decrease histone and DNA methylation which has been shown to allow increased expression of repetitive elements (Wallwork and Duerre, 1985).

It has been suggested that maternal supplementation of either organic or inorganic Zn can epigenetically modify offspring by protecting them from damage caused by stressors. Zn from different sources fed to broiler breeder hens resulted in an 11% increase in healthy chicks and eliminated the embryonic mortality induced by heat stress (Zhu et al., 2017). Both organic and inorganic Zn sources resulted in higher levels of global DNA methylation and lower global levels of histone 3 lysine 9 acetylation within the liver (Zhu et al., 2017). However, it is important to note that some analyzed regions exhibited the opposite effect, such as the promoter region of the *MT4* gene, which is involved in zinc metabolism. Similar to our results, the addition of methyl catalysts, including zinc, does not always result in increased methylation to genes. Currently, little is known as to what regions are affected in birds and how changes in methylation level to a particular genomic region can affect the organism. It could be that more vast difference would have been detected if the control diet vitamin fortification was stripped of methionine vitamins; however the control diet herein was formulated to have adequate vitamins required for typical growth and reproduction. This makes differences in egg sizes more interesting considering typical nutrient requirements should allow for peak performance including egg size and production.

Conclusions

This study is the first to analyze how an environmental effect of dietary methyl catalysts can affect the methylation level of genes directly related to the methylation cycle in the avian for generations. Overall, differences in phenotypic traits such as egg weight were detected in response

to dietary alterations in a preceding generation. Our objective was to analyze the basis of inheritance across multiple generations to detect potential effectors such as CpG gene methylation level of enzymes involved in the methylation cycle. The results reported herein suggested methylation level can vary significantly from generation to generation and between treatments. However, a distinct pattern for this process is not clear considering the lack of consistency between genes, generations, and treatment. Methylation across the entire genome did not appear to result in a vast increase in methylation due to methyl catalysts nor was it maintained generationally in response to increased dietary methyl catalyst supplementation. When CpG methylation levels were analyzed on a more localized affect, distinct changes in the level of methylation to specific genes related to the methylation cycle were detected. Evaluating localized methylation levels which are associated with a genomic region can assist in our understanding of the effect an environmental factor may be having on the regulation of gene transcription.

Epigenetic changes associated with nutrient availability in the offspring leads to further questions regarding the mechanisms behind the alterations of the epigenetic landscape for the expression or suppression of specific genes due to the early embryonic environment that results in long term changes in phenotype. Organisms respond and react to an environment to which they are exposed for generations. Knowledge of this interaction between the environment and the genome could prove beneficial in better understanding how environmental factors are related to the health and disease and possible approaches of how organisms can be predisposed to better handle the environment it is reared in. As oviparous organisms, avian species serve as interesting candidates to evaluate transgenerational inheritance because the embryo is developing in an incubator with many external environmental influences of the embryo's primordial germ cells. This is unlike mammals, wherein the primordial germ cells of the embryo are exposed to the same

environmental conditions as the parent. Understanding of predisposition via epigenetic changes could be a useful tool to the poultry industry by controlling the environmental conditions of the parents to better prepare the offspring for future environmental conditions in the hopes of producing a healthier bird with improved performance. In conclusion, avian species can serve as a useful model for epigenetic studies due to the absence of a uterus and imprinting which may allow for a clearer understanding of the impact of the environment on progeny.

Chapter 3

Transgenerational Comparative Analysis of Genome Wide DNA

Methylation and RNA Sequencing of Methylation Enzymes in Japanese Quail

Abstract

The environmental conditions to which one generation is exposed can result in epigenetic modifications of that genome thereby potentially preprogramming the offspring during development to withstand comparable environmental conditions. Regardless of the environmental stimuli resulting in transgenerational effects, epigenetic modification involves chemical modifications of DNA or of structural and regulatory proteins bound to DNA that result in expression or repression of genes at various loci. Therefore, the changes in expression are not due to changes in the nucleotide sequence itself. The aim of this study was to gain insight into new interactions or alternative processes of transgenerational epigenetics effects and allele specific expression in birds. Herein we characterize mechanisms which may potentially be altered due to a grandparent (G0) dietary treatment of high methyl catalysts during different time points of their life. When dietary methyl catalysts where fed to G0 a transgenerational epigenetic affect occurred in two subsequent generations (G2). Changes in CpG DNA methylation levels along with RPKM gene expression values were altered due to G0 dietary treatment. Correlations between methylation levels and RPKM values were analyzed for molecules associated with the methylation pathway which indicated in many cases increases in methylation level result in decreases in gene expression. Pathway analysis of RNA sequencing data reveled changes in metabolic functions such as amino acid and lipid metabolism. The results from this study are evidence that dietary treatment to a flock could have generational effects.

Introduction

Since the advancement of commercialized poultry production, diet has been an area of focus for those concerned with poultry production agriculture. Nutritionists and geneticists have become essential to growing healthy and efficient birds. It is our hypothesis that birds respond and react to an environment to which they are exposed for generations. Knowledge of this interaction between the environment and the genome could prove beneficial in better understanding how environmental factors such as nutrition and stressors are related to health and disease. This could assist in identifying possible approaches to predispose organisms to handle the environment in which they are reared in and how can a previous generations environmental conditions prepare progeny. One question regarding this could be, how is the diet which is fed to one generation affecting future generations and can grandparent or parental diet alter the requirements needed for the offspring?

Environmental conditions can affect development and thus phenotypes of an organism and these alterations may persist for generations. Environmental epigenomics refers to exposure of environmental factors, such as nutrition, during development that alters gene transcription through epigenetic modifications. While this subject has been extensively studied in other species, evidence has been presented in birds as well. One such example is in the preoptic hypothalamus where satiety and body temperature are regulated, where the epigenetic modification of histone H3 methylation was modified in 3 d old chicks that underwent 24 hr fasting (Xu et al., 2012). Another environmental exposure that has been shown to elicit epigenetic modifications is temperature. Increases in temperature have been shown to alter DNA methylation and histone modifications in the hypothalamus of chicks. Specifically, studies evaluated a thermoregulator gene, BDNF, which changes CpG methylation of the promoter region as well as H3K27 and H3K9

histone modifications (Kisliouk et al., 2010, 2011; Kisliouk and Meiri, 2009; Yossifoff et al., 2008). One additional example which could have immense affects to those in the poultry industry is a study evaluating the individual birds need for calcium and phosphorus. Although only observed in a single generation, calcium and phosphorus restriction during the growth period can result in preprogramming or adaptation of the bird to require less later in life (Ashwell and Angel, 2010; Yan et al., 2005).

Epigenetic changes associated with nutrient availability in the offspring leads to further questions regarding the mechanisms behind the alterations of the epigenetic landscape for the expression or suppression of specific genes due to the early embryonic environment that results in long term changes in phenotype. It could prove beneficial to understand the specific modifications which contribute to changes in phenotypes. As an oviparous organism, the chicken serves as an interesting candidate to evaluate transgenerational inheritance because the embryo is developing in an incubator with many external environmental influences of the embryo's primordial germ cells. This is unlike mammals, wherein the primordial germ cells of the embryo are exposed to the same environmental conditions as the parent. These ideas could be a useful tool to the poultry industry by controlling the environmental conditions of the parents to better prepare the offspring for future environmental conditions in the hopes of producing a healthier bird with improved performance.

Many enzymes, vitamins, and metabolites are involved in the process of DNA methylation. S-Adenosylmethionine (SAM) is known as the universal methyl donor which donates a methyl group directly to DNA with the help of DNA methyl transferases. Once donated SAM becomes homocysteine and therefore must go through the methylation cycle with a donation of a single carbon unit to yield methionine. Methionine is then converted to the biologically active molecule,

SAM, before methylation can occur. CpG DNA methylation and gene expression evaluation of the enzymes involved in the methylation cycle has yet to be examined in birds. Additionally, the supplementation of vitamins known as methyl catalysts, that are essential for DNA methylation, has previously been examined (Chapter 2 of this dissertation).

The aim of this study is to gain insight into new interactions or alternative processes of transgenerational epigenetics effects and allele specific expression in birds. Herein we characterize mechanisms which may potentially be altered due to a grandparent (G0) dietary treatment of high methyl catalysts during different time points of their life. The grandparent flock was fed a methyl diet or control diet during two phases: a starter phase and a layer phase. The analysis was accomplished through RNA sequencing data acquired from Illumina high throughput sequencing platform of liver tissue from the generation 2 (G2) flock and further analyzed using pathway analysis. Furthermore, in this study we compare gene expression analysis with genome wide DNA methylation level of G2 for a holistic understanding of the interaction CpG methylation level could be having with gene expression for various enzymes involved in the methylation cycle. The results from this study are evidence that dietary treatment to a flock could have generationally affects to future offspring.

Materials & Methods

Experimental Birds and Housing

The study was conducted at the Prestage Department of Poultry Science's Animal Facilities in Scott Hall at North Carolina State University. For all three generations, quail chicks were hatched from eggs obtained from North Carolina State University's residential meat type Japanese Quail lines in Scott Hall. At placement, each brooder level was equipped with two side feeders, two plastic jar style drinkers, and one supplemental feed tray. The supplemental feed tray was

removed at 14 d. Birds were raised in thermostatically controlled battery style brooders and later battery style layer cages with raised wire flooring. Room temperature at chick placement was 32.2°C (90°F). Air temperature was reduced daily until 7 d of age and was then maintained at approximately 23.9°C (70°F). Lighting was provided for 23 h from 0 – 3 d, 20 h from 4 – 10 d, 18 h from 11 d until the end of the trial. All birds were cared for according to North Carolina State University Institutional Animal Care and Use Committee (**IACUC**) guidelines.

Generation 0

For the first generation (G0) of this multi-generational trial, 324 Japanese Quail were weighed and randomly divided into four groups and placed in four separate brooder levels. Two brooder levels were given a starter feed that contained high levels of methyl catalysts while the other two levels were given a control feed that contained standard levels of methyl catalysts. 162 chicks received a control diet, while the remaining 162 received a control diet with the addition of methyl catalysts added on top. The methyl catalysts included 7030 mg/kg choline chloride, 5 mg/kg betaine, 1.5 mg/kg Vitamin B12, 7.5 mg/kg folic acid, 12 mg/kg pyridoxine, and 99 mg/kg zinc sulfate (Table 3.1). Chicks were given feed and water *ad libitum*. The control diet was formulated to meet NRC quail requirements for typical growth and reproduction.

Table 3.1. Composition of basal starter and layer diets (HiMet-HiMet, HiMet-Con, Con-HiMet, and Con-Con).

Ingredients (%)	Starter	Layer
Corn	49.18	48.56
Soybean meal (48% CP)	32.29	37.83
Poultry By-Product Meal	14.03	----
Soybean Oil	1.00	3.82
Mono-Dicalcium phosphate	0.04	1.60
Limestone	1.02	5.85
Salt	0.50	0.48
DL-Methionine	0.33	0.30
Choline chloride (60%)	0.10	0.10
L-Threonine	0.01	0.06
Vitamin premix[2]	0.10	0.10
Mineral premix[3]	0.20	0.20
PrimaLac	0.05	----
Lasolocid	0.05	----
Celite/Methyl Package	1.10	1.10
Calculated nutrient content		
Crude protein	27.90	22.60
Calcium	1.00	2.60
Available phosphorus	0.50	0.45
Total methionine	0.74	0.64
Metabolizable energy (kcal/kg)	2,850	2,800

[1]Starter diet was fed to approximately 6 wk of age
[2]Vitamin premix supplied the following per kg of diet: 13,200 IU vitamin A, 4,000 IU vitamin D_3, 33 IU vitamin E, 0.02 mg vitamin B_{12}, 0.13 mg biotin, 2 mg menadione (K_3), 2 mg thiamine, 6.6 mg riboflavin, 11 mg d-pantothenic acid, 4 mg vitamin B_6, 55 mg niacin, and 1.1 mg folic acid.
[3]Mineral premix supplied the following per kg of diet: manganese, 120 mg; zinc, 120 mg; iron, 80 mg; copper, 10 mg; iodine, 2.5 mg; and cobalt, 1 mg.
[4] PrimaLac is a probiotic manufactured by Star-Labs/Forage Research, Inc.
[5]Lasolocid is an anticoccidial with the trade name Avatec

At three weeks of age, birds were moved to 32 cages containing 4 females and 1 male per cage. From the two groups of either high or low methyl starter, 40 birds (32 females and 8 males) from each group were fed high or low methyl layer diets (Table 3.1). This provided four total groups of high methyl starter to low methyl layer, high methyl starter to high methyl layer, low methyl starter to high methyl layer and low methyl starter to low methyl layer. A total of 160 birds were placed in cages. Each generation consisted of four treatment groups indicating the diet they received at two different phases: High Starter High Layer (HiMet-HiMet), High Starter Control Layer (HiMet-Con), Control Starter High Layer (Con-HiMet) and Control Starter and Control

Layer (Con-Con). For all generations bird sex was not noted for the BW measured in the brooder because they are too young to differentiate. However once birds were moved to layer cages, three females and a single male were weighed for each cage.

Generation 1 & 2

Generation 1 (G1) and generation 2 (G2) consisted of 640 chicks from the four G0 treatment groups (HiMet-HiMet, HiMet-CON, CON-HiMet, CON-CON). All chicks were individually weighed and grouped based on G0 treatment (CH2. Figure 2.5). For placement, 10 chicks from each of the 32 parental groups were tagged and two chicks from each group were randomly placed in 5 separate brooder levels. The 10 chicks chosen to be placed in the 5 brooders were selected based on BWs that were closest to the average hatch weight from the previous generations hatch weight (10g). All chicks from the 4 parental treatment groups were tagged and placed together in one of the 5 brooder levels all receiving the same control diet fed to the parental generation low line.

At three wks of age three females from each group were randomly chosen and placed in the same cage group as their maternal line. One male from the same treatment but from a different cage group was assigned to the three females to avoid inbreeding. For G2, when birds were transferred to layer cages liver samples were collected randomly from males and females from each group. Samples were placed in RNAlater and stored at -80ºC.

RNA sequencing analysis

For liver RNA evaluation, samples were pooled for a single sample from the four treatment groups. Total RNA was extracted from the tissue using Qiagen RNeasy Mini protocol (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The RNA quality was assessed by Nanodrop 2000 spectrophotometer (Thermo, USA). Two micrograms of RNA from each sample

were taken to the North Carolina State University Genomics Sciences Laboratory for library preparation and sequencing on the Illumina HiSeq 2500 sequencer. RNA sequencing was analyzed using CLC Genomics Workbench (Qiagen, Valencia, CA, USA; licensed to NCSU) version 11 following the software manual (http://resources.qiagenbioinformatics.com/manuals/clcgenomics workbench/current/User_Manual.pdf). RNA sequencing reads and annotations were mapped to the Coturnix Japonica 2.0 reference genome which was downloaded from NCBI (https://www.ncbi.nlm.nih.gov/assembly/GCF_001577835.1). Raw reads were processed by the default settings of reads' quality control and adapter trimming. The false discovery rate p-value (FDRp) was calculated to correct for multiple testings' and an FDR adjusted $p \leq 0.05$ was considered statistically significant. Fold change and Log_2 fold change differences in gene expression between HiMet-HiMet, HiMet-Con, Con-HiMet and the Con-Con group were calculated. Reads per kilo base per million mapped reads (RPKM) values were used to analyze gene expression values between treatments for generation two (G2).

Bisulfite Conversion & Sequencing

Currently, the best-known method to determine DNA methylation is through genome wide bisulfite-conversion and sequencing. During bisulfite conversion, all unmethylated cytosines are converted into uracil while methylated cytosines are protected from the conversion process. Uracils are read as thymines by DNA polymerase and when bisulfite treated DNA is amplified using PCR the products of unmethylated cytosine appear as thymines. Methylated and unmethylated cytosines can therefore be distinguished after sequencing. A total of 4 pooled liver samples from each generation of female Japanese Quail from the four G0 grandparent treatments: HiMet-HiMet, HiMet-Con, Con-HiMet, Con-Con was analyzed using 100 ng of gDNA for bisulfite conversion. Liver samples for G0 were taken at 16 wk and for G1 and G2 at 3wks.

Bisulfite conversion was accomplished using EZ DNA Methylation-GoldTM Kit by Zymo (Irvine, CA, USA) following the manufacture's protocol. After conversion, 50 ng of single stranded DNA was used to create gDNA libraries using TruSeq DNA Methylation Kit following the manufacture's protocol (Illumina, San Diego, Ca). Libraries were sequenced using Illumina NextSeq with 75bp paired end runs with 800 million paired end reads total. A total of 24 total samples were sequenced with two runs of NextSeq-75 PE run with 800 PE reads total.

Pathway Analysis

Ingenuity Pathway Analysis (IPA; Qiagen, Valencia, CA; http://www.ingenuity.com) software was used for canonical pathway analysis, upstream regulatory analysis, and gene network discovery. G2 RNA sequencing liver samples were analyzed from HiMet-HiMet vs. Con-Con group, Con-HiMet vs. Con-Con and HiMet-Con vs. Con-Con G0 treatments were analyzed using IPA. The top statistical ($p<0.001$) expressed genes were analyzed from each group. IPA calculation of z-scores using the gene expression fold change values measures the state of activation or inhibition of the molecules involved in the molecular networks. The analysis of biological mechanisms occurring in the differentially expressed genes of the chicken in IPA were based on mammalian systems for human, rat, and mouse.

Results

Comparative Analysis of Diseases, Biological Function and Top Canonical Pathways

A comparative analysis using a Venn diagram allows for the visualization of differentially expressed genes from G2 based on G0 treatment groups compared to the control. There were 506 in common with altered gene expression between all three treatment groups (Figure 3.1). There are 1048 differentially expressed only in the HiMet-HiMet vs. Con-Con group, 1115 for Con-HiMet vs. Con-Con and only 395 for the HiMet- Con vs. Con-Con group (Figure 3.1). Of the 2323

total differentially expressed genes identified for the HiMet-HiMet vs. Con-Con, 533 were shared between the Con-HiMet vs. Con-Con which had a total of 2401 differentially expressed genes (Figure 3.1). Analysis of the 1384 total differentially expressed genes for the HiMet-Con vs. Con-Con group presents an overlap of 247 shared with the Con-HiMet vs. Con-Con group and 236 shared with the HiMet-HiMet vs. Con-Con group (Figure 3.1).

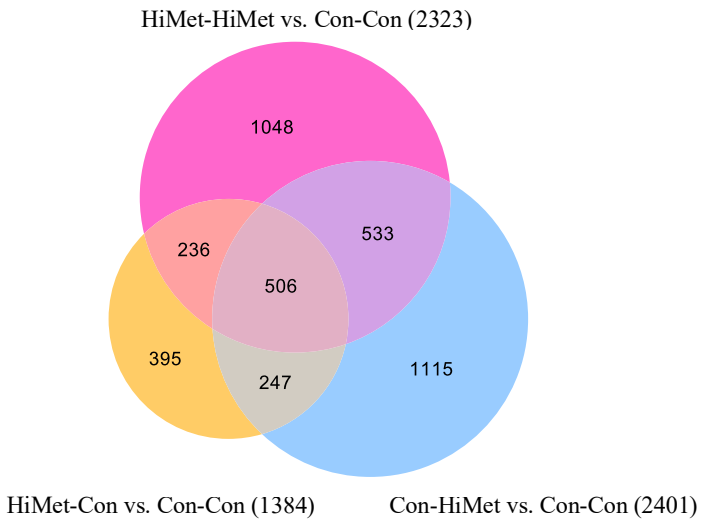

Figure 3.1. Overlap of Differentially Expressed Genes Across G2 Dietary Treatment. Differentially expressed genes between HiMet-HiMet, HiMet-Con, and Con-HiMet when compared to the control (Con-Con).

An in-depth examination of the comparative analysis between G0 treatment groups of the differentially expressed genes in G2 was accomplished using IPA. When comparing the differential expression of the treatment groups together it appears the diseases and biological functions which are significantly activated (Z scores ≥ 2) or repressed (Z score ≤ -2) were similar across groups (Figure 3.2). Similarly, those identified in the top canonical pathways act in similar fashion of repression or inhibition with some variation in intensity (Figure 3.3).

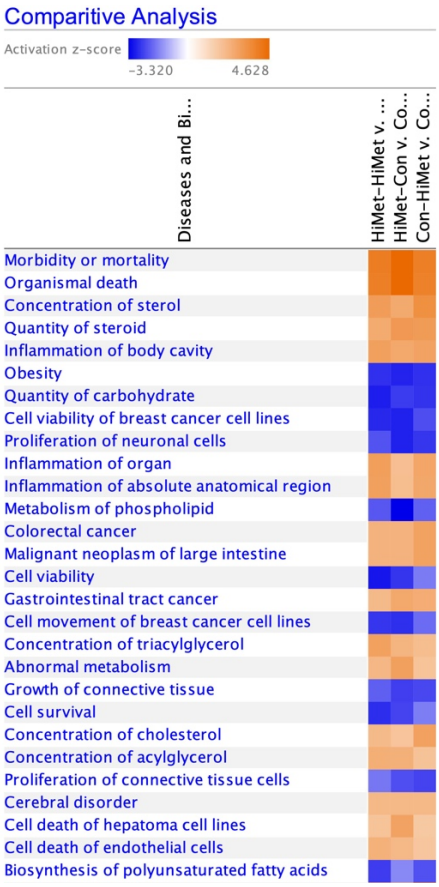

Figure 3.2. IPA Diseases and Biological Functions Comparative Analysis. Comparison of differentially expressed genes associated with diseases and biological functions from G2 HiMet-HiMet vs. Con-Con, HiMet-Con vs. Con-Con, and Con-HiMet vs. Con-Con.

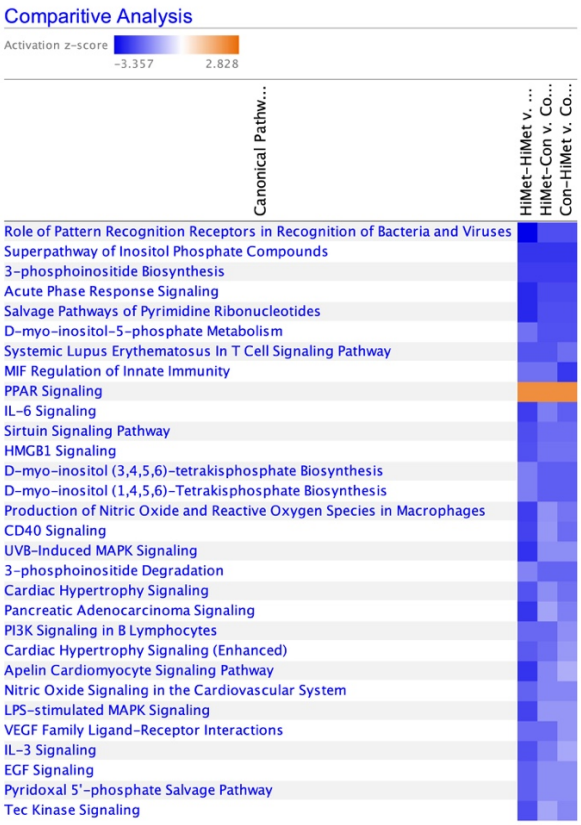

Figure 3.3. IPA Top Canonical Pathways Comparative Analysis. Comparison of differentially expressed genes associated with the top canonical pathways from G2 HiMet-HiMet vs. Con-Con, HiMet-Con vs. Con-Con, and Con-HiMet vs. Con-Con.

CpG DNA Methylation Levels in Relation to RPKM Gene Expression

Evaluating the CpG DNA methylation levels with the RPKM levels from RNA sequencing analysis, it appears that there is a correlation between DNA methylation and gene expression levels in some regions; however this pattern is not absolute, and many other factors could be playing a role (Table 3.2). DNMT3A has the lowest methylation level from the Con-HiMet group which also has the lowest RPKM value. However for DNMT3B, MTRR, and MAT2B the opposite is true for both the highest and lowest values which appear to be inversely related where highest methylation is associated with lowest RPKM and lowest methylation is associated with highest RPKM. Similarly for SHMT1, SHMT2, DHFR, PDXK the lowest methylation level is associated with the highest RPKM value and for MTHFR, RFK, MAT1A, CHDH, and AHCY the highest

methylation level is correlated with the lowest RPKM values. Like DNMT3A, high methylation level was associated with high RPKM values for MTR, MAT2A, FLAD1.

A linear correlation of the CpG methylation level in correlation with the RPKM gene expression of the 16 genes associated with enzymes needed for the methyl catalysts to be used in the methylation pathway revealed 11 which follow the normal trend of an increase in methylation level results in a decrease of gene expression (Figure 3.4). The 11 included AHCY, CHDH, DHFR, DNMT3B, MAT1A, MAT2B, MTHFR, MTRR, PDXK, RFK, and SHMT1 and the 5 not following the trend were DNMT3A, FLAD1, MAT2A, MTR, and SHMT2 (Figure 3.4)

Table 3.2. RNA Sequencing RPKM values and CpG DNA Methylation Levels for G2 Treatments.

Name	HiMet-HiMet RPKM*	HiMet-Con RPKM*	Con-HiMet RPKM*	Con-Con RPKM*	HiMet-HiMet Methylation Level	HiMet-Con Methylation Level	Con-HiMet Methylation Level	Con-Con Methylation Level	***n***
DNMT3A	2.1079	2.0541	1.8234	2.2622	0.7867	0.7224	0.7044	0.7382	15014
DNMT3B	0.1124	0.2300	0.3445	0.2625	0.7528	0.7433	0.7336	0.7464	5204
SHMT1	163.6446	224.9895	215.7287	349.1237	0.7121	0.7329	0.7082	0.6634	1612
SHMT2	57.3563	44.0377	40.6365	56.4316	0.7565	0.7761	0.7591	0.8061	1727
MTRR	0.7019	0.5599	0.6413	0.5118	0.8664	0.8822	0.8835	0.9086	1520
MTR	0.4450	0.3700	0.4020	0.3055	0.8770	0.8383	0.8709	0.8600	2843
MAT2A	2.7319	2.0736	2.0180	2.6126	0.8281	0.7576	0.7634	0.7936	1426
DHFR	1.3012	1.2894	1.6210	0.9894	0.9285	0.9513	0.8613	0.9238	359
MTHFR	2.2391	2.2399	2.1097	1.5645	0.7909	0.7761	0.7588	0.8074	1317
PDXK	5.3389	5.3163	6.9661	6.4969	0.8534	0.8505	0.8189	0.8435	2958
RFK	6.5922	4.8456	5.1786	3.0299	0.7500	0.7460	0.7361	1.0000	157
FLAD1	3.6633	3.5439	2.8687	4.5972	0.6813	0.6698	0.6812	0.6950	2113
MAT1A	42.5716	50.3513	35.1679	59.0595	0.8714	0.8266	0.8985	0.8471	1343
MAT2B	1.6445	1.5105	1.9176	1.4059	0.7862	0.8405	0.7299	0.8584	757
CHDH	4.8200	3.8909	3.8168	4.1845	0.8367	0.8112	0.9242	0.8638	477
AHCY	58.9278	67.9575	58.2173	49.8382	0.7874	0.7940	0.7936	0.8057	1924

**RPKM Reads Per Kilobase of transcript per Million mapped reads*

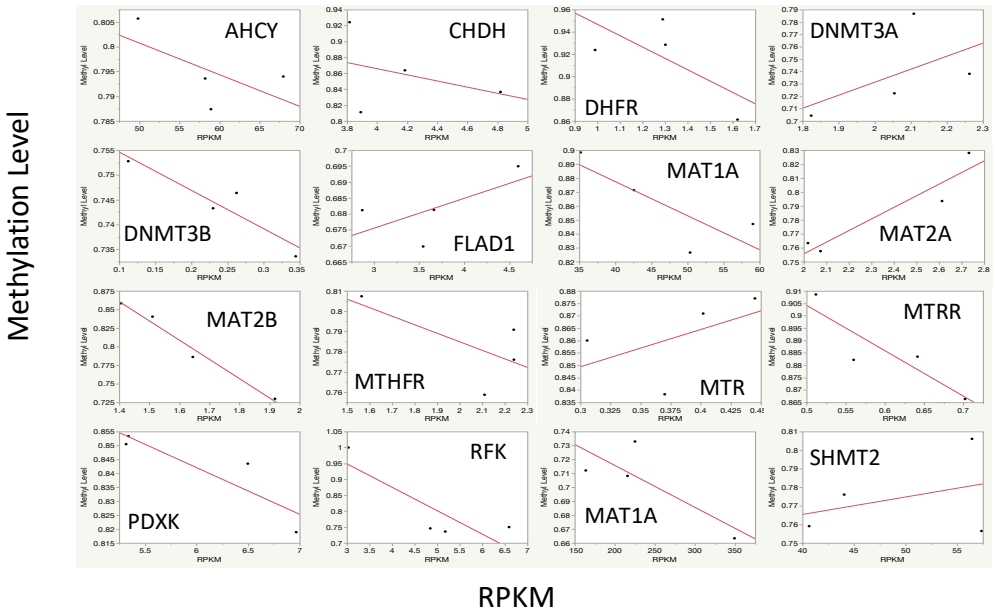

Figure 3.4. RNA Sequencing RPKM values and CpG DNA Methylation Levels for G2 Treatments Linear Graph.

IPA molecular and cellular functions and physiological system development and functional pathways

Analysis of the differentially expressed genes in IPA revealed a number of molecular and cellular functions and physiological system development and functional pathways. The most prominent pathways detected in molecular and cellular functions were carbohydrate metabolism, lipid metabolism, small molecule biochemistry, cellular function and maintenance, and cellular movement (Figure 3.5). The identified pathways for physiological system development and function were organismal development, organismal survival, connective tissue development and function, tissue development and morphology (Figure 3.5). Crossover of similar functions were observed across all three treatment groups compared with the control with the majority being involved in metabolism and cellular properties. A total of 14 functions were observed as being affected for carbohydrate metabolism with quantity of carbohydrate predicted to decrease in HiMet-HiMet compared to the Con-Con group. Lipid metabolism alone resulted in 20 affected functions with increased prediction of triacylglycerol, acyl glycerol, sterol, cholesterol and steroid

concentration in the G2 generation of grandparents (G0) who were fed a treatment group when compared with the control. Biosynthesis of polyunsaturated fatty acids and metabolism of phospholipids was predicted to decrease. Small molecule biochemistry had 30 total affected functions and was made up of energy production, lipid metabolism, amino acid metabolism and carbohydrate metabolism. The majority of the 9 functions for cellular function and maintenance were associated with cell organization and homeostasis. Associations with movement and migration of different cell types was observed for 26 total functions.

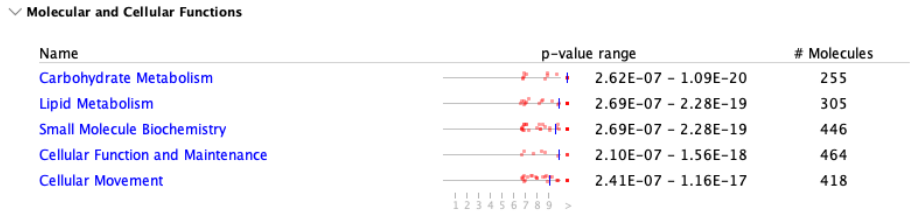

Figure 3.5. Top Molecular and Cellular Functions. Similar pathways were detected for the HiMet-HiMet v. Con-Con, HiMet-Con v. Con-Con, and Con-HiMet v. Con-Con groups.

Mapped Molecular Networks

Functional relationships formed through molecular networks in IPA to examine the interaction of differentially expressed genes with other gene products. These molecular networks were formed based on connections evaluated in previous literature on human, mouse, and rat. Main networks identified tended to surrounded metabolic functions. This comprised of lipid metabolism and amino acid metabolism which were all affected in G2 from grandparent treatment group (G0) when compared with the control. In IPA, the amino acid network had a score of 43 with 30 focus molecules. This included the upregulation of the upstream regulator MYC resulted in the downregulation of many metabolic enzymes including previously mentioned methylation cycle enzymes SHMT1 and MTHFR (Figure 3.6). The IPA molecular network Lipid Metabolism was

detected with a score of 21 with 16 focus molecules (Figure 3.7). This pathway included molecules such as *lipoprotein lipase (LPL)*, known to have epigenetic alterations due to methyl catalyst supplementation (Xing et al., 2011).

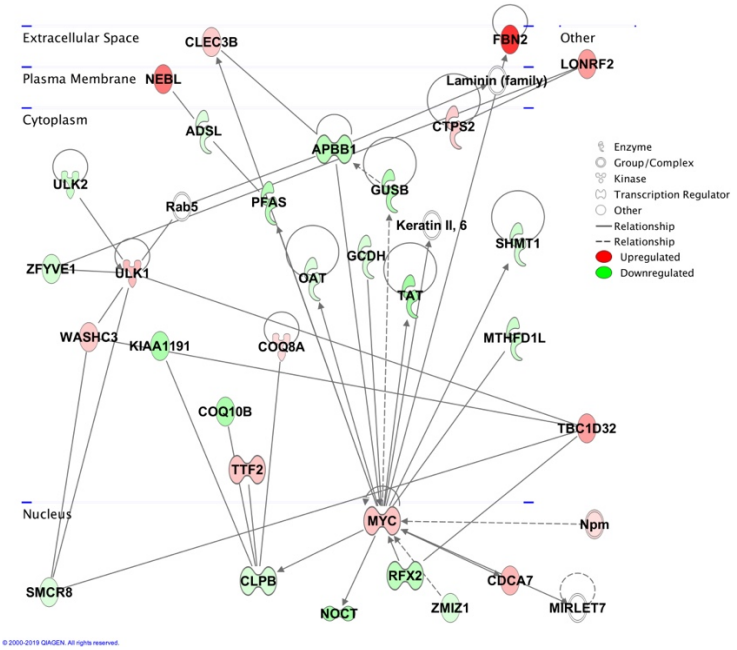

Figure 3.6. IPA Molecular Network Amino Acid Metabolism.

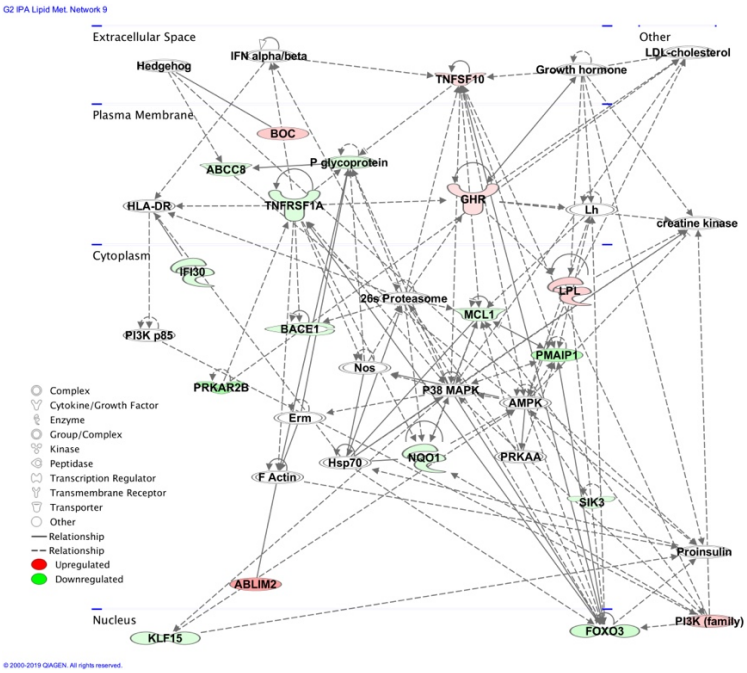

Figure 3.7. IPA Molecular Network Lipid Metabolism.

Gene Function Analysis

The genes listed in Table 3.3 were evaluated in literature to identify biological function. Through this investigation of our gene list from liver of HiMet-HiMet vs. Con-Con, HiMet-Con vs. Con-Con, and Con-HiMet vs. Con-Con, multiple top molecules associated with metabolism, regulation and cellular processes were revealed (Table 3.3). Many of the molecules identified that have increased or decreased expression are present in more than one group. The predicted activated molecules included *ASCL1, BHLHE41, ABLIM2, ANF385B, PLEKHG7, NOTUM, LAPTM4B, DGKB, TMPRSS15, CADM2, CA4, FBN2, WNT11, CCDC3, TENM2, PIK3C2G*. Molecules predicted to be inactive were *EREG, ORM2, NPFFR1, MIOX, CASR, ATP10B, TSPO2, IL4I1, MATN4, DKK2, CEL, TRIM29, STC2*.

Table 3.3. Top Analysis Ready Molecules.

Gene ID	Group	Expression Log Ratio	Description
ASCL1	HiMet-HiMet vs Con-Con, Con-HiMet vs Con-Con	3.698, 2.586	Activates transcription with dimerization of other proteins for the neuronal commitment and differentiation
BHLHE41	HiMet-HiMet vs Con-Con	3.577	Involved in the control of circadian rhythm and cell differentiation
ABLIM2	HiMet-HiMet vs Con-Con, Con-HiMet vs Con-Con	3.272, 3.016	Actin binding
ZNF385B	HiMet-HiMet vs Con-Con	2.976	Nucleic acid binding and p53 binding
PLEKHG7	HiMet-HiMet vs Con-Con	2.909	Rho guanyl-nucleotide exchange factor activity
NOTUM	HiMet-HiMet vs Con-Con, HiMet-Con vs Con-Con	2.82, 1.704	Hydrolase activity and palmitoleyl hydrolase activity
LAPTM4B	HiMet-Con vs Con-Con, Con-HiMet vs Con-Con	3.838, 3.565	Lysosomal function
DGKB	HiMet-Con vs Con-Con	2.16	Regulators of intracellular concentration of the second messenger diacylglycerol and a role in cellular processes
TMPRSS15	HiMet-Con vs Con-Con	2.113	Enzyme that converts the pancreatic proenzyme trypsinogen to trypsin
CADM2	HiMet-Con vs Con-Con	1.888	Crosslinks spectrin and interact with other cytoskeletal proteins
CA4	HiMet-Con vs Con-Con	1.815	Catalyze the reversible hydration of carbon dioxide
FBN2	HiMet-Con vs Con-Con, Con-HiMet vs Con-Con	1.7.4, 3.056	Component of connective tissue microfibrils and possibly elastic fiber assembly
WNT11	Con-HiMet vs Con-Con	3.875	Developmental processes (regulation of cell fate and patterning during embryogenesis)
CCDC3	Con-HiMet vs Con-Con	2.461	Negatively regulates TNF-alpha-induced pro-inflammatory response and positively regulates lipid accumulation
TENM2	Con-HiMet vs Con-Con	2.381	Calcium ion binding and protein heterodimerization activity
PIK3C2G	Con-HiMet vs Con-Con	2.374	Cell proliferation, oncogenic transformation, cell survival, cell migration, and intracellular protein trafficking
EREG	HiMet-HiMet vs Con-Con, Con-HiMet vs Con-Con	-7.147, -4.635	Ligand of epidermal growth factor receptor, may be involved in inflammation, wound healing, oocyte maturation, and cell proliferation
ORM2	HiMet-HiMet vs Con-Con, HiMet-Con vs Con-Con	-6.844, -5.662	Acute phase reactant plasma protein that may be involved in immunosuppression
NPFFR1	HiMet-HiMet vs Con-Con, HiMet-Con vs Con-Con, Con-HiMet vs Con-Con	-6.646, -4.144, -4.993	Protein-coupled receptor activity and neuropeptide receptor activity
MIOX	HiMet-HiMet vs Con-Con	-6.146	Iron ion binding and oxidoreductase activity, acting on NAD(P)H
CASR	HiMet-HiMet vs Con-Con, HiMet-Con vs Con-Con, Con-HiMet vs Con-Con	-6.069, -3.656, -4.026	Role in maintaining mineral ion homeostasis
ATP10B	HiMet-HiMet vs Con-Con, HiMet-Con vs Con-Con	-5.937, -6.021	Nucleotide binding and cation-transporting ATPase activity
TSPO2	HiMet-HiMet vs Con-Con, Con-HiMet vs Con-Con	-5.745, -3.874	Cholesterol binding
IL4I1	HiMet-HiMet vs Con-Con, HiMet-Con vs Con-Con	-5.461, -5.545	Catalysis and binding of flavin adenine dinucleotide (FAD) cofactor
MATN4	HiMet-HiMet vs Con-Con, HiMet-Con vs Con-Con, Con-HiMet vs Con-Con	-5.365, -5.338, -4.597	Formation of filamentous networks in the extracellular matrices tissues
DKK2	HiMet-Con vs Con-Con	-5.388	Involved in embryonic development through interactions with Wnt signaling
CEL	HiMet-Con vs Con-Con	-5.344	Cholesterol and lipid-soluble vitamin ester hydrolysis and absorption
TRIM29	HiMet-Con vs Con-Con	-3.964	Transcriptional regulatory factor
STC2	Con-HiMet vs Con-Con	-4.211	Regulation of renal and intestinal calcium and phosphate transport, cell metabolism, or cellular calcium/phosphate homeostasis

Discussion

Parental nutrition has been identified as a factor that can influence offspring metabolism and alter phenotypes beyond the first generation (Radford et al., 2012, 2014). Hepatic RNA

abundance was analyzed for G2 rather than the previous generations (G0 and G1), because G2 was the first true generation that was not directly influenced by dietary treatment. G1 was not fed the treatment diet, however the oocytes for this generation were formed inside the maternal line which were fed the treatment diets. When analyzing this G2 generation, a large portion of the molecular networks identified by IPA included metabolic functions. These varied from carbohydrate metabolism, energy production, lipid metabolism, and amino acid metabolism which were all affected in G2 from grandparent treatment groups (G0) when compared with the control. Evaluation of the amino acid metabolism network from IPA in Figure 6 demonstrates many molecules affected by MYC.

MYC is a sequence specific transcription factor but is also capable of repressing the transcription of genes through interactions with other transcription factors. MYC recruits DNA methyltransferases, DNMT3A and DNMT3B increasing the methylation to the promoter regions of CpG Islands (Poole and van Riggelen, 2017). Overexpression causes the deregulation of essential cellular processes such as cell cycle progression, metabolism and differentiation. MYC also regulates nutrient acquisition (Stine et al., 2015).

Within the amino acid metabolism network upregulation of the upstream regulator MYC resulted the downregulation of many metabolic enzymes, and two of the enzymes being regulated were serine hydroxymethyltransferase (SHMT1) and methylenetetrahydrofolate dehydrogenase (MTHFRD1L) (Figure 3.5). These were previously mentioned as enzymes involved in the methylation cycle (Table 3.2). MTHFRD1L and SHMT are needed in the methylation cycle to produce the active form of methionine, S-adenosylmethionine, from homocysteine. S-adenosylmethionine is known as the universal methyl donor needed to donate a methyl group to DNA or proteins. For SHMT1 low methylation level was associated with the highest RPKM value

and for MTHFR the highest methylation level was correlated with the lowest RPKM values (Table 3.2). MTHFR and SHMT CpG methylation levels have not been evaluated in birds; however when broiler breeders were fed a diet deficient in folate their offspring had higher serum levels of MTHFR and dihydrofolate reductase (DHFR) when compared to the control offspring (Xing et al., 2018). Gene expression was analyzed from these offspring and MTHFD2L, SHMT1, and LPL was increased from the progeny of folate deficient hens (Xing et al., 2018). Our data show that MTHFR, DHFR, and SHMT follow the typical pattern of an increase in CpG DNA methylation results in a decrease in gene expression values which is most commonly due to the inability for transcription machinery to transcribe the genomic region.

Reductions in the expression of just a single enzyme involved in the methylation pathway can result in biological effects. An example of this is the methionine adenosyltrasferease, MAT1A, in which downregulation has been shown to led to reduced levels of SAM and increase oxidative stress (Aggrey et al., 2018; Niculescu and Zeisel, 2002). MAT1A is also involved in the production of S-adenosylmethionine. In our analysis MAT1A followed the trend of an increase in methylation level resulted in a decrease in methylation (Figure 3.4). Evaluation of the CpG methylation level in correlation with the RPKM gene expression of the 16 genes associated with enzymes needed for the methyl catalysts to be used in the methylation pathway revealed 11 which follow the normal trend of an increase in methylation level results in a decrease of gene expression. These 11 were AHCY, CHDH, DHFR, DNMT3B, MAT1A, MAT2B, MTHFR, MTRR, PDXK, RFK, and SHMT1 (Figure 3.4). It is possible that DNA methylation level to the remaining 5 genes does not affect the binding of transcription machinery or they could be involved in another regulatory process entirely. These 5 were DNMT3A, FLAD1, MAT2A, MTR, and SHMT2 (Figure 3.4). All

of these enzymes are involved in methionine metabolism in the production of S-adenosylmethionine for the direct methylation of DNA.

The most common metabolic pathways studied in humans to be affected due to parental diet has been glucose homeostasis as well as cholesterol and lipid metabolism (Rando and Simmons, 2015). The IPA lipid metabolism molecular network was a pathway discovered as being affected in all three G0 treatment groups when compared to the control in G2 (Figure 3.7). Additionally many of top analysis ready molecules are thought to be involved in lipid metabolism and cellular regulation (Table 3.3). Our study is not the first to detect changes in gene expression and methylation level of genes involved in lipid metabolism of the bird due to the addition of methyl catalysts. Folate was administered to cultured chicken adipocytes to evaluate the effects on CpG methylation and gene expression of essential adipogenic genes in the chicken (Yu et al., 2014). The addition of folate resulted in a decreased gene expression of peroxisome proliferator-activated receptor γ (PPARγ), CCAAt/enhancer binding protein α (C/EBPα) and fatty acid synthase (Yu et al., 2014). CpG methylation level was lower for the gene associated with C/EBPα in the control cells compared to the folate treated cells but methylation of PPARγ was not affected (Yu et al., 2014). Interestingly in our study, when looking at two generations removed from the folate addition, activation of the PPAR pathway was detected in treatment groups compared with the control (Figure 3.3). This difference could be due to the difference in generations evaluated, tissue type, or that we did not evaluate our samples *in vitro*. Additionally, when folate supplementation was only administered to male breeders the most altered offspring metabolites and hepatic transcriptome pathway analysis were involved in lipid and glucose metabolism (Wu et al., 2019). The methyl catalysts for our study were given to both male and female birds in G0 with analysis in G2. It is possible that alterations in the methylation level and gene expression from G2 could be

sex specific. We are unable to conclude if more of the effect was due to the addition of methyl catalysts to the maternal or paternal line of G0 however in the future this distinction would be of interest. Although unlike the other studies, we evaluated a longer generation time (G2 rather than G1) which could indicate a longer time period for transgenerational modifications than anticipated.

Folate is not the only methyl catalyst observed to alter lipid metabolism in the bird. Betaine has been shown to improve the digestibility of methionine and reduce fat deposition (Scott et al., 1982). One possible mechanism for reduced fat alteration was by a change in methylation status at the CpG promoter region of *lipoprotein lipase (LPL)*, ultimately decreasing gene expression in the presence of betaine supplementation (Xing et al., 2011). LPL is a molecule affected in the lipid metabolism molecular networks for the G2 treatment groups compared to the control (Figure 3.7). In rats from E11-17, a choline deficient diet resulted in global hypermethylation which could be due to hypomethylation of the CpGs associated with DNMTS resulting in an increase in the production of DNMT1; however, when choline supplementation was given, there was an increase in S-Adenosylmethionine found in both tissues (Kovacheva et al., 2007).

Folate is known to influence lipid accumulation through the use of S-adenosylmethionine and epigenetic modifications typically by reducing the accumulation (Yu et al., 2014). Lipid metabolism IPA analysis resulted in 20 affected functions with increased prediction of triacylglycerol, acyl glycerol, sterol, cholesterol and steroid concentration in the liver of G2 generation from grandparents (G0) who were fed a treatment group when compared with the control. Perhaps increases in these transcript levels can be correlated with changes observed in the egg size from this generation (Chapter 2). G2 was the first generation the Con-Con group had the largest eggs when compared to the HiMet-HiMet group. It is possible the removal of the methyl catalyst treatment in the next generation affects G1 and G2 differently. Regulation of metabolic

processes could alter G1 in one manner but considering G2 has not been affected by the treatment their epigenetic response could be different.

A comparative analysis of gene expression from G2 based on the three G0 treatment groups compared to the control (HiMet-HiMet vs. Con-Con group, Con-HiMet vs. Con-Con and HiMet-Con vs. Con-Con) revealed similar canonical pathways and diseases and biological functions (Figure 3.2; Figure 3.3). Additionally, many of the individual genes analyzed as the top analysis ready molecules in IPA are present in two groups when compared to the control group. G0 of the HiMet-HiMet was fed the methyl catalyst diet throughout both the growth phase and layer phase. G0 of the Con-HiMet was fed the methyl catalyst diet during the layer phase and HiMet-Con was fed the methyl catalyst diet during the growth phase. By G2, the impacts of treatment seem to be similar in relation to RNA sequencing even though phenotypic differences in egg size were different (Chapter 2). It is possible that the differences are not easily detectable when evaluating the dataset as a whole but localized differences in specific gene transcripts may be altered such as the changes in gene expression listed in the Venn diagram where there are overlaps between groups but there are also specific changes in gene expression found within the treatment group (Figure 3.1). Research has not been conducted in birds to determine how timepoints of environmental factors such as nutrition may alter gene expression generationally. From our data it is difficult to determine if one time point (grower phase or layer phase) has a larger effect on generations, however a study evaluating negative factors such as stress may be a better indicator. In mammals, the development of many diseases later in life have been linked to the intrauterine conditions of the mother (Hales and Barker, 2001; Ravelli et al., 1976, 1998; Valdez et al., 1994). The concept that poor nutrition early in life is correlated with poor fetal growth and later development of metabolic disorders is often denoted as the “thrifty phenotype hypothesis” (Hales and Barker,

2001). Many of these studies have evaluated only the next generation which has directly been affected by the environmental exposure. In mammals, when applying an environmental condition to G0, both G1 and G2 have been exposed to the condition due to the developing oocytes in the embryo. Whereas, in oviparous organisms, G2 would not have been exposed to the G0 environment. Thus gene expression differences due to G0 dietary treatment observed in G2 of the current study presented herein would suggest an epigenetic effect.

Conclusions

The environmental conditions to which one generation is exposed can result in epigenetic modifications of that genome thereby potentially preprogramming the offspring during development to withstand comparable environmental conditions. In certain conditions this could be beneficial to offspring for a quick adaptation to an environmental stimulus that could potentially increase the chance of survival for the next generation. In contrast, poor nutrition during early stages of life could result in persistent negative alterations such as susceptibility to disease. Regardless of the environmental stimuli resulting in transgenerational effects, epigenetic modification involves chemical modifications of DNA or of structural and regulatory proteins bound to DNA that result in expression or repression of genes at various loci. Therefore, the changes in expression are not due to changes in the nucleotide sequence itself. When dietary methyl catalysts including choline chloride, betaine, vitamin B12, folic acid, pyridoxine, and zinc sulfate where fed to G0 a transgenerational epigenetic effect occurred in two generations out G2. Changes in CpG DNA methylation levels along with RPKM gene expression values were altered due to G0 treatment. Correlations between methylation levels and RPKM values were analyzed for molecules associated with the methylation pathway which indicated in many cases increases

in methylation level result in decreases in gene expression. Pathway analysis of RNA sequencing data reveled changes in metabolic functions such as amino acid and lipid metabolism.

Minimal research has been established in birds on transgenerational epigenetic modifications in birds due to environmental factors. This information could be useful for the future of how diets are formulated for generations of birds and could potentially led to the programming of offspring to withstand different dietary treatments. The generation of new approaches to analyze the bird genome and epigenome will help in a deeper understanding of the regulatory events which occur for complex phenotypes. Overall this could lead to an improvement in performance through the understanding of dietary implications from previous generations and by potentially bringing about new parameters in bird selection.

Chapter 4

Evaluation of Transgenerational Epigenetic Effects on

Egg Related Genes as a Result of Grandparent Dietary Treatment

Abstract

One way the environment interacts with the organism is through epigenetic modifications of DNA such as DNA methylation. The addition or subtraction of a methyl group to the 5-carbon ring of cytosine can alter expression of genes without changing the DNA sequence. The maternal epigenome can contribute to optimal embryonic development and chick quality. The aim of our study was to gain insight into the processes of transgenerational epigenetic effects and allele

specific expression of protein and lipid metabolism associated with egg production in birds due to previously detected egg weight differences in response to dietary treatment. The grandparent flock was fed a methyl diet or control diet during two phases: a starter phase and a grower phase, while the following two generations were fed a control diet. Genes analyzed consisted of vitellogenin 1, vitellogenin 2, vitellogenin 2-like, MTTP, APOB and VLDLR. Herein we characterize DNA methylation levels and gene expression values which may potentially be altered due to a grandparent (G0) dietary treatment of high methyl catalysts during different time points of their life. The analysis was accomplished through RNA sequencing data acquired from Illumina high throughput sequencing platform of liver tissue from generation 2 (G2) flock and further analyzed using pathway analysis. The results from this study are evidence that dietary treatment to a flock could alter CpG DNA methylation levels of egg associated genes.

Introduction

Healthy embryos, and later progeny, are dependent on the environment in which they develop. One way the environment interacts with the organism is through epigenetic modifications of DNA such as DNA methylation. The addition or subtraction of a methyl group to the 5-carbon ring of cytosine can alter expression of genes without changing the DNA sequence. The maternal epigenome can contribute to optimal embryonic development and chick quality. The chick spends a significant portion of its life in the egg and many environmental conditions such as humidity, temperature, lighting, and nutrition provided during development can alter the quality of the chick. The avian egg contains all the required nutrients for the embryo to develop during incubation. Changes in lipoprotein metabolism during lay displays a potential mechanism to the offspring-parent conflict in which females must have a balance in the amount of resources allocated to eggs and offspring as well as also meeting her own nutritional demands (Bernardo, 1996). If nutrients are in abundance this may result in more efficient deposition of proteins and nutrients transferred to the embryo by way of the egg while also meeting or exceeding the hen's maintenance requirements.

Yolk serves as the essential nutrient reserve for developing oviparous embryos. The precursor to major yolk proteins is vitellogenin, a glycolipophosphoprotein produced in the liver with production induced by estrogen (Biscotti et al., 2018). Very low density lipoprotein (VLDLy) is the source for yolk lipids (Salvante et al., 2007). The stimulation of hepatocytes by estrogen leads to an accumulation of vitellogenin mRNAs and VLDLy in the blood which is then taken up by receptor-mediated developing follicles. In the laying chicken, vitellogenin production makes up about 50% of the protein production in the liver (Williams, 2019). As a result of increased lipid production in the liver, neutral lipids in the plasma of laying turkeys was shown to increase 7 times

in concentration (Bacon et al., 1974) and plasma triglycerides in laying chickens increased from 0.5-1.5 μmol/ml to 20-50 μmol/ml of plasma (Griffin and Hermier, 1988). Birds have three forms of vitellogenin: VTGI, VTGII, and VTGIII with VTGII being the most common (Wang et al., 1983). Estrogen in the liver transitions VLDV production to smaller yolk-targeted particles (VLDLy) to pass through the pores of the granulosa basal lamina of the ovary and also changes apolipoprotein composition (apoA-I, apoB and apoC) that function in the transport of triglycerides (Speake et al., 1998; Walzem et al., 1999). Considering the change in size for yolk specific VLDL, the two apoproteins used in its transportation are apoB and apoVLDLII (Speake et al., 1998; Walzem et al., 1999).

The most common metabolic pathways in human progeny that have been shown to be affected by parental diet are glucose homeostasis and cholesterol and lipid metabolism (Rando and Simmons, 2015). Comparing the impact of epigenetic regulation on lipid metabolism in the laying bird to that of humans and obesity, diabetes, and high triglycerides may be difficult because the utilization of fats in food animals can be viewed as a positive occurrence in food products such as eggs and marbleized meat. Poultry diets have been formulated to maximize phenotypic parameters such as meat and egg yield. This study was based on the idea that the addition of dietary methyl catalysts provided during one generation (G0) resulted in transgenerational changes in egg size for two subsequent generations (G1 & G2). It is our hypothesis that this phenotypic change is due to alterations in DNA methylation patterns that affect lipid metabolism in future generations. This process has been well documented in mammals. One example of this is in A^{vy} (viable yellow agouti) female mice fed methyl catalysts including vitamin B_{12}, folic acid, betaine and choline (Waterland and Jirtle, 2003). Due to the insertion of a transposable element with CpG islands prior to the start site of the agouti gene, varying amounts of DNA methylation can occur. Phenotypic

changes occur in mice depending on the level of methylation. Unmethylation to this site has resulted in yellow, obese mice but under supplementation of methyl catalyst has resulted in increased DNA methylation and produced mice progeny that were brown and not obese. Maternal treatment of methyl catalysts on obesity of offspring persisted three generations out (Waterland et al., 2008). These results suggest parental nutrition can alter progeny lipid metabolism.

The aim of our study was to gain insight into the processes of transgenerational epigenetic effects and allele specific expression of protein and lipid metabolism associated with egg production in birds due to previous egg weight differences detected (Chapter 2). Herein we characterize DNA methylation levels and gene expression values which may potentially be altered due to a grandparent (G0) dietary treatment of high methyl catalysts during different time points of their life. The grandparent flock was fed a methyl diet or control diet during two phases: a starter phase and a grower phase. The analysis was accomplished through RNA sequencing data acquired from Illumina high throughput sequencing platform of liver tissue from generation 2 (G2) flock and further analyzed using pathway analysis. The results from this study are evidence that dietary treatment to a flock could alter CpG DNA methylation levels and expression levels of egg associated genes.

Materials & Methods

Experimental Birds and Housing

The study was conducted at the Prestage Department of Poultry Science's Animal Facilities in Scott Hall at North Carolina State University. For all three generations, quail chicks were hatched from eggs obtained from North Carolina State University's residential meat type Japanese Quail lines in Scott Hall. At placement, each brooder level was equipped with two side feeders, two plastic jar style drinkers, and one supplemental feed tray. The supplemental feed tray was

removed at 14 d. Birds were raised in thermostatically controlled battery style brooders and later battery style layer cages with raised wire flooring. Room temperature at chick placement was 32.2°C (90°F). Air temperature was reduced daily until 7 d of age and was then maintained at approximately 23.9°C (70°F). Lighting was provided for 23 h from 0 – 3 d, 20 h from 4 – 10 d, 18 h from 11-21 d. All birds were cared for according to North Carolina State University Institutional Animal Care and Use Committee (**IACUC**) guidelines.

Generation 0

For the first generation (G0) of this multi-generational trial, 324 Japanese Quail were weighed and randomly divided into four groups and placed in four separate brooder levels. Two brooder levels were given a starter feed that contained high levels of methyl catalysts while the other two levels were given a control feed that contained standard levels of methyl catalysts (Figure 2.1). 162 chicks received a control diet, while the remaining 162 received a control diet with the addition of methyl catalysts added on top. The methyl catalysts included 7030 mg/kg choline chloride, 5 mg/kg betaine, 1.5 mg/kg Vitamin B12, 7.5 mg/kg folic acid, 12 mg/kg pyridoxine, and 99 mg/kg zinc sulfate (Table 4.1). Chicks were given feed and water ad libitum. The control diet was formulated to meet NRC quail requirements for typical growth and reproduction.

Table 4.1. Composition of basal starter and layer diets (HiMet-HiMet, HiMet-Con, Con-HiMet, and Con-Con).

Ingredients (%)	Starter	Layer
Corn	49.18	48.56
Soybean meal (48% CP)	32.29	37.83
Poultry By-Product Meal	14.03	----
Soybean Oil	1.00	3.82
Mono-Dicalcium phosphate	0.04	1.60
Limestone	1.02	5.85
Salt	0.50	0.48
DL-Methionine	0.33	0.30
Choline chloride (60%)	0.10	0.10
L-Threonine	0.01	0.06
Vitamin premix[2]	0.10	0.10
Mineral premix[3]	0.20	0.20
PrimaLac	0.05	----
Lasolocid	0.05	----
Celite/Methyl Package	1.10	1.10
Calculated nutrient content		
Crude protein	27.90	22.60
Calcium	1.00	2.60
Available phosphorus	0.50	0.45
Total methionine	0.74	0.64
Metabolizable energy (kcal/kg)	2,850	2,800

[1]Starter diet was fed to approximately 6 wk of age
[2]Vitamin premix supplied the following per kg of diet: 13,200 IU vitamin A, 4,000 IU vitamin D_3, 33 IU vitamin E, 0.02 mg vitamin B_{12}, 0.13 mg biotin, 2 mg menadione (K_3), 2 mg thiamine, 6.6 mg riboflavin, 11 mg d-pantothenic acid, 4 mg vitamin B_6, 55 mg niacin, and 1.1 mg folic acid.
[3]Mineral premix supplied the following per kg of diet: manganese, 120 mg; zinc, 120 mg; iron, 80 mg; copper, 10 mg; iodine, 2.5 mg; and cobalt, 1 mg.
[4] PrimaLac is a probiotic manufactured by Star-Labs/Forage Research, Inc.
[5]Lasolocid is an anticoccidial with the trade name Avatec

At three weeks of age, birds were moved to 32 cages containing 4 females and 1 male per cage. From the two groups of either high or low methyl starter 40 birds (32 females and 8 males) from each group were fed high or low methyl layer diets (Table 4.1). This provided four total groups of high methyl starter to low methyl layer, high methyl starter to high methyl layer, low methyl starter to high methyl layer and low methyl starter to low methyl layer. A total of 160 birds were placed in cages. Each generation consisted of four treatment groups indicating the diet they received at two different phases: High Starter High Layer (HiMet-HiMet), High Starter Control

Layer (HiMet-Con), Control Starter High Layer (Con-HiMet) and Control Starter and Control Layer (Con-Con) (Figure 2.1). For all generations bird sex was not separated for the BW measured in the brooder because they are too young to differentiate. However once birds were moved to layer cages three females and a single male were weighed for each cage.

Generation 1 & 2

Generation 1 (G1) and generation 2 (G2) consisted of 640 chicks from the four G0 treatment groups (HiMet-HiMet, HiMet-CON, CON-HiMet, CON-CON). All chicks were individually weighed and grouped based on G0 treatment (Figure 2.1). For placement, 10 chicks from each of the 32 parental groups were tagged and two chicks from each group were randomly placed in 5 separate brooder levels. The 10 chicks chosen to be placed in the 5 brooders were selected based on BWs that were closest to the average hatch weight from the previous generations hatch weight (10g). All chicks from the 4 parental treatment groups were tagged and placed together in one of the 5 brooder levels all receiving the same control diet fed to the parental generation low line.

At three wks of age three females from each group were randomly chosen and placed in the same cage group as their maternal line. One male from the same treatment but from a different cage group was assigned to the three females to avoid inbreeding. For G2, when birds were transferred to layer cages liver samples were collected randomly from males and females from each group. Samples were placed in RNAlater and stored at -80ºC.

RNA sequencing analysis

For liver RNA evaluation, samples were pooled for a single sample from the four treatment groups. Total RNA was extracted from the tissue using Qiagen RNeasy Mini protocol (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The RNA quality was assessed by

Nanodrop 2000 spectrophotometer (Thermo, USA). Two micrograms of RNA from each sample were taken to the North Carolina State University Genomics Sciences Laboratory for library preparation and sequencing on the Illumina HiSeq 2500 sequencer. RNA sequencing was analyzed using CLC Genomics Workbench (Qiagen, Valencia, CA, USA; licensed to NCSU) version 11 following the software manual (http://resources.qiagenbioinformatics.com/manuals/clcgenomics workbench/current/User_Manual.pdf). RNA sequencing reads and annotations were mapped to the Coturnix Japonica 2.0 reference genome which was downloaded from NCBI (https://www.ncbi.nlm.nih.gov/assembly/GCF_001577835.1). Raw reads were processed by the default settings of reads' quality control and adapter trimming. The false discovery rate p-value (FDRp) was calculated to correct for multiple testings' and an FDR adjusted $p \leq 0.05$ was considered statistically significant. Fold change and Log_2 fold change differences in gene expression between HiMet-HiMet, HiMet-Con, Con-HiMet and the Con-Con group were calculated.

Statistical analysis for CpG DNA methylation sites for the three generations of quail from HiMet-HiMet, HiMet-Con, Con-HiMet, Con-Con treatments in this study were analyzed as a 3 x 4 factorial arrangement with the Standard Least Squares ANOVA within the Fit Model platform of JMP 14 (SAS Institute, Cary, NC). Differences among means were considered statistically significant when $P \leq 0.05$ and means were separated using Tukey HSD-test in JMP 14. A total of 12 samples were utilized in this study, therefore $n=3$ for each treatment main effect, $n=4$ for each generation main effect, and $n=1$ for each interaction.

Bisulfite Conversion & Sequencing

Currently, the best-known method to determine DNA methylation is through genome wide bisulfite-conversion and sequencing. During bisulfite conversion, all unmethylated cytosines are

converted into uracil while methylated cytosines are protected from the conversion process. Uracils are read as thymines by DNA polymerase and when bisulfite treated DNA is amplified using PCR the products of unmethylated cytosine appear as thymines. Methylated and unmethylated cytosines can therefore be distinguished after sequencing. A total of 4 pooled liver samples from each generation of female Japanese Quail from the four G0 grandparent treatments: HiMet-HiMet, HiMet-CON, CON-HiMet, CON-CON was analyzed using 100 ng of gDNA for bisulfite conversion. Liver samples for G0 were taken at 16 wk and for G1 and G2 at 3wks.

Bisulfite conversion was accomplished using EZ DNA Methylation-GoldTM Kit by Zymo following the manufacture's protocol. After conversion, 50 ng of single stranded DNA was used to create gDNA libraries using TruSeq DNA Methylation Kit following the manufacture's protocol (Illumina, San Diego, Ca). Libraries were sequenced using Illumina NextSeq with 75bp paired end runs with 800 million paired end reads total. A total of 24 total samples were sequenced with two runs of NextSeq-75 PE run with 800 PE reads total.

Results

Methylation Level of Egg Related Genes

Genes involved in egg yolk accumulation, which could potentially be responsible for changes in egg size were evaluated for the three generations. DNA methylation levels were significantly different between generation (G0), which was fed the dietary treatment, when compared with G1 and G2. The addition of methyl catalysts in G0 resulted in an increase in DNA methylation levels within the next generations (G1 and G2). These genes evaluated with significant increases in G1 and G2 DNA methylation levels were *vitellogenin 1, vitellogenin 2, vitellogenin 2-like, MTTP* and *APOB* (Figure 4.2). G0 dietary treatment resulted in increased *Vitellogenin 2* DNA methylation levels for the Con-HiMet group and decreased DNA methylation levels for the

Con-Con group. The addition of dietary methyl catalysts in G0 also resulted in increased DNA methylation levels at the *VLDLR* gene in the HiMet-HiMet treatment group and decreased DNA methylation for the Con-HiMet group for this genomic region (Table 4.2). Genes *APOB* and *VLDLR* had significant changes in DNA methylation levels for the interaction of generation and treatment group (Table 4.2). *APOB* DNA methylation level was detected to be the lowest for the G0 Con-Con group and highest for G0 Con-HiMet, G1 Con-Con, and G2 Con-Con. DNA methylation levels for *VLDLR* were decreased in G2 Con-HiMet and increased in G0 HiMet-HiMet, G0 Con-Con, and G2 HiMet-HiMet.

When comparing G2 methylation level to gene expression data, the gene expression values were extremely low for *vitellogenin 1, vitellogenin 2, vitellogenin 2-like* (likely due to the age sampled) and DNA methylation level at these CpG sites were over 80% methylated (Table 4.3). It is possible different methylation levels would be detected once the birds are in lay. Gene expression was highest for *MTTP* in the HiMet-HiMet group, which also displayed the lowest DNA methylation level (Table 4.3). *APOB* gene expression was highest for the HiMet-Con group, however this group also displayed a high DNA methylation level when compared to the other groups (Table 4.3).

Table 4.2. CpG DNA Methylation Level of Genes Involved in Yolk Accumulation.

Generation[1]	Treatment[2]	Locus					
		Vitellogenin 1	Vitellogenin 2	Vitellogenin 2-like	APOB	MTTP	VLDLR
		CpG DNAMethylation Level					
G0	-	0.7894 ^{B}	0.7456^{B}	0.7522^{B}	0.8070^{B}	0.7343 ^{B}	0.8677
G1	-	0.8216 ^{A}	0.8431^{A}	0.8388 ^{A}	0.8575^{A}	0.7788 ^{A}	0.8375
G2	-	0.8250 ^{A}	0.8406^{A}	0.8475 ^{A}	0.8564^{A}	0.7971 ^{A}	0.8229
P-value		**0.0021**	**0.0001**	**0.0001**	**0.0008**	**0.0009**	0.1286
SEM[4]				0.01	0.01	0.01	0.01
-	HiMet-HiMet	0.8264	0.8126^{AB}	0.8064	0.8251	0.7769	0.8882^{A}
-	HiMet-Con	0.7964	0.7864^{B}	0.8138	0.8437	0.7524	0.8308^{AB}
-	Con-HiMet	0.8057	0.8510^{A}	0.8114	0.8511	0.7794	0.7965^{B}
-	Con-Con	0.8196	0.7890^{B}	0.8198	0.8413	0.7716	0.8552^{AB}
	P-value	0.0671	**0.0001**	0.8804	0.4887	0.4634	**0.0042**
	SEM[4]	0.008	0.01	0.01	0.01	0.01	0.01
G0	HiMet-HiMet	0.8331	0.7430	0.7590	0.7920 ^{AB}	0.7263	0.9245^{A}
G0	HiMet-Con	0.7529	0.7243	0.7504	0.8096 ^{AB}	0.7205	0.8069^{AB}
G0	Con-HiMet	0.7947	0.8009	0.7197	0.8668 ^{A}	0.7544	0.8119^{AB}
G0	Con-Con	0.7769	0.7142	0.7796	0.7596 ^{B}	0.7360	0.9274^{A}
G1	HiMet-HiMet	0.8240	0.8312	0.8398	0.8408 ^{AB}	0.7781	0.8495^{AB}
G1	HiMet-Con	0.8160	0.8136	0.8516	0.8433 ^{AB}	0.7766	0.8423^{AB}
G1	Con-HiMet	0.8037	0.8846	0.8345	0.8611 ^{AB}	0.7839	0.8480^{AB}
G1	Con-Con	0.8429	0.8428	0.8293	0.8848 ^{A}	0.7768	0.8104^{AB}
G2	HiMet-HiMet	0.8220	0.8636	0.8303	0.8426 ^{AB}	0.8264	0.8908^{A}
G2	HiMet-Con	0.8203	0.8211	0.8393	0.8782 ^{A}	0.7603	0.8432^{AB}
G2	Con-HiMet	0.8188	0.8675	0.8801	0.8252 ^{AB}	0.8000	0.7298^{B}
G2	Con-Con	0.8388	0.8100	0.8503	0.8795 ^{A}	0.8019	0.8279^{AB}
	p-value	0.0641	0.5274	0.2246	**0.0076**	0.7873	**0.0182**
	N[3]	3308	2096	1788	1697	1712	775
	SEM[4]	0.01	0.01	0.02	0.02	0.02	0.03

[A,B] Connecting letters report using Tukey HSD

[1] Three total generations (G0, G1 and G2), where only G0 was provided the high methyl catalyst diet

[2] Treatment: Four total treatment groups of high methyl catalysts during the starter phase and/or layer phase (HiMet-HiMet, HiMet-Con, Con-HiMet, Con-Con)

[3] N is number of individual unique CpG sites measured for DNA methylation level

[4] Standard error of mean (SEM) for n=12 samples [4]All connecting letters report was calculated using Tukey HSD

Table 4.3. RNA Sequencing RPKM values and CpG DNA Methylation Levels for G2 Treatments of Egg Related Genes.

Name	HiMet-HiMet RPKM*	HiMet-Con RPKM*	Con-HiMet RPKM*	Con-Con RPKM*	HiMet-HiMet Methylation Level	HiMet-Con Methylation Level	Con-HiMet Methylation Level	Con-Con Methylation Level	CpG Sites *n*
Vitellogenin 1	0.0009	0.0037	0.0012	0.0030	0.8220	0.8203	0.8188	0.8388	3307
Vitellogenin 2	0.0330	0.0203	0.0137	0.0133	0.8636	0.8211	0.8675	0.8100	2096
Vitellogenin 2-like	0.0627	0.0736	0.0586	0.0467	0.8303	0.8393	0.8801	0.8503	1787
APOB	110.6580	119.5987	107.3672	113.7334	0.8426	0.8782	0.8252	0.8795	1696
MTTP	16.5187	10.7414	9.1354	8.4060	0.8264	0.7603	0.8000	0.8019	1711
VLDLR	0.0799	0.2025	0.1843	0.1643	0.8908	0.8432	0.7298	0.8279	774

**RPKM Reads Per Kilobase of transcript per Million mapped reads*

Discussion

Many nutritional factors are known to affect egg weights such as increasing egg yolk weight by the addition of fat or reducing egg weight by decreasing energy levels (Sell et al., 1987; Whitehead, 1981) or the limiting amino acids (Morris and Gous, 1988). Along with nutritional factors many genes are known to be involved in egg production and lipid metabolism. One such gene is vitellogenin II (VTGII), where lower VTGII concentrations have been associated with decreased in egg production under poor nutritional conditions (Sekimoto et al., 1990). If nutritional requirements are not being met to maintain energy demands, reproductive capabilities will suffer, thus nutritional conditions require a close communication with the genome. One way this can be accomplished is through DNA methylation. Many nutrients are thought to alter the expression of genes (Anderson et al., 2012; Choi and Friso, 2010; Murdoch et al., 2016; Waterland and Jirtle, 2003). The evaluation of methyl catalysts has provided evidence of influencing promoter DNA methylation and ultimately gene expression. The addition of dietary betaine has been shown to alleviate hepatic triglyceride accumulation in mice through decreasing promoter methylation of PPARα and MTTP (Wang et al., 2013). In chickens the addition of betaine increased egg production, hepatic and plasma VTGII concentrations, and protein concentrations of methylation enzymes BHMT, DNMT1 resulting in an increased SAM:SAH ratios (Omer et al., 2018). Microsomal triglyceride transfer protein (MTTP) is essential for the assembly and secretion of VLDL from the liver and the incorporation of triglycerides into apolipoprotein (APOB). MTTP DNA CpG methylation level was found to be different in groups in G0 when compared to G2, which is the generation fed the methyl diet and the first generation not associated with the treatments of the methyl catalyst diet (Table 4.2). Evaluation of egg production in G0 and G2 also demonstrated the most drastic shifts in egg weights where the Con-Con treatment began to have

increased egg sizes when compared to the HiMet-HiMet and HiMet-Con (Chapter 2). Treatment of folic acid in broilers has resulted in an increase in MTTP mRNA and believed to stimulate triglyceride export from the liver to other tissue for energy maintenance (Liu et al., 2019). MTTP mRNA levels has been found to be negatively correlated with promoter methylation levels (Liu et al., 2016). Our analysis from birds two generations removed from the methyl catalyst treatment revealed that the G2 HiMet-HiMet group had double the MTTP mRNA when compared with the Con-Con however no differences in DNA CpG methylation were observed between treatments in G2 (Table 4.3).

In Xenopus hepatocytes, despite expression of the vitellogenin genes, hypermethylation was detected at regions associated with vitellogenin genes indicating gene expression may occur despite methylation (Gerber-Huber et al., 1983). Similarly, in the liver of chickens with estrogen treatment and non-treatment, the vitellogenin gene was heavily methylated except for the 5' region of this gene where demethylation occurs under estrogen treatment (Wilks et al., 1982). Data have indicated demethylation of the CpG region within the promoter of VTGII resulted in hypermethylation when birds were not in lay and hypomethylated during lay in Japanese quail (Gupta et al., 2006). The CpG region is located in between the estrogen responsive element (ERE) and progesterone responsive element (PRE) binding sites (Geiser et al., 1983) and is thought to be essential for ERE and PRE binding for transcription (Gupta et al., 2006). This is evidence to indicate that certain genes can be actively transcribed even though DNA methylation is present and the sensitivity to CpG DNA methylation levels of specific regions within the promoter region could play a role in gene expression. Changes in patterns of DNA methylation are not always required for changes in gene expression. Likewise, our data indicates a heavily methylated region in evaluation of the vitellogenin genes (Table 4.2). It should be noted that low RPKM values for

the vitellogenin genes were detected which is likely due to the time at which the livers were sampled. Data were collected from the livers of birds at the 3 wk time point when they were transferred to layer cages and quail do not reach sexual maturity until 6 wks of age. It could be that the CpG methylation levels to VTGI, VTGII and VTGII-like would change once birds begin to lay. No differences were detected in CpG DNA methylation between G1 or G2 or any of the groups within; however, differences were detected when compared to G0 which received the dietary treatment (Table 4.2). This indicates the possibility that CpG DNA methylation level of the VTG genes may not be maintained transgenerationally. Although, a memory effect has been documented within the same generation for vitellogenesis from birds which were first given exogenous estrogens and then later the second treatment resulted in a more rapid and increased production of vitellogenin (Bergink, 1974). This memory effect is associated with changes in expression of the estrogen receptor, chromatin remodeling and changes in the promoter regions of VTGI, VTGII and VLDLII genes (Edinger et al., 1997). Changes were not observed in transcript levels of apolipoprotein B (APOB) because it is consistently expressed in males and females (Edinger et al., 1997). Similarly, APOB had the greatest RPKM values of the egg related genes measured for birds which were not in production (Table 4.3). Once the lay period begins, APOB has been measured at 6 times normal levels (Kirchgessner et al., 1987). Avian APOB is a part of VLDL that transport cholesterol and triglycerides to the ovary for yolk synthesis (Kirchgessner et al., 1987).

Conclusions

Much is to be learned about the epigenetic effects on lipid metabolism, egg production, and the mechanism behind resource allocation in birds. We sought to determine if CpG DNA methylation levels of vitellogenin genes were responsible for the changes in egg weights observed,

however CpG DNA methylation levels displayed no changes between any of the genes for G1 and G2 except for the VLDL receptor. CpG DNA methylation levels of the genes evaluated did not clearly explain the changes observed in egg size between generations and treatment groups. Alterations in CpG DNA methylation level of the evaluated genes may not be transgenerational or an entirely different process could be occurring. However, evidence does suggest epigenetic changes in CpG DNA methylation level within G0 receiving the dietary treatment. Gene expression for G2 prior to the lay period displays low levels of all egg-related transcripts evaluated excluding APOB which is continuously expressed. Given that these birds had not reached reproductive maturity, and had not yet begun to lay, could explain the lack of vitellogenin and MTTP gene expression. Upon reaching the laying period, gene expression for the genes evaluated would be predicted to increase and changes in the methylation levels could potentially change as well.

Chapter 5

Evaluation via Supervised Machine Learning of the Broiler Pectoralis Major and Liver Transcriptome in Association with the Muscle Myopathy Wooden Breast

Abstract

The muscle myopathy wooden breast (WB) has recently appeared in broiler production and has a negative impact on meat quality. WB is described as hard/firm consistency found within the pectoralis major (PM). In the present study, we use machine learning from our PM and liver transcriptome dataset to capture the complex relationships that are not typically revealed by traditional statistical methods. Gene expression data was evaluated between the PM and liver of birds with WB and those that were normal. Two separate machine learning algorithms were performed to analyze the data set including the sequential minimal optimization (SMO) of support vector machines (SVMs) and Multilayer Perceptron (MLP) Artificial Neural Network (ANN). Machine learning algorithms were compared to identify genes within a gene expression data set of approximately 16,000 genes for both liver and PM, which can be correctly classified from birds with or without WB. The performance of both machine learning algorithms SMO and MLP was determined using percent correct classification during the cross-validations. By evaluating the WB transcriptome datasets by 5x cross-validation using ANNs, the expression of nine genes ranked based on Shannon Entropy (Information Gain) from PM were able to correctly classify if the individual bird was normal or exhibited WB 100% of the time. These top nine genes were all protein coding and potential biomarkers. When PM gene expression data were evaluated between normal birds and those with WB using SVMs they were correctly classified 95% of the time using 450 of the top genes sorted ranked based on Shannon Entropy (Information Gain) as a preprocessing step. When evaluating the 450 attributes that were 95% correctly classified using SVMs through Ingenuity Pathway Analysis (IPA) there was an overlap in top genes identified through MLP. This analysis allowed the identification of critical transcriptional responses for the first time in both liver and muscle during the onset of WB. The information provided has revealed

many molecules and pathways making up a complex molecular mechanism involved with the progression of wooden breast and suggests that the etiology of the myopathy is not limited to activity in the muscle alone but is an altered systemic pathology.

Introduction

The occurrence of wooden breast (WB) in commercial poultry production is rising, leaving producers with an inferior product and, ultimately, unsatisfied consumers (Mudalal et al., 2014; Petracci et al., 2015). Much of the incidence is thought to be attributed to artificial selection that has led to the development of broilers with greater muscle yield, better feed conversion rates, and faster growth. Frequent detection of muscle myopathies has been thought to be associated with increased growth rates and breast muscle yields (Abasht et al., 2019; Kuttappan et al., 2016; Sihvo et al., 2014; Trocino et al., 2015). WB is described as a hard or firm consistency deep within the pectoralis major (PM) muscle (Sihvo et al., 2014). Alterations of the meat composition have been observed including increased moisture, collagen, sodium, calcium and fat content (Zambonelli et al., 2016a). When compared to PM without the myopathy, the PM meat quality with WB is lower due to greater cooking losses and greater shear force when compared to PM without the myopathy (Trocino et al., 2015; Zambonelli et al., 2016a). Consequently, the meat texture of the breast is tougher and less desirable to the consumer. Extensive histological evaluation of WB has been characterized by necrosis, chronic fibrosis, infiltration of fat and connective tissue, and the presence of inflammatory cells and macrophages (Sihvo et al., 2014; Trocino et al., 2015; Kuttappan et al., 2016; Papah et al., 2017).

The severity of WB is often categorized on a scale ranging from 0 to 3 (0, normal; 1, mild; 2, moderate; 3, severe) (Trocino et al., 2015). WB lesions can be detected through manual palpation of the PM as early as 3 wks of age (Mutryn et al., 2015). Research covering muscle myopathies in broilers reveals that myopathies have increased in recent years and have been correlated with the selection of larger breast muscle (Petracci et al., 2015). The cause of WB is currently unknown, but it is more prevalent in older, heavier male broilers than young birds

(Brothers et al., 2019; Kuttappan et al., 2017). Larger breast muscle has been associated with hypertrophied muscle fibers which is thought to impact blood supply and number of satellite cells that are needed in muscle regeneration (Daughtry et al., 2017; Malila et al., 2019).

The increase in prevalence and severity of WB has the potential to result in excessive economic loses. It has been projected that these losses could exceed more than $200 million USD/yr (Kuttappan et al., 2016). Often, the PM muscle of birds with WB can appear pale, bulging, and covered in a clear viscous fluid (Sihvo et al., 2014). Considering consumers frequently purchase chicken breasts based on visual appearance of the meat, this unpleasant appearance will undoubtedly have a negative impact on sales (Petracci et al., 2015). Even if WB is undetectable by the outward appearance prior to purchase, the hardness of the inner muscle will become evident once the meat is handled or consumed. Ultimately, this is bad for the consumer, the company, and poultry production as a whole by breaking consumer trust and potentially initiating the desire for an alternative product.

Currently, researchers are investigating the nutritional, physiological, and genetic factors that surround this myopathy through the use of many common techniques that span from the inclusion of feed additives to molecular approaches using gene expression analysis and histology (Velleman and Clark, 2015; Kuttappan et al., 2016; Papah et al., 2018; Petracci et al., 2019). In the present study, we use machine learning analysis of PM and liver transcriptome datasets to capture the complex relationships that are not typically revealed by traditional statistical methods. This was achieved through the use of algorithms to identify genes within an extensive RNA sequencing dataset whose expression can be used to correctly distinguish normal tissue apart from severe/moderate WB. Previous gene expression datasets have characterized many molecular relationships present in birds with WB (Mutryn et al., 2015; Papah et al., 2018; Brothers et al.,

2019). This study uses a different mechanism to evaluate gene expression data in the hopes for a more concise evaluation of the WB myopathy.

Materials and Methods

Facilities and Rearing

This experiment was conducted at the North Carolina State University Chicken Education Unit between the months of March and April 2019. All procedures used in this study were reviewed and approved by the Institutional Animal Care and Use Committee. Eggs were collected from a resident 25-wk-old broiler breeder flock of known, similar genetic background and stored for no more than 7 d at 15ºC. Incubation was performed based on the methods described by Livingston (Livingston et al., 2018a). Day of hatch chicks were sex-sorted and a total of 128 male chicks were individually neck tagged and placed into 8 replicate pens (1.2 m x 4.0 m; 4.8 m^2) with 16 chicks per pen (blocked by location within the house). Each pen was supplied with one bell water drinker, two tube feeders, and bedded with fresh pine shavings (15 cm deep). Broilers were provided *ad libitum* access to a common commercial starter diet (1-14 d) and a common grower diet (14-45 d) manufactured at the NC State University Feed Mill (Table 5.1). Eight broilers from each pen, for a total of 64, were selected (based on experiment-wise mean BW) for processing at 45 d. Birds were evaluated for WB and samples of PM and liver tissue were collected for analysis of gene expression and stored at -80°C.

Table 5.1. Composition of basal starter and grower diets[1]

Ingredients	Starter	Grower
Corn	55.22	57.45
Soybean meal (48% CP)	36.9	31.87
Poultry fat	2.36	5
Dicalcium phosphate (18.5% P)	2.02	2.39
Glycine	1.25	1.25
Limestone	0.77	0.6
Salt	0.5	0.5
DL-Methionine	0.28	0.24
Choline chloride (60%)	0.2	0.2
L-Threonine	0.1	0.09
L-Lysine	0.05	0.07
Selenium premix[2]	0.05	0.05
Vitamin premix[3]	0.05	0.05
Mineral premix[4]	0.2	0.2
Coccidiostat[5]	0.05	0.05
Total	100	100
Calculated nutrient content		
Crude protein	22.5	20.2
Calcium	0.9	0.9
Available phosphorus	0.45	0.5
Potassium	0.89	0.82
Total lysine	1.27	1.14
Total methionine	0.62	0.55
Total threonine	0.85	0.76
Total methionine + cysteine	0.97	0.87
Sodium	0.21	0.2
Metabolizable energy (kcal/g)	2.85	3.03

[1]Starter diet was fed to approximately 14 d of age, 1820 g per bird.
[2]Selenium premix provided 0.2 mg Se (as Na_2SeO_3) per kg of diet.
[3]Vitamin premix supplied the following per kg of diet: 13,200 IU vitamin A, 4,000 IU vitamin D_3, 33 IU vitamin E, 0.02 mg vitamin B_{12}, 0.13 mg biotin, 2 mg menadione (K_3), 2 mg thiamine, 6.6 mg riboflavin, 11 mg d-pantothenic acid, 4 mg vitamin B_6, 55 mg niacin, and 1.1 mg folic acid.
[4]Mineral premix supplied the following per kg of diet: manganese, 120 mg; zinc, 120 mg; iron, 80 mg; copper, 10 mg; iodine, 2.5 mg; and cobalt, 1 mg.
[5]Coccidiostat supplied monensin sodium at 90 mg/kg of food.

Processing

At 45 d, selected broilers were collected and transported to the North Carolina State University Chicken Education Unit's broiler processing facility followed by shackling and stunning in a salt saturated saline head stun cabinet. Birds were head stunned with a 110v/60hz CF2000 poultry stun knife set to 150 mA for 10 sec. Broilers were exsanguinated for 120 s by opening of the jugular vein and carotid artery with a single knife cut by a trained technician followed by scalding in hot water (60ºC) for 120 s. This was followed by feather picking for 30 s (Meyn Food Processing Technology B.V., Westeinde Amsterdam, The Netherlands). Head and feet were removed, vent opened (VC Poultry Vent Cutter, Jarvis Product Corp., Middleton, CT,

USA), and viscera and giblets removed manually. Liver and PM tissue were removed and snap frozen on liquid nitrogen for RNA sequencing analysis. Hot carcass weights (HCW) were collected prior to carcasses being air chilled at 3.0ºC for approximately 24 h. At 24 h postmortem examination of the PM muscle was evaluated after breast muscle was removed from carcass and bones by a trained and experienced technician for WB using a one to four-point ordinal scale of measurement in accordance with the methods described previously by Livingston with a score of one being normal and 4 being most severe (Livingston et al., 2018b).

RNA sequencing analysis

PM and liver tissue samples from 45 d broilers with varying severities of WB scores were obtained and preserved in RNALater. RNA extracted from the PM of 15 birds with moderate to severe WB was compared to RNA extracted from 5 normal PM. For liver RNA evaluation, 10 samples from birds with moderate to severe WB and 5 livers from birds with normal PM were used. RNA was extracted from the tissues using Qiagen RNeasy Mini protocol (Qiagen, Valencia, CA, USA) following the manufacturer's instructions. The RNA quality was assessed by Nanodrop 2000 spectrophotometer (Thermo, USA). Two micrograms of RNA from each sample were taken to the North Carolina State University Genomics Sciences Laboratory for library preparation and sequencing on the Illumina HiSeq 2500 sequencer. RNA sequencing was analyzed using CLC Genomics Workbench (Qiagen, Valencia, CA, USA; licensed to NCSU) version 11 following the software manual (http://resources.qiagenbioinformatics.com/manuals/clcgenomicsworkbench/current/User_Manual.pdf). RNA sequencing reads and annotations were mapped to the chicken genome (*galgal5*) from NCBI. Raw reads were processed by the default settings of reads' quality control and adapter trimming. The false discovery rate p-value (FDRp) was calculated to correct for multiple testings' and an FDR adjusted $p \leq 0.05$ was considered statistically significant. Fold

change and Log_2 fold change differences in gene expression between WB scores moderate to severe and normal were also calculated.

Machine Learning Analysis

Gene expression data were analyzed from the PM and liver using the Waikato Environment for Knowledge Analysis (WEKA) version 3.8.3. Two different pattern recognition machine learning algorithms were performed to analyze the data set: sequential minimal optimization support vector machines (SVMs) and artificial neural network multilayer perceptron (MLP) (Cortes and Vapnik, 1995; Eibe et al., 2016). The machine learning algorithms were compared to identify gene expression patterns within the data set of 15,569 genes, which could be used to correctly classify birds as either exhibiting moderate to severe WB or normal (dichotomous class assignment). Briefly, the 15,569 genes from the gene expression dataset were ranked based on Shannon Entropy (Information Gain) in dichotomous classification assignment by SVMs (Eibe et al., 2016; McCarthy J, Minsky M, Rochester N, 1955). Information gain ranking was then used to identify those gene expression patterns most relevant to assignment of each bird as having WB or normal by either MLP or SVMs. Reduction of data dimensionality for each machine learning algorithm was then performed by sequential exclusion of those gene expression patterns least relevant to the class assignment (50-2,000 per sequence). This step eliminates overfitting of the machine learning classifiers. To identify the minimum number of gene expression patterns required for classification, 1-50 genes were sequentially excluded from the dataset until only the top 2-7 remained. This step identifies underfitting of the machine learning classifiers and the point of optimal classification for the MLP and SVMs was determined to be the intersection between the underfitting and overfitting curve plots.

Class assignment of all machine learning algorithms was evaluated *vis-à-vis* by two cross-validation strategies (classification as either WB or normal). The first being a percentage split, where 66% of the total data were randomly used for training and the remaining 34% of the data were used in testing. The second cross-validation was a stratified hold-out (*n*-fold) method with 5-folds, where 4-folds of the randomized gene expression data were used for the training and 1-fold was used for testing. This was repeated 5 times, such that all normal replicate samples were used at least once in testing and the average model performance was recorded.

The performance of the two machine learning algorithms SVMs and MLP was determined using percent correct classification during the cross-validations, which indicated the likelihood that each individual biological replicate could accurately be assigned into the classes of WB or normal based on the gene expression data provided. Kappa statistic and ROC score also were recorded. Any kappa statistic greater than 0 indicated that the machine learning classifier is performing better than random chance along with a ROC score of greater than 0.500. The random probability of chance for dichotomous assignment was assumed to be 50% based on the Law of Probability.

A negative control of machine learning was created through 10 separate randomizations of the individual birds within the dataset. The SVMs and MLP were unable to predict WB or normal, indicating the machine learning herein is true.

Pathway Analysis

Ingenuity Pathway Analysis (IPA; Qiagen, Valencia, CA; http://www.ingenuity.com) software was used for canonical pathway analysis, upstream regulatory analysis, and gene network discovery. SVM analysis of the top 450 performing differentially expressed genes from the PM of birds with moderate/severe to normal WB were used in IPA and the top 150 were used

from the liver dataset. IPA calculation of z-scores using the gene expression fold change values measures the state of activation or inhibition of the molecules involved in the molecular networks. The analysis of biological mechanisms occurring in the differentially expressed genes of the chicken in IPA are based on mammalian systems for human, rat, and mouse.

Results

Multilayer Perceptron

By using the novel approach of evaluating the WB PM transcriptome dataset by 5-fold cross-validation using MLP, the expression of nine genes (*NUP43, KPNA7, DEAF1, NUD19, CCDC85A, SLC25A30, ENSGAL00000015075, PACSIN3, and RPL19*) were able to correctly classify if the PM tissue from an individual bird was normal or exhibited WB in 100% of the individual genomes when using the top nine genes ranked based on Shannon Entropy (Information Gain) (Figure 5.1). MLP is an artificial neural network, which can distinguish data that are not linearly separable, but instead is a feed forward mechanism that maps a dataset into suitable outputs. The top nine genes in the PM were further analyzed for their individual biological roles (Table 5.2). When the liver transcriptome dataset was evaluated by 66% split using MLP, the expression of 75 genes were capable of correctly classifying the PM tissue of an individual bird as exhibiting WB or normal in 100% of the individual genomes (Figure 5.2). The kappa statistic and ROC score were optimal at 1 for both PM and liver. The individual expression of transcripts *CARD19* and *ITCH* predicted WB or normal using MLP 5-fold cross validation 93.333% of the time whereas *BUD13* and *ENSGALG00000039590* individually predicted WB or normal 100% of the time using 66% split (Table 5.3).

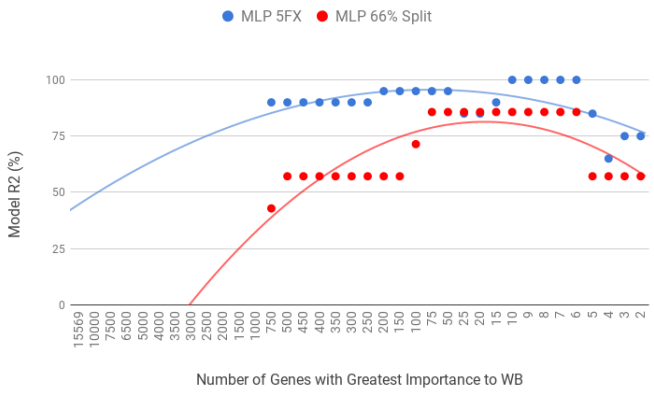

Figure 5.1. WB PM transcriptome dataset by 5x cross validation and 66% percent split using Multilayer Perceptron (MLP).

Table 5.2. Top nine regulators of differential expressed genes in the PM of birds with normal PM compared to severe/moderate WB by 5-fold cross-validation with 100% correct classification and percent split with 85.71% correct classification using MLP.

Gene	*Gene Type*	*Short Description*	*Log_2 fold change*	*P-value*
NUP43	Protein Coding	Forms Nuclear Pore Complex (NPC)	0.2005	0.4900
KPNA7	Protein Coding	Forms Nuclear Pore Complex (NPC)	-3.4539	2.62E-06
DEAF1	Protein Coding	Zinc Finger Domain Transcription Regulator; Inhibits Cell Proliferation	0.3406	0.2972
NUDT19	Protein Coding	Enzyme involved in Peroxisomal Lipid Metabolism	0.9608	1.28E-04
CCDC85A	Protein Coding	Unknown	2.0774	0.0009
SLC25A30	Protein Coding	Renal Mitochondrial Carrier	0.1223	0.9574
ENSGALG00000015075	Protein Coding	Beta-1,3-glucuronyltransferase 1	-0.3370	0.5767
PACSIN3	Protein Coding	Links Actin Cytoskeleton with Vesicle Formation	-0.9920	1.58E-04
RPL19	Protein Coding	Ribosomal Protein Component of the 60S Subunit	-0.8323	0.0066

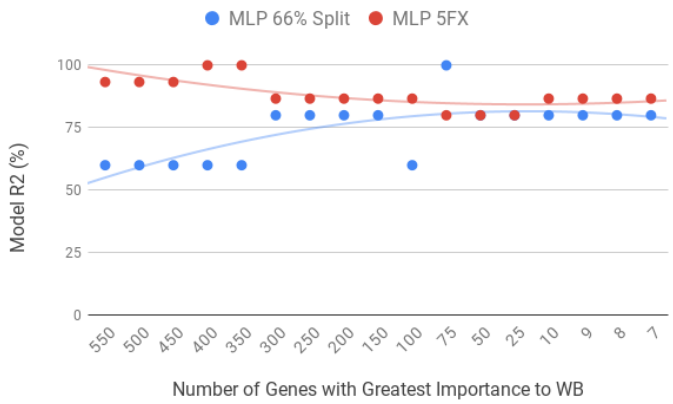

Figure 5.2. WB Liver transcriptome dataset by 5x cross validation and 66% percent split using Multilayer Perceptron (MLP).

Table 5.3. Top Regulators of differential expressed genes in the liver of birds with normal PM compared to severe/moderate WB tissues using MLP.

Gene & Validation Method	***Gene Type***	***Short Description***	***Log_2 fold change***	***P-value***
CARD19 *Stratified hold-out (93.333%)*	Protein Coding	Caspase Recruitment Domain Family Member 19; Negative Regulation of IKKß /NFKB Cascade; Regulation of Apoptosis	-2.5547	0.2831
ITCH *Stratified hold-out (93.333%)*	Protein Coding	Related pathways are signaling by Sonic Hedgehog and TNF signaling pathway. Plays a role in erythroid and lymphoid cell differentiation and regulation of immune responses.	-1.2210	0.7238
BUD13 *Percent split (100%)*	Protein Coding	Component of the active spliceosome involved in pre-mRNA splicing	1.2022	0.6601
ENSGALG00000039590 *Stratified hold-out (93.333%) & Percent Split (100%)*	Protein Coding	Unknown	-2.0511	0.1209

Sequential Minimal Optimization

When the PM gene expression data set was evaluated between birds with WB and those that were normal using WEKA SMO function of SVMs by a 5-fold cross-validation method they were correctly classified 95% of the time using 450 of the top genes ranked based on Shannon Entropy (Information Gain) as a preprocessing step (Figure 5.3). The kappa statistic and ROC score were optimal at 0.8571 and 0.9000, consecutively. The liver gene expression data set was evaluated using the SMO function of SVMs by both a 5-fold cross-validation method and the 66% split method was capable of predicting WB or normal 100% of the time with 100 to 200 of the top genes ranked based on Shannon Entropy (Information Gain) (Figure 5.4). Optimal kappa statistic and ROC scores of 1 were achieved for both methods. In both machine learning algorithms, the stratified hold-out method appeared to accurately estimate the machine learning classifier correctly more often than the percentage split method.

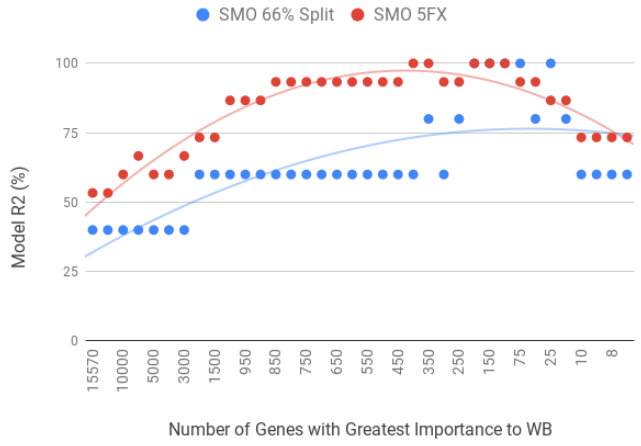

Figure 5.3. WB PM transcriptome dataset by 5x cross validation and 66% percent split using Sequential Minimal Optimization (SMO).

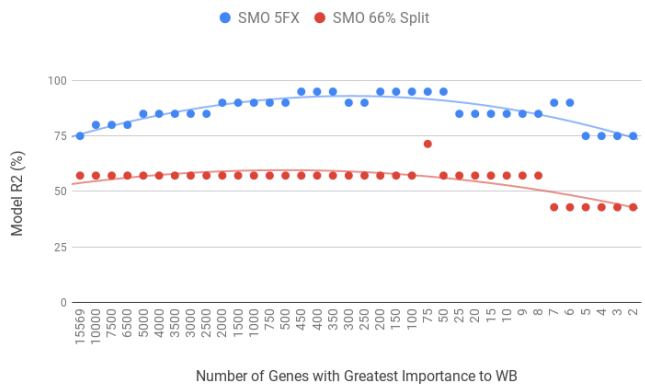

Figure 5.4. WB Liver transcriptome dataset by 5x cross validation and 66% percent split using Sequential Minimal Optimization (SMO).

Ingenuity Pathway Analysis

Lastly, Ingenuity Pathway Analysis (IPA) was used to evaluate the top 450 genes ranked based on Shannon Entropy (Information Gain) from the PM transcriptome using SMO and 150 of the top genes ranked based on Shannon Entropy (Information Gain) for the liver transcriptome using SMO. Interestingly, many of the top analysis ready molecules detected in IPA were the same molecules detected using the completely separate machine learning approach of MLP. The repeatability in identification of these genes leads to greater confidence in the role they are having in the WB myopathy. These included *BUD13* from the liver transcriptome dataset and *CCDC85A* and *KPNA7* from the PM dataset (Tables 5.2 & 5.3).

The top associated network function in IPA for the PM transcriptome dataset was for RNA Damage and Repair, Protein Synthesis. This pathway involves *RPL19* which is a component of the 60S ribosomal subunit detected through MLP of WEKA (Figure 5.5). When evaluating the liver transcriptome dataset three top network associations identified were Skeletal and Muscular System Development and Function, Developmental Disorder, Hereditary Disorder (Figure 5.6) Connective Tissue Disorders, Hematological Disease, Hereditary Disorder (Figure 5.7) and Cell Cycle, Embryonic Development, Cellular Movement (Figure 5.8). Figure 5.6

depicts *CARD19* which was also detected using MLP of WEKA. This network is an association of the relationship this group of molecules has in association with the skeletal and muscular system development and function. Figure 5.8 represents molecules in the cell cycle such as *TGF-β*, *MAP2K1/2*, and calcineurin proteins which are important in skeletal muscle myoblast regulation and differentiation.

When comparing the performance of the top ranked genes between MLP and SVM, many genes were identified as top performing in both ML models. In the PM data set, evaluation of the top nine molecules with increased expression, *KPNA7* was observed in both MLP (100% classification) and the top analysis ready molecules in IPA from the best performing data set using SVM (Table 5.2). *RPL19* was observed as a top regulator in MLP with the ability to correctly classify WB or normal 100% of the time and was an affected molecule in the IPA analysis of the top associated network (Figure 5.5). In the same data set *CCDC85A* was observed as a molecule with decreased expression in both ML models and able to correctly classify WB 100% of the time using MLP (Table 5.2).

Assessing the liver dataset there were many overlaps using the two ML models. *BUD13* was a top regulator in MLP and a top analysis ready molecule with increased expression in IPA (Table 5.3). The associated network related to skeletal and muscular system development and function from IPA had *CARD19* as a downregulated molecule in IPA and a top regulator in MLP that was able to predict WB or normal 93.33% of the time (Figure 5.6). Lastly, the IPA network associated with cell cycle, embryonic development, and cellular movement included the down regulation of the *ITCH* molecule which was also detected in MLP as being able to correctly classify 93.33% of the time (Figure 5.8).

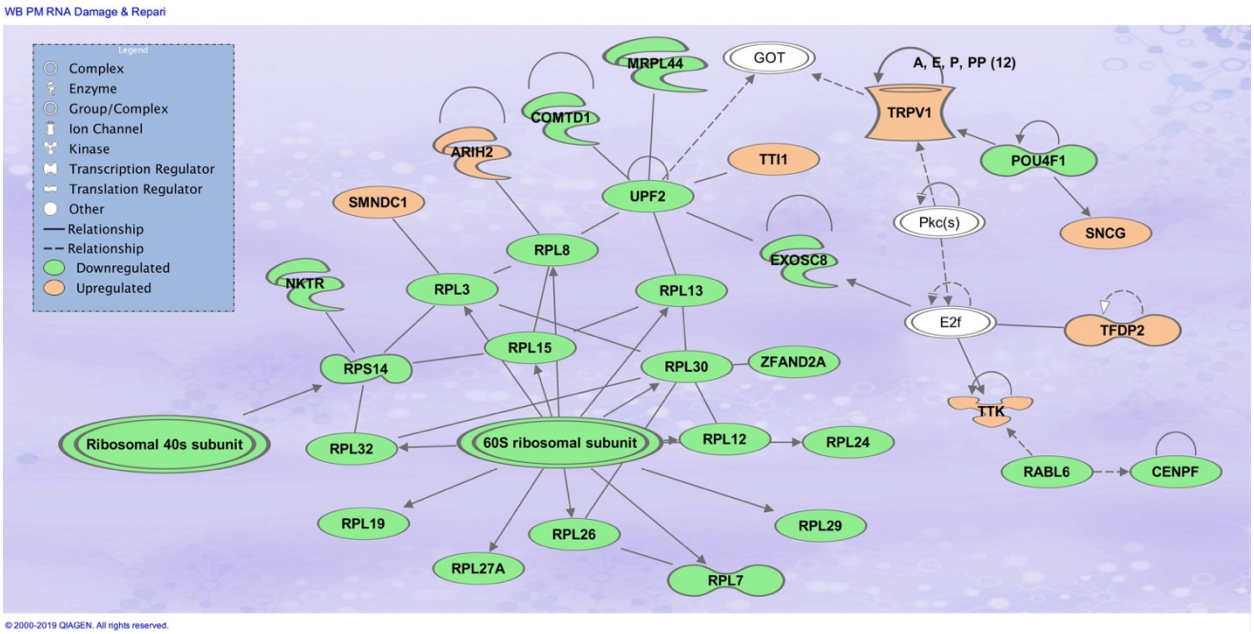

Figure 5.5. Top associated network function in IPA for the PM transcriptome dataset of birds with normal PM compared to severe/moderate WB: RNA Damage and Repair and Protein Synthesis.

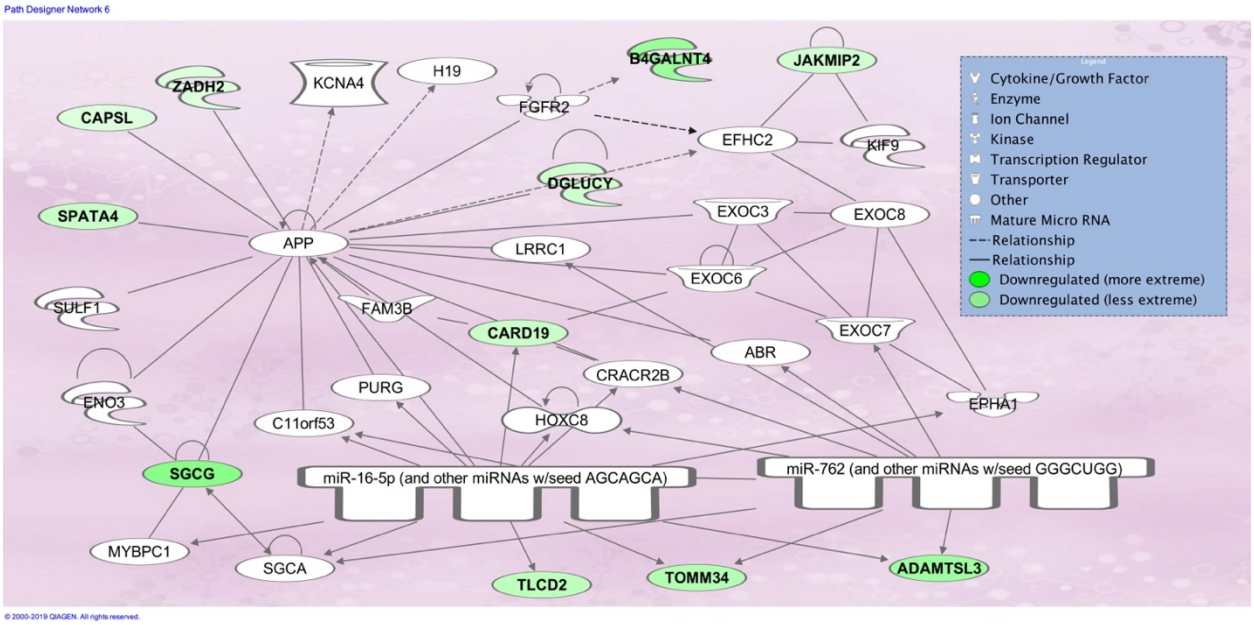

Figure 5.6. Associated network function in IPA for the liver transcriptome dataset of birds with normal PM compared to severe/moderate WB: Skeletal and Muscular System Development and Function, Developmental Disorder, Hereditary Disorder.

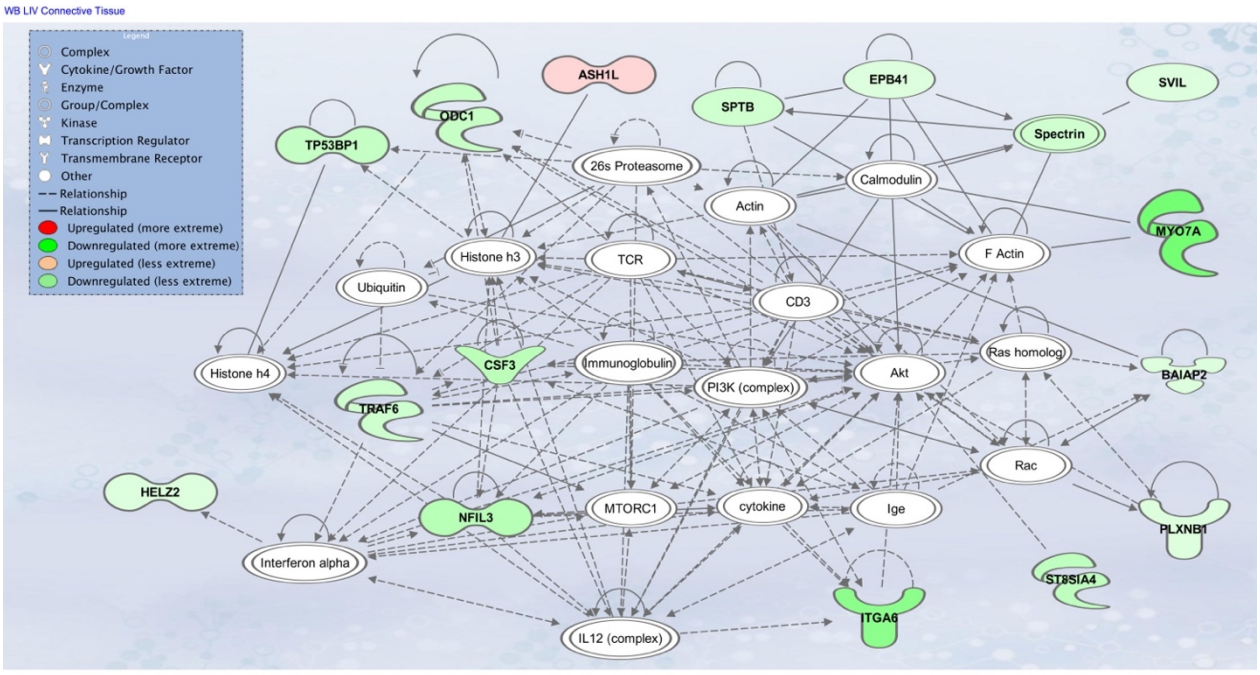

Figure 5.7. Associated network function in IPA for the liver transcriptome dataset of birds with normal PM compared to severe/moderate WB: Connective Tissue Disorders, Hematological Disease, Hereditary Disorder.

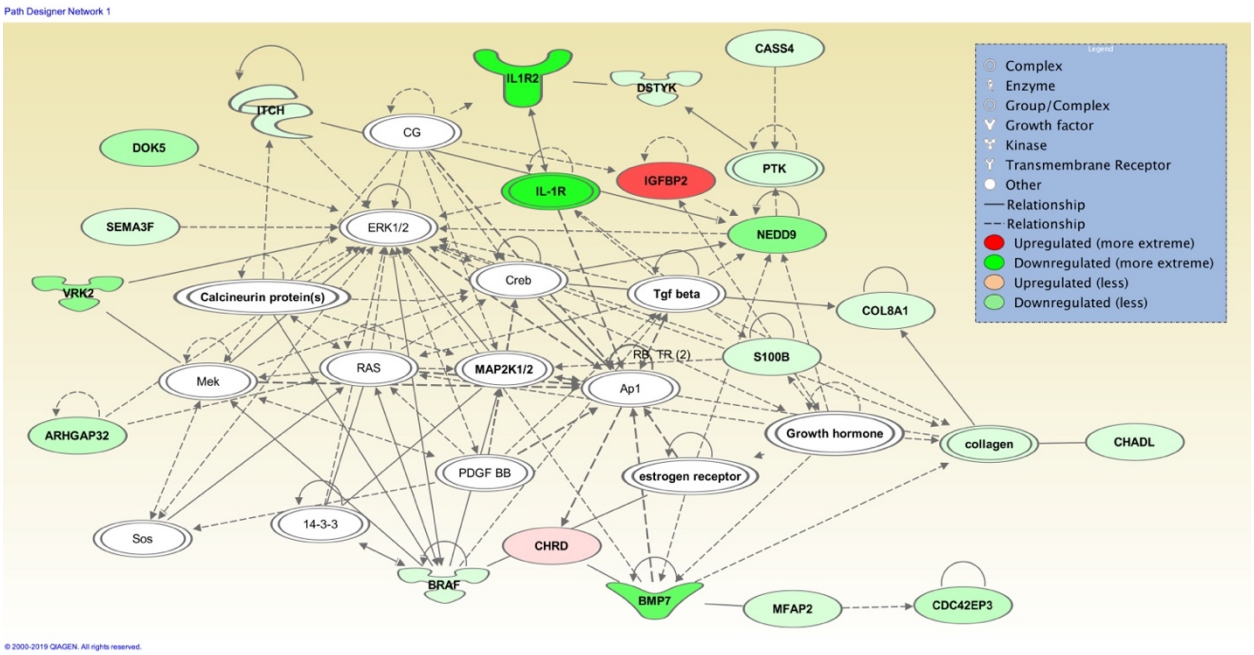

Figure 5.8. Associated network function in IPA for the liver transcriptome dataset of birds with normal PM compared to severe/moderate WB: Cell Cycle, Embryonic Development, Cellular Movement.

Discussion

Prior systematic studies of the abnormal muscle phenotype referred to as wooden breast using gene expression measurements has identified multiple processes that may contribute to the development of the myopathy. Processes including muscle fiber regeneration, inflammatory response, myodegeneration, hypoxia, fibrosis, lipidosis, and altered energy metabolism are likely involved in the manifestation of wooden breast (Malila et al., 2019; Mutryn et al., 2015; Papah et al., 2018; Petracci et al., 2019; Zambonelli et al., 2016b). This study attempts to connect organismal level physiology and metabolism with the activity in the breast muscle by including the evaluation of liver gene expression by a novel analysis method.

Machine Learning Approach

Traditional statistics is not well designed to handle datasets which have more variables than observations therefore this is an alternative method to analyze and make interpretations of datasets. Using the traditional statistical approach on this dataset resulted in almost 1000 genes being identified as differentially expressed (FDR corrected $p<0.05$) across the etiology of WB in the PM and only one gene in the liver. This large number of targets (or too few) when subjected to pathway analysis in IPA results in more pathways (or none) than can be interpreted, therefore an alternative approach was warranted. The use of machine learning on the transcriptome datasets allows for the recognition of consistent patterns or systematic relationships within the datasets and therefore can be used to make predictions. Through this process, the machine learns by building a model from example inputs and then makes predictions on new data by the learned pattern recognition. This is the first report using machine learning to identify gene expression patterns associated with WB in muscle as well as peripheral tissues that may be influencing the myopathy development.

PM Fibrosis

Evaluating the top nine genes ranked based on Shannon Entropy (Information Gain), all the genes are protein coding genes (Table 5.2). The top two genes *NUP43* and *KPNA7* are two essential Nuclear Pore Complexes (NPC). *KPNA7* was also observed as one of the top nine upregulated genes with a fold-change for birds with WB (Mutryn et al., 2015). NPC's are macromolecular proteins found within the nuclear envelope in eukaryotic cells. These complexes are surrounded by decondensed chromatin and are responsible for the exchange of large molecules such as proteins and RNA between the nucleus and cytoplasm (Kelley et al., 2010). Prior to cell division there is an increase in expression of NPC found in the nuclear envelope (NE). Once mitosis begins the NE is broken down and NPCs form subcomplexes, which are essential for later reassembly of the NPC. This process of reconstruction is unknown; however, it has been shown when the KPNA7 NPC is depleted, mitotic defects and deformation of the nucleus occur (Vuorinen et al., 2018). In the mouse, *KPNA7* is mostly expressed in oocytes and zygotes and is responsible for epigenetic reprogramming which occurs during fertilization and zygotic gene activation (Hu et al., 2010). Recently, NPCs have been observed in regulation of gene expression and have been associated with both gene silencing and activation (Casolari et al., 2004; Galy et al., 2000). The expression of nucleoporins, which make up the NPCs, vary depending on cell types and changes in the structure of NPCs are used in the regulation of cellular differentiation (D'Angelo, 2018). In mammals, the tissue specific NPC, Nup210 has an effect on the regulation muscle development and maintenance. Nup210 regulates myofiber maturation, growth and even survival through the use of a dependent transcription factor Mef2C in the regulation of structural and maturation related muscle genes (D'Angelo et al., 2012; Raices et al., 2017). In the absences of Nup210 the initial formation of muscle fibers occurs during

development, however in older animals abnormal muscle structure develops and muscle degeneration can even occur (Raices et al., 2017).

PM Regeneration

Another top gene, *DEAF1*, is a part of the SAND domain in the molecule Ski. The activity of Ski was originally identified in the chicken as a transduced retroviral oncogene; however, research has indicated homologs which are not associated with endogenous viral loci (Li et al., 1986). The c-Ski residue is primarily found in the nucleus and is highly conserved in many species. The SAND domain is involved in protein-protein interactions and is responsible for the interactions of SKI with Smad4, FHL2, and MeCP2 (Engle, K. M.; Mei, T-S.; Wasa, M.; Yu, 2008). Ski can act as an activator or repressor to gene transcription depending on the transcription factor it interacts with. C-Ski has been shown to bind with Smad4 and block activation of transforming growth factor (TGF-β). TGF-β leads to an increase in β-catenin within the cytoplasm and β-catenin is an activator of canonical Wnt signaling (Staal, 2016). TGF-β, TNF-α, and IGF-2 are growth factors that regulate myoblast differentiation rather than activation (Carter et al., 2009; Li et al., 2005; Rosenthal et al., 1991). The key role of TGF-β in muscle repair is to regulate the balance between fibrosis and regeneration. In Japanese quail, myogenic differentiation is induced through the activation of myogenic regulatory factors (MRFs), MyoD, and myogenin as well as inhibiting HDAC activity (Colmenares and Stavnezer, 1989; Kobayashi et al., 2007). Satellite cells are activated by expression of early myogenic regulatory factors, MyoD and Myf5. Later, myogenin and MRF4 are expressed. The Ski response element to myogenin is located upstream of the promoter region.

The gene RPL19 was discovered as one of the top nine regulators in the MLP model able to 100% correctly classify PM samples as WB or normal sample. This protein coding gene encodes

a ribosomal protein that makes up the 60S ribosomal subunit (Davies and Fried, 1995). Like many of the genes observed, RPL19 also plays a role in genetic regulation. In mammals, this protein contains a CpG island at the 5' transcriptional start site, which would indicate an area for modifications to the expression of this gene (Davies and Fried, 1995). RPL19 was also involved in the top molecular network associated with RNA damage and repair protein synthesis when evaluating the 450 attributes that were 95% correctly classified using SMO through Ingenuity Pathway Analysis (IPA) (Figure 5.5). Transcripts related to spliceosomes were also detected in IPA analysis in (Figure 5.6), involving the previously mentioned gene *BUD13* as a component of the spliceosome. Similar to this finding previous research evaluating gene expression data in the PM has indicated differential expression of small nucleolar RNAs including snoRNAs and miRNAs, which are often involved in ribosomal and protein synthesis (Zambonelli et al., 2016a). *CCDC85A*, another top regulator identified with MLP, was able to correctly classify as a single attribute WB and normal PM birds 95% of the time using stratified cross-validation. *CCDC85A* was also a top analysis molecule when evaluating the 450 attributes that were 95% correctly classified using SMO through Ingenuity Pathway Analysis (IPA). *CCDC85A* is a protein coding gene for coiled coil domain containing 85a (Iwai et al., 2008). In humans, *CCDC85A* is regulated by p53 and results in the degradation of β-catenin. This protein suppresses β-catenin activity through interaction with T-cell factors to result in Wnt signaling (Iwai et al., 2008). β-catenin is an activator of canonical Wnt signaling (Staal, 2016). Wnt signaling is responsible for the activation of satellite cells in adult skeletal muscle and perturbations of this pathway can result in muscle fibrosis (Cisternas et al., 2014). If Wnt signaling occurs too often, the satellite cells become exhausted and eventually lose the ability to renew (Ryall et al., 2008). This has been characterized by increased extracellular matrix molecules such as fibronectin, collagen, and macrophages

leading to the inability of muscles to regenerate and ultimately the loss of activity, leading to similar traits associated with WB (Cisternas et al., 2014). The addition of Wnt3A protein in mice has been shown to increase the rate at which progenitor cells are converted from a myogenic to a fibrogenic state resulting in increased deposition of connective tissue (Brack et al., 2007). The expression of myogenic regulatory factors (MRF) responsible for normal formation of new myotubes, such as MyoD, Myf5, myogenin, and Pax3/7, are activated by Wnt (Yokoyama and Asahara, 2011). However, in the event that these progenitor cells lose the ability to mediate repair, the muscle tissue is replaced by adipose and fibrotic tissue, which also appears to be a phenotype associated with WB (Laumonier and Menetrey, 2016). Our findings were not the first to detect changes in Wnt signaling due to WB. Others have shown that WB results in statistically significant differential expression of *WNT7A* (Zambonelli et al., 2016a). Wnt7a is responsible for stimulating skeletal muscle growth and repair through the induction of satellite cells via the mTOR pathway (Bentzinger et al., 2014). In contrast, others have found that there is an increase in gene expression of the MRF's however they tend to vary depending on the lineage of the bird used (Velleman and Clark, 2015). This has led to a different understanding of the disease state which may not be entirely genetic or environmental but rather both, which is commonly referred to as epigenetic. It could be that environmental conditions stimulate pathways leading to genomic modifications, potentially resulting in phenotypic alterations.

Our working hypothesis, based on the ML analysis, is that the underlying mechanism resulting in fibrosis and hence, WB, is related to genetic regulation, possibly through NPCs, CCDC85A, and β-catenin. These activate the Wnt signaling pathway via TGF-β, mTOR and IGF-II pathways, potentially resulting in WB pathology. It is possible that a pattern of Mendelian

inheritance does not result in direct causation of WB, but rather modifications that result in changes in the expression of genes such as histone modifications and DNA methylation.

Following damage or rapid growth, skeletal muscle satellite cells are failing to regenerate myoblasts and results in fibrotic scar tissue; overall, because stem cells are restricted to a limited number of divisions, we hypothesize satellite cells are being exhausted and eventually resulting in WB (Sacco et al., 2010). This is similar to the hypothesis presented by Daughtry et al. (2017), who thought that a disruption in satellite cell homeostasis was involved in muscle myopathies. Throughout the life of an organism, the number of satellite cells available for regeneration of cells decreases. For satellite cells, aging has been characterized by delayed activation and the inability to proliferate and differentiate. A decrease in the efficiency of Wnt, TGF and IGF signaling pathways has been shown to limit satellite cell proliferation and myoblast differentiation (Barton-Davis et al., 1998; Carlson et al., 2009). It is known that fast twitch muscle is the leading muscle type found in the PM of broilers. Fast twitch muscle has fewer satellite cells than those of slow twitch resulting in differences in the course of muscle regeneration (Collins and Partridge, 2005). Differences depicted in fast twitch fibers include the TGF-β expression pattern, early activation of the myogenic regulatory factors, and better regeneration efficiency (Zimowska et al., 2009, 2017). After injury, satellite cells are activated by expression of early myogenic regulatory factors, MyoD and Myf5. Next, late myogenic regulatory factors are expressed, which consist of myogenin and MRF4. Pax 3/7 are paired box transcription factors that directly and indirectly regulate myogeneic regulatory factors as skeletal muscle progenitor cells. Together these altered pathways are likely contributing to the development of the PM myopathy.

Organismal Metabolic Influence – Liver Transcriptome

The liver was considered as a tissue of importance related to WB due to the vast array of metabolic functions including the responsibility to synthesize, metabolize and excrete many molecules (Zaefarian et al., 2019). In the bird, 11% of all protein synthesis occurs in the liver which are then transported via systemic circulation to other tissues (Denbow, 2000). Evaluation of the liver transcriptome dataset using IPA resulted in the identification of Skeletal and Muscular System Development and Function, Developmental Disorder, Hereditary Disorder (Figure 5.6), Connective Tissue Disorders, Hematological Disease, Hereditary Disorder (Figure 5.7) and Cell Cycle, Embryonic Development, Cellular Movement (Figure 5.8) network associations. Similarly when evaluating the PM through IPA analysis of differential gene expression data, connective tissue disorders, embryonic development and cell cycle pathways have previously been detected (Mutryn et al., 2015).

The association network Skeletal and Muscular System Development and Function, Developmental Disorder, Hereditary Disorder (Figure 5.6) depicts molecules involved in muscle function and development such as SGCG which protects and maintains the structure of muscle cells through the sarcoglycan protein. In mammals, mutations of this gene result in the loss of y-sarcoglycan protein and ultimatley muscle dystrophy and fibrosis (Heydemann et al., 2009). Disfunction in SCCG has been shown to result in enhanced TGF-β availability and therefore increased SMAD signaling leading to fibrosis (Heydemann et al., 2009). It is thought that proteins from this gene could be mediating their effect by regulating myostatin activity. Myostatin (MSTN), a family member of TGF-β, inhibits myoblast differentiation by repressing myogenic regulatory factors (Langley et al., 2002). MSTN prevents differentiation via the transcription factor SMAD3, which can be activated by both TGF-β and MSTN. Other molecules identified in IPA with changes

in expression due to WB were JAKMIP2, which is invovlved in microtubule binding and CAPSL which is involved in calcium ion binding. This IPA pathway has also been detected when evaluating the PM of differential gene experssion between male and females birds with WB (Brothers et al., 2019).

CARD19 in this molecular network was also detected in MLP of WEKA as being able to predict normal or WB 93.333% using the cross-validation method (Figure 5.8, Table 5.3). CARD proteins (caspase recruitment domain) are a domain of proteins which regulate apoptosis and inflammation (Jang et al., 2015). Studies evaluating CARD19 and its role in the IKKß and NF-kB pathway have been contradictory. Early data suggests CARD19 is a negative regulator of NF-kB, which is a transcription factors that signals IKK; however a more recent study in mice suggests in the absence of CARD19 there was an increase in TNF-α which would subsequently increase IKKß and NF-kB (Rios et al., 2018). IKKß has been shown to decrease β-catenin activation which as previously mentioned is an activator canonical Wnt signaling (Lamberti et al., 2001; Staal, 2016).

Downstream TNF-α kinases IKKß and NF-kB play many roles in regulating physiological reactions including regulators of tuberous sclerosis complex (TSC), which repress the mechanistic Target of Rapamycin (mTOR) pathway (Lee et al., 2007). IKKß in association with TSC allows for the activation of mTOR. *MTOR* coordinates cell growth and is the major regulator of metabolic processes (Figure 5.7). Many factors are responsible for the activation of mTORC1 including Wnt signaling, growth factors, and TNF-α through interaction with TSC (Laplante and Sabatini, 2009). Activation of mTORC1 positively results in cell growth and proliferation through the activation of protein and lipid synthesis pathways. Disruptions in these pathways is associated with tumor development and fibrogenesis, and macrophage regulation. Ribosome biogenesis has been observed to be promoted through activation of mTORC1 by transcription of ribosomal RNA which

can be observed as being downregulated in Figure 5.5 and Table 5.3 of the PM dataset (Mayer et al., 2004). Much of the IPA network association Connective Tissue Disorders, Hematological Disease, Hereditary Disorder involves molecules related to cell adhesion and cytoskeleton (Figure 7). Interestingly, mTORC2 regulates cytoskeleton organization (Jacinto et al., 2004).

Cell Cycle, Embryonic Development, Cellular Movement (Figure 5.8) network association represents molecules in the cell cycle such as: ITCH, NEDD9, DOK5, and IGFBP2. ITCH in this molecular network was also detected in MLP of WEKA as being able to predict normal or WB 93.333% using the cross-validation method. The Itch protein encodes a member of the Nedd4 family ubiquitin ligases that targets specific proteins for lysosomal degradation. Itch plays a role in lymphoid cell differentiation and the regulation of immune response and pathways related to this protein include the TNF-α signaling pathway. NEDD9 plays a role in the TGF-β pathway and growth signals initiating cellular proliferation and has been identified as upregulated in hepatic fibrosis (Dooley et al., 2008). *DOK5* in humans is strongly expressed in muscle and is involved in the positive activation of MAPK and possibly insulin activation (Cai et al., 2003; Grimm et al., 2001). DOK5 has been identified as a membrane associated protein triggered by insulin-like growth factor binding protein-5 (IGFBP-5) for intracellular signaling resulting in pro-fibrotic effects and is thought to promote fibrosis (Yasuoka et al., 2014). IGFBP2 is an insulin-like growth factor binding protein involved in cellular signaling. While muscle is the primarily a site for glucose disposal, the avian liver does function to control muscle growth through the allocation of resources by regulating the birds nutritional balance. The muscle has a paracrine effect, whereas the liver has an endocrine role in circulating IGFs and IGFBPs. Signaling pathways in the liver play an important role in regulating many aspects of energy metabolism and cell cycle processes (Nguyen et al., 2014). Circulating molecules have been identified that play a role of signaling

between the liver and muscle (Liu et al., 2013). Some signaling pathways are only responsive within the liver while others are only responsive in the muscle (proliferation and differentiation). Most of the IPA pathways identified resulted in an effect which would be detected within skeletal muscle. Liver and muscle are important tissues in understanding regulation of metabolic homeostasis, genes involved in glycogenesis, glycolysis and lipogenesis are responsive in both liver and muscle even though the expression patterns are very different between the two tissues. It is not unexpected to observe effects of the liver on muscle development since the liver is the major location of protein, lipid, and carbohydrate metabolism supporting the rapid growth of broiler chickens.

Histological evaluation of WB has revealed multifocal degeneration and necrosis of fibers and accumulation of immune related cells such as macrophages, heterophils and lymphocytes (Kuttappan et al., 2016; Sihvo et al., 2014; Trocino et al., 2015). Affected areas have characteristics of fibrosis separating muscle fibers and thickening of the interstitium. Fibrosis has been characterized as hardening or scaring of tissue as a result of the accumulation of the extra cellular matrix proteins, including collagen and fibronectin eventually leading to loss of activity to the tissue (Wynn, 2008). Fibrosis has been detected in various tissues lung, liver, kidney and skeletal muscle, however the mechanism resulting in fibrosis has been similar in tissues (Cisternas et al., 2014). Pathways detected through IPA of the liver and PM have indicated many molecules which directly and indirectly lead to tissue fibrosis. The fibrosis associated with WB may be driven by signal originating in the liver or other tissues.

Conclusion

In conclusion, using a machine learning approach, we were able to identify predictors that were able to accurately differentiate normal tissue from WB tissue using liver and PM

transcriptomes from individual birds. Through the use of IPA, predictors from both PM and liver tissue identified gene networks associated with skeletal muscle disorders and other networks that could be associated with the development of WB. Given that gene expression data from both PM and liver transcriptomes were able to predict WB or normal tissue using select genes, with some redundancy between tissues, suggests that WB is the result of systematic disruptions in one or more regulatory pathways involving abnormal muscle development, deposition, or maintenance. The data herein suggests that WB phenotype could be potentially be mediated through genes which ultimately result in the up- or down-regulation of pathways that are largely involved with metabolic regulation and basic cellular maintenance, such as Wnt and mTOR, respectively. In mammals, dysregulation in either of these canonical pathways has been shown to result in similar characteristics identified in WB and further investigation of these pathways in chickens exhibiting WB is warranted.

Overall Conclusions

The research herein was conducted in an attempt to better understand epigenetic mechanisms occurring in the avian through a detailed analysis of CpG DNA methylation of genomic regions. It was of interest to evaluate how environmental exposures can alter modifications to the genome and to understand if specific modifications can be altered for many generations. This research was designed to fill voids in our current knowledge of epigenetics in the avian using a molecular approach of bisulfite conversion and whole genome sequencing to provide information about the occurrences of molecular mechanisms and interactions. It was found that providing additional dietary resources of methyl catalysts in one generation can have effects on two subsequent generations. Phenotypic variations included changes in egg size for the three generations evaluated when only the first generation was provided with additional dietary resources. This raises many questions regarding resource allocation in the avian. With the understandign from prior literature that imprinting has not been identified in the avian, it is of interest to understand how nutrients are deposited in the egg and the mechanism behind the amount of nutrients which are given to the embryo. The assessment of genes involved in yolk accumulation resulted in differences calculated for CpG DNA methylation level between generation, treatment and their interaction.

To better understand how CpG DNA methylation may alter transcription, transcriptome analysis was accomplished with RNA sequencing in the second generation removed from dietary treatment. This generation was of importance because this is the first true generation not exposed to dietary treatment. Gene expression differences were detected between the dietary treatment groups. When using Ingenuity Pathway Analysis, the most relevant pathways detected were

involved in metabolic functions, including amino acid metabolism, lipid metabolism, energy production, and carbohydrate metabolism.

Methyl catalysts are directly involved in the DNA methylation pathway, which uses the universal methyl donor S-adenosylmethionine to methylate DNA with DNA methyl transferases. Many coenzymes and cofactors use the dietary methyl donors and methyl catalysts to recycle homocysteine to methionine and then the active form S-adenosylmethionine. CpG DNA methylation level of genes responsible for the enzymes within this pathway were evaluated for the three generations. Differences were detected between generations, treatments, and their interactions. CpG DNA methylation level was compared with gene expression and the majority of the genes involved resulted in the typical trend reported in literature where increased DNA methylation level results in decreased expression.

Many diseases in mammals have been linked to the nutrition of the previous parental generation through alterations in epigenetic modifications. These modifications have been shown to alter metabolic function in the offspring due to parental environmental exposures. Many diseases, including wooden breast, are known to impact the poultry industry which could potentially be a result from the interaction of both environmental influences and genetics. Future research will be needed determine if the susceptibility or severity of diseases affecting poultry production is correlated with epigenetic modifications, resulting in changes in gene regulation and disease incidence. In the future of poultry production, it will be essential to utilize new tools and technologies to make improvements in production practices and meet consumer demands. The knowledge that birds can be preprogrammed to withstand environmental conditions could lead to the creation of useful tools or strategies in the poultry industry to potentially help progeny positively withstand environmental conditions such as temperature, disease susceptibility, or

improved utilization of nutrients. Implementation of parental factors such as these could be used within the poultry industry to help producers maximize the potential for the birds. It is my belief that epigenetic programming of breeders in poultry production presents a new field for realizing potential gains in health and performance of the offspring.

References

Abasht, B., Zhou, N., Lee, W. R., Zhuo, Z., and Peripolli, E. (2019). The metabolic characteristics of susceptibility to wooden breast disease in chickens with high feed efficiency. *Poult. Sci.* 98, 3246–3256. doi:10.3382/ps/pez183.

Aggrey, S. E., González-Cerón, F., Rekaya, R., and Mercier, Y. (2018). Gene expression differences in the methionine remethylation and transsulphuration pathways under methionine restriction and recovery with D,L-methionine or D,L-HMTBA in meat-type chickens. *J. Anim. Physiol. Anim. Nutr. (Berl).* 102, e468–e475. doi:10.1111/jpn.12779.

Allfrey, VG Faulkner, R Mirsky, A. (1964). Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. *Proc. Natl. Acad. Sci. U. S. A.* 51, 786–94.

Anderson, L. M., Riffle, L., Wilson, R., Travlos, G. S., Lubomirski, M. S., and Alvord, W. G. (2006). Preconceptional fasting of fathers alters serum glucose in offspring of mice. *Nutrition* 22, 327–331. doi:10.1016/J.NUT.2005.09.006.

Anderson, O. S., Sant, K. E., and Dolinoy, D. C. (2012). Nutrition and epigenetics: An interplay of dietary methyl donors, one-carbon metabolism, and DNA methylation. *J Nutr Biochem* 23, 853–859. doi:10.1016/j.jnutbio.2012.03.003.

Ashwell, C. M., and Angel, R. (2010). Nutritional genomics: a practical approach by early life conditioning with dietary phosphorus. *Rev. Bras. Zootec.* 39, 268–278. doi:10.1590/s1516-35982010001300030.

Asp, P., Blum, R., Vethantham, V., Parisi, F., Micsinai, M., Cheng, J., et al. (2011). Genome-wide remodeling of the epigenetic landscape during myogenic differentiation. *PNAS* 108, 149–158. doi:10.1073/pnas.1102223108.

Auclair, G., Guibert, S., Bender, A., and Weber, M. (2014). Ontogeny of CpG island methylation and specificity of DNMT3 methyltransferases during embryonic development in the mouse. *Genome Biol.* 15, 545. doi:10.1186/s13059-014-0545-5.

Bacon, W. L., Musser, M. A., and Brown, K. I. (1974). Plasma free fatty acid and neutral lipid concentrations in immature, laying and broody turkey hens. *Poult. Sci.* 53, 1154–1160. doi:10.3382/ps.0531154.

Barau, J., Teissandier, A., Zamudio, N., Roy, S., Nalesso, V., Hérault, Y., et al. (2016). The DNA methyltransferase DNMT3C protects male germ cells from transposon activity. *Science (80-.).* 354, 909–912. doi:10.1126/science.aah5143.

Barile, M., Giancaspero, T. A., Leone, P., Galluccio, M., and Indiveri, C. (2016). Riboflavin transport and metabolism in humans. *J. Inherit. Metab. Dis.* 39, 545–557. doi:10.1007/s10545-016-9950-0.

Barton-Davis, E. R., Shoturma, D. I., Musaro, A., Rosenthal, N., and Sweeney, H. L. (1998). Viral mediated expression of insulin-like growth factor I blocks the aging-related loss of skeletal muscle function. 95, 15603–15607. Available at: www.pnas.org. [Accessed July 7, 2018].

Bentzinger, C. F., Maltzahn, J. von, Dumont, N. A., Stark, D. A., Wang, Y. X., Nhan, K., et al. (2014). Wnt7a stimulates myogenic stem cell motility and engraftment resulting in improved muscle strength. *J. Cell Biol.* 205, 97–111. doi:10.1083/JCB.201310035.

Bird, A. (2002). DNA methylation patterns and epigenetic memory. *Genes Dev* 16, 6–21. doi:10.1101/gad.947102.6.

Bird, A. P. (1978). Use of restriction enzymes to study eukaryotic DNA methylation: II. The symmetry of methylated sites supports semi-conservative copying of the methylation

pattern. *J. Mol. Biol.* 118, 49–60. doi:10.1016/0022-2836(78)90243-7.
Biscotti, M. A., Barucca, M., Carducci, F., and Canapa, A. (2018). New perspectives on the evolutionary history of vitellogenin gene family in vertebrates. *Genome Biol. Evol.* 10, 2709–2715. doi:10.1093/gbe/evy206.
Brack, A. S., Conboy, M. J., Roy, S., Lee, M., Kuo, C. J., Keller, C., et al. (2007). Increased Wnt Signaling During Aging Alters Muscle Stem Cell Fate. *Science (80-.).* 129, 2865. doi:10.1126/science.1144090.
Brothers, B., Zhuo, Z., Papah, M. B., and Abasht, B. (2019). RNA-seq analysis reveals spatial and sex differences in pectoralis major muscle of broiler chickens contributing to difference in susceptibility to wooden breast disease. *Front. Physiol.* 10, 764. doi:10.3389/fphys.2019.00764.
Cabrero, C., Duce, A. M., Ortiz, P., Alemany, S., and Mato, J. M. (1988). Specific loss of the high-molecular-weight form ofS-adenosyl-L-methionine synthetase in human liver cirrhosis. *Hepatology* 8, 1530–1534. doi:10.1002/hep.1840080610.
Cai, D., Dhe-Paganon, S., Melendez, P. A., Lee, J., and Shoelson, S. E. (2003). Two new substrates in insulin signaling, IRS5/DOK4 and IRS6/DOK5. *J. Biol. Chem.* 278, 25323–25330. doi:10.1074/jbc.M212430200.
Carlson, M. E., Conboy, M. J., Hsu, M., Barchas, L., Jeong, J., Agrawal, A., et al. (2009). Relative roles of TGF-β1 and Wnt in the systemic regulation and aging of satellite cell responses. *Aging Cell* 8, 676–689. doi:10.1111/j.1474-9726.2009.00517.x.
Carone, B. R., Fauquier, L., Habib, N., Shea, J. M., Hart, C. E., Li, R., et al. (2010). Paternally induced transgenerational environmental reprogramming of metabolic gene expression in mammals. *Cell* 143, 1084–1096. doi:10.1016/j.cell.2010.12.008.
Carrell, D. T., and Hammoud, S. S. (2009). The human sperm epigenome and its potential role in embryonic development. *Mol. Hum. Reprod.* 16, 37–47. doi:10.1093/molehr/gap090.
Carter, E. J., Cosgrove, R. A., Gonzalez, I., Eisemann, J. H., Lovett, F. A., Cobb, L. J., et al. (2009). MEK5 and ERK5 are mediators of the pro-myogenic actions of IGF-2. *J. Cell Sci.* 122, 3104–3112. doi:10.1242/jcs.045757.
Casolari, J. M., Brown, C. R., Komili, S., West, J., Hieronymus, H., and Silver, P. A. (2004). Genome-Wide Localization of the Nuclear Transport Machinery Couples Transcriptional Status and Nuclear Organization. *Cell* 117, 427–439. doi:10.1016/S0092-8674(04)00448-9.
Choi, S.-W., and Friso, S. (2010). Epigenetics: A New Bridge between Nutrition and Health. *Adv. Nutr.* 1, 8–16. doi:10.3945/an.110.1004.
Christman, J. K., Sheikhnejad, G., Dizik, M., Abileah, S., and Wainfan, E. (1993). Reversibility of changes in nucleic acid methylation and gene expression induced in rat liver by severe dietary methyl deficiency. *Carcinogenesis* 14, 551–557. doi:10.1093/carcin/14.4.551.
Cisternas, P., Henriquez, J. P., Brandan, E., and Inestrosa, N. C. (2014). Wnt signaling in skeletal muscle dynamics: Myogenesis, neuromuscular synapse and fibrosis. *Mol. Neurobiol.* 49, 574–589. doi:10.1007/s12035-013-8540-5.
Clarke, H. J., McLay, D. W., and Mohamed, O. A. (1998). Linker histone transitions during mammalian oogenesis and embryogenesis. *Dev. Genet.* 22, 17–30. doi:10.1002/(SICI)1520-6408(1998)22:1<17::AID-DVG3>3.0.CO;2-A.
Collins, C. A., and Partridge, T. A. (2005). Self-renewal of the adult skeletal muscle satellite cell. *Cell Cycle* 4, 1338–1341. doi:10.4161/cc.4.10.2114.
Colmenares, C., and Stavnezer, E. (1989). The ski oncogene induces muscle differentiation in quail embryo cells. *Cell* 59, 293–303. doi:10.1016/0092-8674(89)90291-2.

Colwell, M., Drown, M., Showel, K., Drown, C., Palowski, A., and Faulk, C. (2018). Evolutionary conservation of DNA methylation in CpG sites within ultraconserved noncoding elements. *Epigenetics* 13, 49–60. doi:10.1080/15592294.2017.1411447.
Cortes, C., and Vapnik, V. (1995). Support-Vector Networks. *Mach. Learn.* 20, 273–297.
Curley, J. P., Mashoodh, R., and Champagne, F. A. (2011). Epigenetics and the origins of paternal effects. *Horm. Behav.* 59, 306–14. doi:10.1016/j.yhbeh.2010.06.018.
D'Angelo, M. A. (2018). Nuclear pore complexes as hubs for gene regulation. *Nucleus* 9, 142–148. doi:10.1080/19491034.2017.1395542.
D'Angelo, M. A., Gomez-Cavazos, J. S., Mei, A., Lackner, D. H., and Hetzer, M. W. (2012). A Change in Nuclear Pore Complex Composition Regulates Cell Differentiation. *Dev. Cell* 22, 446–458. doi:10.1016/J.DEVCEL.2011.11.021.
Daughtry, M. R., Berio, E., Shen, Z., Suess, E. J. R., Shah, N., Geiger, A. E., et al. (2017). Satellite cell-mediated breast muscle regeneration decreases with broiler size. *Poult. Sci.* 96, 3457–3464. doi:10.3382/ps/pex068.
David, S.-A., Mersch, M., Foissac, S., Collin, A., Pitel, F., and Coustham, V. (2017). Genome-Wide Epigenetic Studies in Chicken: A Review. *Epigenomes* 1, 20. doi:10.3390/epigenomes1030020.
Davies, B., and Fried, M. (1995). The L19 ribosomal protein gene (RPL19): gene organization, chromosomal mapping, and novel promoter region. *Genomics* 25, 372–380. doi:10.1016/0888-7543(95)80036-L.
Denbow, D. M. (2000). *Gastrointestinal anatomy and physiology. In Sturkie's Avian Physiology*. 5th ed. , ed. E. Whittow, G.C. New York, NY, USA,: Academic Press.
Dooley, S., Hamzavi, J., Ciuclan, L., Godoy, P., Ilkavets, I., Ehnert, S., et al. (2008). Hepatocyte-Specific Smad7 Expression Attenuates TGF-β–Mediated Fibrogenesis and Protects Against Liver Damage. *Gastroenterology* 135, 642-659.e46. doi:10.1053/j.gastro.2008.04.038.
Duhl, D. M. J., Vrieling, H., Miller, K. A., Wolff, G. L., and Barsh, G. S. (1994). Neomorphic agouti mutations in obese yellow mice. *Nat. Genet.* 8, 59–65. doi:10.1038/ng0994-59.
Edinger, R. S., Mambo, E., and Evans, M. I. (1997). Estrogen-dependent transcriptional activation and vitellogenin gene memory. *Mol. Endocrinol.* 11, 1985–1993. doi:10.1210/mend.11.13.0037.
Eibe, F., Hall, M. A., and Witten, I. H. (2016). *The WEKA Workbench. Online Appendix for "Data Mining: Practical Machine Learning Tools and Techniques."* Fourth. , ed. M. Kaufmann.
Ekmay, R. D., De Beer, M., Mei, S. J., Manangi, M., and Coon, C. N. (2013). Amino acid requirements of broiler breeders at peak production for egg mass, body weight, and fertility. *Poult. Sci.* 92, 992–1006. doi:10.3382/ps.2012-02554.
Engle, K. M.; Mei, T-S.; Wasa, M.; Yu, J.-Q. (2008). NIH Public Access. *Acc. Chem. Res.* 45, 788–802. doi:10.1038/jid.2014.371.
Felsenfeld, G. (2014). A Brief History of Epigenetics. *Cold Spring Harb. Perspect. Biol.* 6, a018200–a018200. doi:10.1101/cshperspect.a018200.
Finkelstein, J. D. (1998). The metabolism of homocysteine: pathways and regulation. *Eur. J. Pediatr.* 157, S40–S44. doi:10.1007/PL00014300.
Finkelstein, J. D., and Martin, J. J. (1984). Methionine Metabolism in Mammals Ftz : 259, 9508–9513.
Frésard, L., Leroux, S., Servin, B., Gourichon, D., Dehais, P., Cristobal, M. S., et al. (2014). Transcriptome-wide investigation of genomic imprinting in chicken. *Nucleic Acids Res.* 42,

3768–3782. doi:10.1093/nar/gkt1390.
Friedel, H. A., Goa, K. L., and Benfield, P. (1989). S-Adenosyl-L-Methionine. *Drugs* 38, 389–416. doi:10.2165/00003495-198938030-00004.
Galy, V., Olivo-Marin, J.-C., Scherthan, H., Doye, V., Rascalou, N., and Nehrbass, U. (2000). Nuclear pore complexes in the organization of silent telomeric chromatin. *Nature* 403, 108–112. doi:10.1038/47528.
Gardiner-Garden, M., and Frommer, M. (1987). CpG Islands in vertebrate genomes. *J. Mol. Biol.* 196, 261–282. doi:10.1016/0022-2836(87)90689-9.
Gartler, S. M., and Goldman, M. A. (1994). Reactivation of inactive X-linked genes. *Dev. Genet.* 15, 504–514. doi:10.1002/dvg.1020150609.
Geiman, T. M., Sankpal, U. T., Robertson, A. K., Zhao, Y., Zhao, Y., and Robertson, K. D. (2004). DNMT3B interacts with hSNF2H chromatin remodeling enzyme, HDACs 1 and 2, and components of the histone methylation system. *Biochem. Biophys. Res. Commun.* 318, 544–555. doi:10.1016/j.bbrc.2004.04.058.
Geiser, M., MattajS, I. W., Wilks, A. F., Seldran, M., and Jost, J.-P. (1983). Structure and Sequence of the Promoter Area and of a 5' Upstream Demethylation Site of the Estrogen-regulated Chicken Vitellogenin I1. Available at: http://www.jbc.org/ [Accessed October 30, 2019].
Gerber-Huber, S., May, F. E. B., Westley, B. R., Felber, B. K., Hosbach, H. A., Andres, A. C., et al. (1983). In contrast to other Xenopus genes the estrogen-inducible vitellogenin genes are expressed when totally methylated. *Cell* 33, 43–51. doi:10.1016/0092-8674(83)90333-1.
Griffin, H., and Hermier, D. (1988). "Plasma lipoprotein metabolism and fattening in poultry," in *Leanness in Domestic Birds*, eds. B. Leclercq and C. C. Whitehead (New York, NY, USA,: Elsevier), 175–201. doi:10.1016/B978-0-408-01036-8.50020-8.
Grimm, J., Sachs, M., Britsch, S., Di Cesare, S., Schwarz-Romond, T., Alitalo, K., et al. (2001). Novel p62dok family members, dok-4 and dok-5, are substrates of the c-Ret receptor tyrosine kinase and mediate neuronal differentiation. *J. Cell Biol.* 154, 345–54. doi:10.1083/jcb.200102032.
Grossniklaus, U., and Paro, R. (2014). Transcriptional Silencing by Polycomb-Group Proteins. *Cold Spring Harb. Perspect. Biol.* 6, a019331–a019331. doi:10.1101/cshperspect.a019331.
Guibert, F., Richard-Yris, M. A., Lumineau, S., Kotrschal, K., Bertin, A., Petton, C., et al. (2011). Unpredictable mild stressors on laying females influence the composition of Japanese quail eggs and offspring's phenotype. *Appl. Anim. Behav. Sci.* 132, 51–60. doi:10.1016/j.applanim.2011.03.012.
Gupta, S., Pathak, R. U., and Kanungo, M. S. (2006). DNA methylation induced changes in chromatin conformation of the promoter of the vitellogenin II gene of Japanese quail during aging. *Gene* 377, 159–168. doi:10.1016/j.gene.2006.04.020.
Hales, B. F., Grenier, L., Lalancette, C., and Robaire, B. (2011). Epigenetic programming: From gametes to blastocyst. *Birth Defects Res. Part A - Clin. Mol. Teratol.* 91, 652–665. doi:10.1002/bdra.20781.
Hales, C. N., and Barker, D. J. (2001). The thrifty phenotype hypothesis. *Br. Med. Bull.* 60, 5–20. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11809615.
Hammoud, S. S., Nix, D. A., Hammoud, A. O., Gibson, M., Cairns, B. R., and Carrell, D. T. (2011). Genome-wide analysis identifies changes in histone retention and epigenetic modifications at developmental and imprinted gene loci in the sperm of infertile men. *Hum. Reprod.* 26, 2558–2569.

Hanson, M. A., and Skinner, M. K. (2016). Developmental origins of epigenetic transgenerational inheritance. *Environ. Epigenetics* 2, dvw002. doi:10.1093/eep/dvw002.
Harry, D. E., Brown, McElroy, J. P., Lamont, S. J., Kim, J.-J., and Dekkers, J. C. M. (2014). Identification of Trait Loci Affecting White Meat Percentage and Other Growth and Carcass Traits in Commercial Broiler Chickens. *Poult. Sci.* 85, 593–605. doi:10.1093/ps/85.4.593.
Heard, G. S., and Annison, E. F. (1986). Gastrointestinal absorption of vitamin B-6 in the chicken (Gallus domesticus). *J. Nutr.* 116, 107–120. doi:10.1093/jn/116.1.107.
Hebert, K., House, J. D., and Guenter, W. (2005). Effect of dietary folic acid supplementation on egg folate content and the performance and folate status of two strains of laying hens. *Poult. Sci.* 84, 1533–1538. doi:10.1093/ps/84.10.1533.
Heydemann, A., Ceco, E., Lim, J. E., Hadhazy, M., Ryder, P., Moran, J. L., et al. (2009). Latent TGF-beta-binding protein 4 modifies muscular dystrophy in mice. *J. Clin. Invest.* 119, 3703–12. doi:10.1172/JCI39845.
Hillier, L. W., Miller, W., Birney, E., Warren, W., Hardison, R. C., Ponting, C. P., et al. (2004). Sequence and comparative analysis of the chicken genome provide unique perspectives on vertebrate evolution. *Nature* 432, 695. Available at: https://www.nature.com/articles/nature03154.pdf.
Hirasawa, R., Chiba, H., Kaneda, M., Tajima, S., Li, E., Jaenisch, R., et al. (2008). Maternal and zygotic Dnmt1 are necessary and sufficient for the maintenance of DNA methylation imprints during preimplantation development. *Genes Dev.* 22, 1607–1616. doi:10.1101/gad.1667008.
Ho, D. H., Reed, W. L., and Burggren, W. W. (2011). Egg yolk environment differentially influences physiological and morphological development of broiler and layer chicken embryos. *J. Exp. Biol.* 214, 619–628. doi:10.1242/jeb.046714.
Hoey, L., McNulty, H., McCann, E. M. E., McCracken, K. J., Scott, J. M., Marc, B. B., et al. (2009). Laying hens can convert high doses of folic acid added to the feed into natural folates in eggs providing a novel source of food folate. *Br. J. Nutr.* 101, 206. doi:10.1017/S0007114508995647.
Hu, J., Wang, F., Yuan, Y., Zhu, X., Wang, Y., Zhang, Y., et al. (2010). Novel importin-α family member Kpna7 is required for normal fertility and fecundity in the mouse. *J. Biol. Chem.* 285, 33113–33122. doi:10.1074/jbc.M110.117044.
Hu, Y., Xu, H., Li, Z., Zheng, X., Jia, X., Nie, Q., et al. (2013). Comparison of the Genome-Wide DNA Methylation Profiles between Fast-Growing and Slow-Growing Broilers. *PLoS One* 8, e56411. doi:10.1371/journal.pone.0056411.
Huang, Y., Pastor, W. A., Shen, Y., Tahiliani, M., Liu, D. R., and Rao, A. (2010). The Behaviour of 5-Hydroxymethylcytosine in Bisulfite Sequencing. *PLoS One* 5, e8888. doi:10.1371/journal.pone.0008888.
Hustad, S., Schneede, J., and Ueland, P. M. (2013). Riboflavin and Methylenetetrahydrofolate Reductase. Available at: https://www.ncbi.nlm.nih.gov/books/NBK6145/ [Accessed April 18, 2019].
Hyun, K., Jeon, J., Park, K., and Kim, J. (2017). Writing, erasing and reading histone lysine methylations. *Exp. Mol. Med.* 49, e324–e324. doi:10.1038/emm.2017.11.
Ikeobi, C. O. N., Woolliams, J. A., Morrice, D. R., Law, A., Windsor, D., Burt, D. W., et al. (2002). Quantitative trait loci affecting fatness in the chicken. *Anim. Genet.* 33, 428–435. doi:10.1046/j.1365-2052.2002.00911.x.

Iwai, A., Hijikata, M., Hishiki, T., Isono, O., Chiba, T., and Shimotohno, K. (2008). Coiled-coil domain containing 85B suppresses the β-catenin activity in a p53-dependent manner. *Oncogene* 27, 1520–1526. doi:10.1038/sj.onc.1210801.

Jablonka, E. V. A., and Raz, G. A. L. (2009). Transgenerational epigenetic inheritance: prevalence, mechanisms, and implications for the study of heredity and evolution. *Q. Rev. Biol.* 84, 131–176. doi:10.1086/598822.

Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Rüegg, M. A., Hall, A., et al. (2004). Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat. Cell Biol.* 6, 1122–1128. doi:10.1038/ncb1183.

Jaenicke, L. (1970). The Biochemistry of Folic Acid and Related Pteridines. *Angew. Chemie* 82, 780–780. doi:10.1002/ange.19700821818.

Jahan, S., Xu, W., He, S., Gonzalez, C., Delcuve, G. P., and Davie, J. R. (2016). The chicken erythrocyte epigenome. *Epigenetics Chromatin* 9, 19. doi:10.1186/s13072-016-0068-2.

Jang, T. H., Kim, S. H., Jeong, J. H., Kim, S., Kim, Y. G., and Park, H. H. (2015). Crystal structure of caspase recruiting domain (CARD) of apoptosis repressor with CARD (ARC) and its implication in inhibition of apoptosis. *Sci. Rep.* 5. doi:10.1038/srep09847.

Jendryczko, A., Drozdz, M., and Sieron, G. (1989). Effect of zinc deficiency on methionine metabolism, methylation reactions and protein synthesis in isolated perfused rat placenta. *Rev. Roum. Biochim.* 26, 131–138. Available at: https://academic.oup.com/jn/article-abstract/115/2/252/4763038 [Accessed May 3, 2019].

Jeppesen, P., and Turner, B. M. (1993). The inactive X chromosome in female mammals is distinguished by a lack of histone H4 acetylation, a cytogenetic marker for gene expression. *Cell* 74, 281–289. doi:10.1016/0092-8674(93)90419-Q.

Jiao, F., Wang, X., Yan, Z., Liu, C., Yue, Z., Li, Z., et al. (2013). Effect of dynamic DNA methylation and histone acetylation on cPouV expression in differentiation of chick embryonic germ cells. *Stem Cells Dev.* 22, 2725–2735. doi:10.1089/scd.2013.0046.

Jones, PA Taylor, SM Wilson, V. (1983). Inhibition of DNA methylation by 5-azacytidine. *Recent Results Cancer Res.*, 202–211.

Jones, P. L., Jan Veenstra, G. C., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., et al. (1998). Methylated DNA and MeCP2 recruit histone deacetylase to repress transcription. *Nat. Genet.* 19, 187–191. doi:10.1038/561.

Kaneda, M., Okano, M., Hata, K., Sado, T., Tsujimoto, N., Li, E., et al. (2004). Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. *Nature* 429, 900–903. doi:10.1038/nature02633.

Kato, Y., Tani, T., Sotomaru, Y., Kurokawa, K., Kato, J. Y., Doguchi, H., et al. (1998). Eight calves cloned from somatic cells of a single adult. *Science (80-.).* 282, 2095–2098. doi:10.1126/science.282.5396.2095.

Katz, D. J., Edwards, T. M., Reinke, V., and Kelly, W. G. (2009). A C. elegans LSD1 Demethylase Contributes to Germline Immortality by Reprogramming Epigenetic Memory. *Cell* 137, 308–320. doi:10.1016/J.CELL.2009.02.015.

Kelley, J. B., Talley, A. M., Spencer, A., Gioeli, D., and Paschal, B. M. (2010). Karyopherin alpha7 (KPNA7), a divergent member of the importin alpha family of nuclear import receptors. *BMC Cell Biol.* 11, 63. doi:10.1186/1471-2121-11-63.

Kirchgessner, T. G., Heinzmann, C., Svenson, K. L., Gordon, D. A., Nicosia, M., Lebherz, H. G., et al. (1987). Regulation of chicken apolipoprotein B: cloning, tissue distribution, and estrogen induction of mRNA. *Gene* 59, 241–251. doi:10.1016/0378-1119(87)90332-5.

Kisliouk, T., and Meiri, N. (2009). A critical role for dynamic changes in histone H3 methylation at the Bdnf promoter during postnatal thermotolerance acquisition. *Eur. J. Neurosci.* 30, 1909–1922. doi:10.1111/j.1460-9568.2009.06957.x.
Kisliouk, T., Yosefi, S., and Meiri, N. (2011). MiR-138 inhibits EZH2 methyltransferase expression and methylation of histone H3 at lysine 27, and affects thermotolerance acquisition. *Eur. J. Neurosci.* 33, 224–235. doi:10.1111/j.1460-9568.2010.07493.x.
Kisliouk, T., Ziv, M., and Meiri, N. (2010). Epigenetic control of translation regulation: Alterations in histone H3 lysine 9 post-translation modifications are correlated with the expression of the translation initiation factor 2B (Eif2b5) during thermal control establishment. *Dev. Neurobiol.* 70, 100–113. doi:10.1002/dneu.20763.
Klose, R. J., and Bird, A. P. (2006). Genomic DNA methylation: The mark and its mediators. *Trends Biochem. Sci.* 31, 89–97. doi:10.1016/j.tibs.2005.12.008.
Klosin, A., Casas, E., Hidalgo-Carcedo, C., Vavouri, T., and Lehner, B. (2017). Transgenerational transmission of environmental information in C. elegans. *Science (80-.).* 356, 320–323. doi:10.1126/science.aah6412.
Kobayashi, N., Goto, K., Horiguchi, K., Nagata, M., Kawata, M., Miyazawa, K., et al. (2007). c-Ski activates MyoD in the nucleus of myoblastic cells through suppression of histone deacetylases. *Genes to Cells* 12, 375–385. doi:10.1111/j.1365-2443.2007.01052.x.
Kornberg, R. D., and Thonmas, J. O. (1974). Chromatin Structure: Oligomers of the Histones. *Science (80-.).* 184, 865–868. doi:10.1126/science.184.4139.865.
Kouzarides, T. (2007). Chromatin Modifications and Their Function. *Cell* 128, 693–705. doi:10.1016/j.cell.2007.02.005.
Kovacheva, V. P., Mellott, T. J., Davison, J. M., Wagner, N., Lopez-Coviella, I., Schnitzler, A. C., et al. (2007). Gestational Choline Deficiency Causes Global and Igf2 Gene DNA Hypermethylation by Up-regulation of Dnmt1 Expression. *J. Biol. Chem.* 282, 31777–31788. doi:10.1074/jbc.M705539200.
Kuttappan, V. A., Hargis, B. M., and Owens, C. M. (2016). White striping and woody breast myopathies in the modern poultry industry: A review. *Poult. Sci.* 95, 2724–2733. doi:10.3382/ps/pew216.
Kuttappan, V. A., Owens, C. M., Coon, C., Hargis, B. M., and Vazquez-A Non, M. (2017). Research Note Incidence of broiler breast myopathies at 2 different ages and its impact on selected raw meat quality parameters. *Poult. Sci.* 96, 3005–3009. doi:10.3382/ps/pex072.
Lamberti, C., Lin, K. M., Yamamoto, Y., Verma, U., Verma, I. M., Byers, S., et al. (2001). Regulation of β-Catenin Function by the IκB Kinases. *J. Biol. Chem.* 276, 42276–42286. doi:10.1074/jbc.M104227200.
Lamers, Y., Coats, B., Ralat, M., Quinlivan, E. P., Stacpoole, P. W., and Gregory, J. F. (2011). Moderate Vitamin B-6 Restriction Does Not Alter Postprandial Methionine Cycle Rates of Remethylation, Transmethylation, and Total Transsulfuration but Increases the Fractional Synthesis Rate of Cystathionine in Healthy Young Men and Women. *J. Nutr.* 141, 835–842. doi:10.3945/jn.110.134197.
Langley, B., Thomas, M., Bishop, A., Sharma, M., Gilmour, S., and Kambadur, R. (2002). Myostatin Inhibits Myoblast Differentiation by Down-regulating MyoD Expression. *J. Biol. Chem.* 277, 49831–49840. doi:10.1074/JBC.M204291200.
Laplante, M., and Sabatini, D. M. (2009). mTOR signaling at a glance. *J. Cell Sci.* 122, 3589–3594. doi:10.1242/jcs.051011.
Laskey, R. A., and Gurdon, J. B. (1970). Genetic Content of Adult Somatic Cells tested by

Nuclear Transplantation from Cultured Cells. *Nature* 228, 1332. Available at: https://doi.org/10.1038/2281332a0.
Latshaw, J. D., and Jensen, L. S. (1971). Choline level and its effect on egg weight in the Japanese quail. *Poult. Sci.* 50, 790–794. doi:10.3382/ps.0500790.
Lattuada, D., Colleoni, F., Martinelli, A., Garretto, A., Magni, R., Radaelli, T., et al. (2008). Higher Mitochondrial DNA Content in Human IUGR Placenta. *Placenta* 29, 1029–1033. doi:10.1016/j.placenta.2008.09.012.
Laumonier, T., and Menetrey, J. (2016). Muscle injuries and strategies for improving their repair. *J. Exp. Orthop.* 3. doi:10.1186/s40634-016-0051-7.
Lee, D. F., Kuo, H. P., Chen, C. Te, Hsu, J. M., Chou, C. K., Wei, Y., et al. (2007). IKKβ Suppression of TSC1 Links Inflammation and Tumor Angiogenesis via the mTOR Pathway. *Cell* 130, 440–455. doi:10.1016/j.cell.2007.05.058.
Leeson, S., Summers, J. D., and Scott, M. L. (2001). *Nutrition of the chicken.* University Books Available at: https://books.google.com/books/about/Nutrition_of_the_Chicken.html?id=JlhnQgAACAAJ [Accessed March 15, 2019].
Leksrisompong, N., Romero-Sanchez, H., Plumstead, P. W., Brannan, K. E., and Brake, J. (2007). Broiler Incubation. 1. Effect of Elevated Temperature During Late Incubation on Body Weight and Organs of Chicks. *Poult. Sci.* 86, 2685–2691. doi:10.3382/ps.2007-00170.
Li, C., Fan, Y., Li, G., Xu, X., Duan, J., Li, R., et al. (2018). DNA methylation reprogramming of functional elements during mammalian embryonic development. *Cell Discov.* 4, 41. doi:10.1038/s41421-018-0039-9.
Li, C., Guo, S., Gao, J., Guo, Y., Du, E., Lv, Z., et al. (2015a). Maternal high-zinc diet attenuates intestinal inflammation by reducing DNA methylation and elevating H3K9 acetylation in the A20 promoter of offspring chicks. *J. Nutr. Biochem.* 26, 173–183. doi:10.1016/j.jnutbio.2014.10.005.
Li, C., Guo, S., Zhang, M., Gao, J., and Guo, Y. (2015b). DNA methylation and histone modification patterns during the late embryonic and early postnatal development of chickens. *Poult. Sci.* 94, 706–721. doi:10.3382/ps/pev016.
Li, Q., Li, N., Hu, X., Li, J., Du, Z., Chen, L., et al. (2011). Genome-wide mapping of dna methylation in chicken. *PLoS One* 6, 1–7. doi:10.1371/journal.pone.0019428.
Li, Y. P., Chen, Y., John, J., Moylan, J., Jin, B., Mann, D. L., et al. (2005). TNF-α acts via p38 MAPK to stimulate expression of the ubiquitin ligase atrogin1/MAFbx in skeletal muscle. *FASEB J.* 19, 362–370. doi:10.1096/fj.04-2364com.
Li, Y., Turck, C. M., Teumer, J. K., and Stavnezert, E. (1986). Unique Sequence, ski, in Sloan-Kettering Avian Retroviruses with Properties of a New Cell-Derived Oncogene Downloaded from. *J. Virol.* 57, 1065–1072. Available at: http://jvi.asm.org/.
Lillycrop, K. A., Hoile, S. P., Grenfell, L., and Burdge, G. C. (2014). DNA methylation, ageing and the influence of early life nutrition. in *Proceedings of the Nutrition Society*, 413–421. doi:10.1017/S0029665114000081.
Liu, S., Brown, J. D., Stanya, K. J., Homan, E., Leidl, M., Inouye, K., et al. (2013). A diurnal serum lipid integrates hepatic lipogenesis and peripheral fatty acid use. *Nature* 502, 550–554. doi:10.1038/nature12710.
Liu, Y., Liu, X., Zhou, J., Ren, Z., Yang, X., Cao, Y., et al. (2019). Folic acid perfusion administration reduced abdominal fat deposition in starter Arbor Acres broilers. *Poult. Sci.* doi:10.3382/ps/pez413.

Liu, Z., Li, Q., Liu, R., Zhao, G., Zhang, Y., Zheng, M., et al. (2016). Expression and methylation of microsomal triglyceride transfer protein and acetyl-CoA carboxylase are associated with fatty liver syndrome in chicken. *Poult. Sci.* 95, 1387–1395. doi:10.3382/ps/pew040.

Livingston, M. L., Ferket, P. R., Brake, J., and Livingston, K. A. (2018a). Dietary amino acids under hypoxic conditions exacerbates muscle myopathies including wooden breast and white stripping. *Poult. Sci.* doi:10.3382/ps/pey463.

Livingston, M. L., Landon, C., Barnes, H. J., and Brake, J. (2018b). White striping and wooden breast myopathies of broiler breast muscle is affected by time-limited feeding, genetic background, and egg storage. *Poult. Sci.* doi:10.3382/ps/pey333.

Luger, K., Mäder, A. W., Richmond, R. K., Sargent, D. F., and Richmond, T. J. (1997). Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature* 389, 251. Available at: https://doi.org/10.1038/38444.

Luo, J., Mitra, A., Tian, F., Chang, S., Zhang, H., Cui, K., et al. (2012). Histone Methylation Analysis and Pathway Predictions in Chickens after MDV Infection. *PLoS One* 7, e41849. doi:10.1371/journal.pone.0041849.

Lyko, F. (2018). The DNA methyltransferase family: A versatile toolkit for epigenetic regulation. *Nat. Rev. Genet.* 19, 81–92. doi:10.1038/nrg.2017.80.

Malila, Y., Thanatsang, K., Arayamethakorn, S., Uengwetwanit, T., Srimarut, Y., Petracci, M., et al. (2019). Absolute expressions of hypoxia-inducible factor-1 alpha (HIF1A) transcript and the associated genes in chicken skeletal muscle with white striping and wooden breast myopathies. *PLoS One* 14, e0220904. doi:10.1371/journal.pone.0220904.

Mandel, J. L., and Chambon, P. (1979). DNA methylation: Organ specific variations in the methylation pattern within and around ovalbumin and other chicken genes. *Nucleic Acids Res.* 7, 2081–2103. doi:10.1093/nar/7.8.2081.

Mayer, C., Zhao, J., Yuan, X., and Grummt, I. (2004). mTOR-dependent activation of the transcription factor TIF-IA links rRNA synthesis to nutrient availability. *Genes Dev.* 18, 423–34. doi:10.1101/gad.285504.

McCarthy J, Minsky M, Rochester N, S. C. (1955). A Proposal for the Dartmouth Summer Research Project on Artificial Intelligence. *AI Mag.*

McLay, D. W., and Clarke, H. J. (2003). Remodelling the paternal chromatin at fertilization in mammals. *Reproduction* 125, 625–633. doi:10.1530/rep.0.1250625.

McQueen, H. A., McBride, D., Miele, G., Bird, A. P., and Clinton, M. (2001). Dosage compensation in birds. *Curr. Biol.* 11, 253–257. doi:10.1016/S0960-9822(01)00070-7.

Messerschmidt, D. M., Knowles, B. B., and Solter, D. (2014). DNA methylation dynamics during epigenetic reprogramming in the germline and preimplantation embryos. *Genes Dev.* 28, 812–28. doi:10.1101/gad.234294.113.

Mitra, A., Luo, J., He, Y., Gu, Y., Zhang, H., Zhao, K., et al. (2015). Histone modifications induced by MDV infection at early cytolytic and latency phases. *BMC Genomics* 16, 311. doi:10.1186/s12864-015-1492-6.

Mitra, A., Luo, J., Zhang, H., Cui, K., Zhao, K., and Song, J. (2012). Marek's disease virus infection induces widespread differential chromatin marks in inbred chicken lines. *BMC Genomics* 13, 557. doi:10.1186/1471-2164-13-557.

Moore, S. E., Cole, T. J., Poskitt, E. M. E., Sonko, B. J., Whitehead, R. G., McGregor, I. A., et al. (1997). Season of birth predicts mortality in rural Gambia [7]. *Nature* 388, 434. doi:10.1038/41245.

Morris, J. G. (2001). Vitamins in Animal and Human Nutrition. *Am. J. Clin. Nutr.* 74, 413–413. doi:10.1093/ajcn/74.3.413.
Morris, T. R., and Gous, R. M. (1988). Partitioning of the response to protein between egg number and egg weight. *Br. Poult. Sci.* 29, 93–99. doi:10.1080/00071668808417030.
Mudalal, S., Babini, E., Cavani, C., and Petracci, M. (2014). Quantity and functionality of protein fractions in chicken breast fillets affected by white striping. *Poult. Sci.* 93, 2108–2116. doi:10.3382/ps.2014-03911.
Murdoch, B. M., Murdoch, G. K., Greenwood, S., and McKay, S. (2016). Nutritional influence on epigenetic marks and effect on livestock production. *Front. Genet.* 7, 182. doi:10.3389/fgene.2016.00182.
Mutryn, M. F., Brannick, E. M., Fu, W., Lee, W. R., and Abasht, B. (2015). Characterization of a novel chicken muscle disorder through differential gene expression and pathway analysis using RNA-sequencing. *BMC Genomics* 16, 1–19. doi:10.1186/s12864-015-1623-0.
Naber, E. C. (1979). The Effect of Nutrition on the Composition of Eggs,. *Poult. Sci.* 58, 518–528. doi:10.3382/ps.0580518.
Naito, M., Mori, M., Inagawa, M., Miyata, K., Hashimoto, N., Tanaka, S., et al. (2016). Dnmt3a Regulates Proliferation of Muscle Satellite Cells via p57Kip2. *PLoS Genet.* 12, 1–23. doi:10.1371/journal.pgen.1006167.
Nakamura, T., Arai, Y., Umehara, H., Masuhara, M., Kimura, T., Taniguchi, H., et al. (2007). PGC7/Stella protects against DNA demethylation in early embryogenesis. *Nat. Cell Biol.* 9, 64–71. doi:10.1038/ncb1519.
Nakamura, T., Liu, Y.-J., Nakashima, H., Umehara, H., Inoue, K., Matoba, S., et al. (2012). PGC7 binds histone H3K9me2 to protect against conversion of 5mC to 5hmC in early embryos. *Nature* 486, 415–419. doi:10.1038/nature11093.
Ng, S.-F., Lin, R. C. Y., Laybutt, D. R., Barres, R., Owens, J. A., and Morris, M. J. (2010). Chronic high-fat diet in fathers programs β-cell dysfunction in female rat offspring. *Nature* 467, 963–966. doi:10.1038/nature09491.
Nguyen, T. T., Almon, R. R., Dubois, D. C., Sukumaran, S., Jusko, W. J., and Androulakis, I. P. (2014). Tissue-specific gene expression and regulation in liver and muscle following chronic corticosteroid administration. *Gene Regul. Syst. Bio.* 8, 75–87. doi:10.4137/GRSB.S13134.
Niculescu, M. D., and Zeisel, S. H. (2002). Diet, Methyl Donors and DNA Methylation: Interactions between Dietary Folate, Methionine and Choline. *J. Nutr.* 132, 2333S-2335S. doi:10.1093/jn/132.8.2333S.
Nolan, C. M., Killian, J. K., Petitte, J. N., and Jirtle, R. L. (2001). Imprint status of M6P/IGF2R and IGF2 in chickens. *Dev. Genes Evol.* 211, 179–183. doi:10.1007/s004270000132.
Obeid, R. (2013). The metabolic burden of methyl donor deficiency with focus on the betaine homocysteine methyltransferase pathway. *Nutrients* 5, 3481–95. doi:10.3390/nu5093481.
Ohno, S., Kaplan, W. D., and Kinosita, R. (1959). Formation of the sex chromatin by a single X-chromosome in liver cells of Rattus norvegicus. *Exp. Cell Res.* 18, 415–418. doi:10.1016/0014-4827(59)90031-X.
Okano, M., Xie, S., and Li, E. (1998). Dnmt2 is not required for de novo and maintenance methylation of viral DNA in embryonic stem cells. *Nucleic Acids Res.* 26, 2536–2540. doi:10.1093/nar/26.11.2536.
Omer, N. A., Hu, Y., Hu, Y., Idriss, A. A., Abobaker, H., Hou, Z., et al. (2018). Dietary betaine activates hepatic VTGII expression in laying hens associated with hypomethylation of GR

gene promoter and enhanced GR expression. *J. Anim. Sci. Biotechnol.* 9, 2. doi:10.1186/s40104-017-0218-9.
Papah, M. B., Brannick, E. M., Schmidt, C. J., and Abasht, B. (2017). Evidence and role of phlebitis and lipid infiltration in the onset and pathogenesis of Wooden Breast Disease in modern broiler chickens. *Avian Pathol.* 46, 623–643. doi:10.1080/03079457.2017.1339346.
Papah, M. B., Brannick, E. M., Schmidt, C. J., and Abasht, B. (2018). Gene expression profiling of the early pathogenesis of wooden breast disease in commercial broiler chickens using RNA-sequencing. *PLoS One* 13, e0207346. doi:10.1371/journal.pone.0207346.
Pejznochova, M., Tesarova, M., Hansikova, H., Magner, M., Honzik, T., Vinsova, K., et al. (2010). Mitochondrial DNA content and expression of genes involved in mtDNA transcription, regulation and maintenance during human fetal development. *Mitochondrion* 10, 321–329. doi:10.1016/j.mito.2010.01.006.
Pelizzola, M., and Ecker, J. R. (2011). The DNA methylome. *FEBS Lett.* 585, 1994–2000. doi:10.1016/j.febslet.2010.10.061.
Pesti, G. M. (1991). Response Surface Approach to Studying the Protein and Energy Requirements of Laying Hens. *Poult. Sci.* 70, 103–114. doi:10.3382/ps.0700103.
Pesti, G. M. (1995). Nutrient requirements of poultry. *Anim. Feed Sci. Technol.* 56, 177–178. doi:10.1016/0377-8401(95)90024-1.
Petersen, K. F., Dufour, S., Befroy, D., Garcia, R., and Shulman, G. (2004). Impaired Mitochondrial Activity in the Insulin-Resistant Offspring of Patients with Type 2 Diabetes. *N Engl J Med* 350, 664–671. doi:10.1056/NEJMoa031314.
Petracci, M., Mudalal, S., Soglia, F., and Cavani, C. (2015). Meat quality in fast-growing broiler chickens. *Worlds. Poult. Sci. J.* 71, 363–374. doi:10.1017/S0043933915000367.
Petracci, M., Soglia, F., Madruga, M., Carvalho, L., Ida, E., and Estévez, M. (2019). Wooden-Breast, White Striping, and Spaghetti Meat: Causes, Consequences and Consumer Perception of Emerging Broiler Meat Abnormalities. *Compr. Rev. Food Sci. Food Saf.* 18, 565–583. doi:10.1111/1541-4337.12431.
Pinto, J. T., and Zempleni, J. (2016). Riboflavin. *Adv. Nutr.* 7, 973–5. doi:10.3945/an.116.012716.
Poole, C. J., and van Riggelen, J. (2017). MYC—Master Regulator of the Cancer Epigenome and Transcriptome. *Genes (Basel).* 8, 142. doi:10.3390/genes8050142.
Powers, H. J. (2003). Riboflavin (vitamin B-2) and health. *Am. J. Clin. Nutr.* 77, 1352–1360. doi:10.1093/ajcn/77.6.1352.
Pufulete, M., Al-Ghnaniem, R., Khushal, A., Appleby, P., Harris, N., Gout, S., et al. (2005). Effect of folic acid supplementation on genomic DNA methylation in patients with colorectal adenoma. *Gut* 54, 648–653. doi:10.1136/gut.2004.054718.
Radford, E. J., Isganaitis, E., Jimenez-Chillaron, J., Schroeder, J., Molla, M., Andrews, S., et al. (2012). An Unbiased Assessment of the Role of Imprinted Genes in an Intergenerational Model of Developmental Programming. *PLoS Genet.* 8, e1002605. doi:10.1371/journal.pgen.1002605.
Radford, E. J., Ito, M., Shi, H., Corish, J. A., Yamazawa, K., Isganaitis, E., et al. (2014). In utero undernourishment perturbs the adult sperm methylome and intergenerational metabolism. *Science (80-.).* 345, 1255903–1255903. doi:10.1126/science.1255903.
Raices, M., Bukata, L., Sakuma, S., Borlido, J., Hernandez, L. S., Hart, D. O., et al. (2017). Nuclear Pores Regulate Muscle Development and Maintenance by Assembling a Localized Mef2C Complex. *Dev. Cell* 41, 540-554.e7. doi:10.1016/j.devcel.2017.05.007.

Rampersaud, G., Kauwell, G., Hutson, A., Cerda, J., and Bailey, L. (2000). Genomic DNA methylation decreases in response to moderate folate depletion in elderly women. *Am. J. Clin. Nutr.* 72, 998-1003 6p. Available at: http://search.ebscohost.com/login.aspx?direct=true&db=rzh&AN=106992235&site=ehost-live.

Rando, O. J. (2012). Daddy issues: Paternal effects on phenotype. *Cell* 151, 702–708. doi:10.1016/j.cell.2012.10.020.

Rando, O. J., and Simmons, R. A. (2015). I'm eating for two: Parental dietary effects on offspring metabolism. *Cell* 161, 93–105. doi:10.1016/j.cell.2015.02.021.

Ravelli, A. C., van der Meulen, J. H., Michels, R. P., Osmond, C., Barker, D. J., Hales, C. N., et al. (1998). Glucose tolerance in adults after prenatal exposure to famine. *Lancet (London, England)* 351, 173–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9449872.

Ravelli, G.-P., Stein, Z. A., and Susser, M. W. (1976). Obesity in Young Men after Famine Exposure in Utero and Early Infancy. *N. Engl. J. Med.* 295, 349–353. doi:10.1056/NEJM197608122950701.

Razin, A., and Riggs, A. (1980). DNA Methylation and Gene Function. *Science (80-.).* 210, 604–610. Available at: https://www.jstor.org/stable/1685145.

Recoquillay, J., Pitel, F., Arnould, C., Leroux, S., Dehais, P., Moréno, C., et al. (2015). A medium density genetic map and QTL for behavioral and production traits in japanese quail. *BMC Genomics* 16. doi:10.1186/s12864-014-1210-9.

Regina, M., Korhonen, V. P., Smith, T. K., Alakuijala, L., and Eloranta, T. O. (1993). Methionine Toxicity in the Rat in Relation to Hepatic Accumulation of S-Adenosylmethionine: Prevention by Dietary Stimulation of the Hepatic Transsulfuration Pathway. *Arch. Biochem. Biophys.* 300, 598–607. doi:10.1006/abbi.1993.1083.

Reik, W., Constância, M., Fowden, A., Anderson, N., Dean, W., Ferguson-Smith, A., et al. (2003). Regulation of supply and demand for maternal nutrients in mammals by imprinted genes. *J. Physiol.* 547, 35–44. doi:10.1113/jphysiol.2002.033274.

Reik, W., and Walter, J. (2001). Genomic imprinting: Parental influence on the genome. *Nat. Rev. Genet.* 2, 21–32. doi:10.1038/35047554.

Rios, K. E., Liem, A., Kashyap, A., Maynard, S., Paul, S., and Schaefer, B. C. (2018). CARD19: a mitochondrial caspase recruitment domain protein with a role in regulating pro-inflammatory innate immune responses. *J. Immunol.* 200, 169.18 LP-169.18. Available at: http://www.jimmunol.org/content/200/1_Supplement/169.18.abstract.

Robel, E. J. (1993). Evaluation of Egg Injection of Folic Acid and Effect of Supplemental Folic Acid on Hatchability and Poult Weight. *Poult. Sci.* 72, 546–553. doi:10.3382/ps.0720546.

Rosenthal, S. M., Brunetti, A., Brown, E. J., Mamula, P. W., and Goldfine, I. D. (1991). Regulation of insulin-like growth factor (IGF) I receptor expression during muscle cell differentiation. Potential autocrine role of IGF-II. *J. Clin. Invest.* 87, 1212–1219. doi:10.1172/JCI115121.

Rossant, J., Sanford, J. P., Chapman, V. M., and Andrews, G. K. (1986). Undermethylation of structural gene sequences in extraembryonic lineages of the mouse. *Dev. Biol.* 117, 567–573. doi:10.1016/0012-1606(86)90325-8.

Rowling, M. J., McMullen, M. H., Chipman, D. C., and Schalinske, K. L. (2002). Hepatic Glycine N-Methyltransferase Is Up-Regulated by Excess Dietary Methionine in Rats. *J. Nutr.* 132, 2545–2550. doi:10.1093/jn/132.9.2545.

Ryall, J. G., Schertzer, J. D., and Lynch, G. S. (2008). Cellular and molecular mechanisms

underlying age-related skeletal muscle wasting and weakness. *Biogerontology* 9, 213–228. doi:10.1007/s10522-008-9131-0.
Sacco, A., Mourkioti, F., Tran, R., Choi, J., Llewellyn, M., Kraft, P., et al. (2010). Short telomeres and stem cell exhaustion model duchenne muscular dystrophy in mdx/mTR mice. *Cell* 143, 1059–1071. doi:10.1016/j.cell.2010.11.039.
Salvante, K. G., Lin, G., Walzem, R. L., and Williams, T. D. (2007). Characterization of very-low density lipoprotein particle diameter dynamics in relation to egg production in a passerine bird. *J. Exp. Biol.* 210, 1064–1074. doi:10.1242/jeb.02724.
Sasazaki, S., Hinenoya, T., Lin, B., Fujiwara, A., and Mannen, H. (2006). A comparative map of macrochromosomes between chicken and Japanese quail based on orthologous genes. *Anim. Genet.* 37, 316–320. doi:10.1111/j.1365-2052.2006.01454.x.
Schilling, J., Nepomuceno, A. I., Planchart, A., Yoder, J. A., Kelly, R. M., Muddiman, D. C., et al. (2015). Machine learning reveals sex-specific 17β-estradiol-responsive expression patterns in white perch (Morone americana) plasma proteins. *Proteomics* 15, 2678–2690. doi:10.1002/pmic.201400606.
Schmid, M., Nanda, I., Guttenbach, M., Steinlein, C., Hoehn, M., Schartl, M., et al. (2000). First report on chicken genes and chromosomes 2000. *Cytogenet. Genome Res.* 90, 169–218. doi:10.1159/000056772.
Schulz, L. C. (2010). The Dutch Hunger Winter and the developmental origins of health and disease. *Proc. Natl. Acad. Sci.* 107, 16757–16758. doi:10.1073/pnas.1012911107.
Scott, L. M., Nesheim, M. C., and Young, R. . (1982). *Nutrition of the Chicken*.
Sekimoto, K., Imai, K., Kato, Y., and Takikawa, H. (1990). Acute decrease in vitellogenin synthesis by deprivation of food and water in laying hens. *Endocrinol. Jpn.* 37, 319–330. doi:10.1507/endocrj1954.37.319.
Selhub, J. (1999). Homocysteine metabolism. 19, 217–46. Available at: https://search-proquest-com.prox.lib.ncsu.edu/docview/204148246/fulltext/2BAA16832A2D4A64PQ/1?accountid=12725.
Sell, J. L., Angel, C. R., and Escribano, F. (1987). Influence of supplemental fat on weights of eggs and yolks during early egg production. *Poult. Sci.* 66, 1807–1812. doi:10.3382/ps.0661807.
Sewalem, A., Morrice, D. M., Law, A., Windsor, D., Haley, C. S., Ikeobi, C. O. N., et al. (2002). Mapping of quantitative trait loci for body weight at three, six, and nine weeks of age in a broiler layer cross. *Poult. Sci.* 81, 1775–1781. doi:10.1093/ps/81.12.1775.
Seyoum, E., and Selhub, J. (1998). Properties of Food Folates Determined by Stability and Susceptibility to Intestinal Pteroylpolyglutamate Hydrolase Action. *J. Nutr.* 128, 1956–1960. doi:10.1093/jn/128.11.1956.
Shane, B. (2008). Folate and vitamin B12, metabolism: Overview and interaction with riboflavin, vitamin B6, and polymorphisms. in *Food and Nutrition Bulletin* doi:10.1177/15648265080292s103.
Shibusawa, M., Minai, S., Nishida-Umehara, C., Suzuki, T., Mano, T., Yamada, K., et al. (2001). A comparative cytogenetic study of chromosome homology between chicken and Japanese quail. *Cytogenet. Genome Res.* 95, 103–109. doi:10.1159/000057026.
Sihvo, H. K., Immonen, K., and Puolanne, E. (2014). Myodegeneration With Fibrosis and Regeneration in the Pectoralis Major Muscle of Broilers. *Vet. Pathol.* 51, 619–623. doi:10.1177/0300985813497488.
Speake, B. K., Murray, A. M. B., and C. Noble, R. (1998). Transport and transformations of yolk

lipids during development of the avian embryo. *Prog. Lipid Res.* 37, 1–32. doi:10.1016/S0163-7827(97)00012-X.
Squires, M. W., and Naber, E. C. (1993). Vitamin profiles of eggs as indicators of nutritional status in the laying hen: riboflavin study. *Poult. Sci.* 72, 483–494. doi:10.3382/ps.0720483.
Staal, F. J. T. (2016). Wnt signalling meets epigenetics. *Stem Cell Investig.* 3, 38–38. doi:10.21037/sci.2016.08.01.
Stine, Z. E., Walton, Z. E., Altman, B. J., Hsieh, A. L., and Dang, C. V (2015). MYC, Metabolism, and Cancer MYC function. *Cancer Discov.* 5, 1024–39. doi:10.1158/2159-8290.CD-15-0507.
Sun, Y.-C., Wang, Y.-Y., Ge, W., Cheng, S.-F., Dyce, P. W., and Shen, W. (2017). Epigenetic regulation during the differentiation of stem cells to germ cells. *Oncotarget* 8, 57836–57844. doi:10.18632/oncotarget.18444.
Tactacan, G. B., Jing, M., Thiessen, S., Rodriguez-Lecompte, J. C., O'Connor, D. L., Guenter, W., et al. (2010). Characterization of folate-dependent enzymes and indices of folate status in laying hens supplemented with folic acid or 5-methyltetrahydrofolate. *Poult. Sci.* 89, 688–696. doi:10.3382/ps.2009-00417.
Takahashi, K., Yamanaka, S., Zhang, Y., Li, Y., Feng, C., Li, X., et al. (2006). Induction of Pluripotent Stem Cells from Mouse Embryonic and Adult Fibroblast Cultures by Defined Factors. *Cell* 126, 663–676. doi:10.1016/j.cell.2006.07.024.
Tian, X., and Diaz, F. J. (2013). Acute dietary zinc deficiency before conception compromises oocyte epigenetic programming and disrupts embryonic development. *Dev. Biol.* 376, 51–61. doi:10.1016/j.ydbio.2013.01.015.
Trocino, A., Piccirillo, A., Birolo, M., Radaelli, G., Bertotto, D., Filiou, E., et al. (2015). Effect of genotype, gender and feed restriction on growth, meat quality and the occurrence of white striping and wooden breast in broiler chickens. *Poult. Sci.* 94, 2996–3004. doi:10.3382/ps/pev296.
Tuiskula-Haavisto, M., De Koning, D. J., Honkatukia, M., Schulman, N. F., M??ki-Tanila, A., and Vilkki, J. (2004). Quantitative trait loci with parent-of-origin effects in chicken. *Genet. Res.* 84, 57–66. doi:10.1017/S0016672304006950.
Tuiskula-Haavisto, M., Honkatukia, M., Vilkki, J., De Koning, D. J., Schulman, N. F., and Mäki-Tanila, A. (2002). Mapping of quantitative trait loci affecting quality and production traits in egg layers. *Poult. Sci.* 81, 919–927. doi:10.1093/ps/81.7.919.
Tycko, B., and Morison, I. M. (2002). Physiological functions of imprinted genes. *J. Cell. Physiol.* 192, 245–258. doi:10.1002/jcp.10129.
Ueland, P. M., Ulvik, A., Rios-Avila, L., Midttun, Ø., and Gregory, J. F. (2015). Direct and Functional Biomarkers of Vitamin B6 Status. *Annu. Rev. Nutr.* 35, 33–70. doi:10.1146/annurev-nutr-071714-034330.
Uriu-Adams, J. Y., and Keen, C. L. (2010). Zinc and reproduction: Effects of zinc deficiency on prenatal and early postnatal development. *Birth Defects Res. Part B - Dev. Reprod. Toxicol.* 89, 313–325. doi:10.1002/bdrb.20264.
Valdez, R., Athens, M. A., Thompson, G. H., Bradshaw, B. S., and Stern, M. P. (1994). Birthweight and adult health outcomes in a biethnic population in the USA. *Diabetologia* 37, 624–31. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7926349.
Vallee, B. L., and Falchuk, K. H. (1993). The biochemical basis of zinc physiology. *Physiol. Rev.* 73, 79–118. doi:10.1152/physrev.1993.73.1.79.
Van Kaam, J. B. C. H. M., Van Arendonk, J. A. M., Groenen, M. A. M., Bovenhuis, H.,

Vereijken, A. L. J., Crooijmans, R. P. M. A., et al. (1998). Whole genome scan for quantitative trait loci affecting body weight in chickens using a three generation design. Available at: https://ac-els-cdn-com.prox.lib.ncsu.edu/S0301622697001711/1-s2.0-S0301622697001711-main.pdf?_tid=cc94f1b1-8d35-4049-8f54-5d32a6130c36&acdnat=1552607168_1ec48c24a6856031c60f7752d913be06 [Accessed March 14, 2019].

Vasicek, T. J., Zeng, L., Guan, X. J., Zhang, T., Costantini, F., and Tilghman, S. M. (1997). Two dominant mutations in the mouse Fused gene are the result of transposon insertions. *Genetics* 147, 777–786.

Velleman, S. G., and Clark, D. L. (2015). Histopathologic and Myogenic Gene Expression Changes Associated with Wooden Breast in Broiler Breast Muscles. *Avian Dis.* 59, 410–418. doi:10.1637/11097-042015-reg.1.

Vuorinen, E. M., Rajala, N. K., Ihalainen, T. O., and Kallioniemi, A. (2018). Depletion of nuclear import protein karyopherin alpha 7 (KPNA7) induces mitotic defects and deformation of nuclei in cancer cells. *BMC Cancer* 18, 325. doi:10.1186/s12885-018-4261-5.

Waddington, C. H. (1942). Canalization of Development and teh Inheritance of Aquired Characteristics. *Nature* 150, 336. doi:10.1038/153621a0.

Waddington, C. H. (1957). *The Strategy Of The Genes*.

Wakayama, T., Perry, A. C. F., Zuccotti, M., Johnson, K. R., and Yanagimachi, R. (1998). Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* 394, 369–374. doi:10.1038/28615.

Walzem, R. L., Hansen, R. J., Williams, D. L., and Hamilton, R. L. (1999). Estrogen Induction of VLDLy Assembly in Egg-Laying Hens. *J. Nutr.* 129, 467S-472S. doi:10.1093/jn/129.2.467s.

Wang, L., Chen, L., Tan, Y., Wei, J., Chang, Y., Jin, T., et al. (2013). Betaine supplement alleviates hepatic triglyceride accumulation of apolipoprotein e deficient mice via reducing methylation of peroxisomal proliferator-activated receptor alpha promoter. *Lipids Health Dis.* 12, 34. doi:10.1186/1476-511X-12-34.

Wang, S. Y., Smith, D. E., and Williams, D. L. (1983). Purification of Avian Vitellogenin III: Comparison with Vitellogenins I and II. *Biochemistry* 22, 6206–6212. doi:10.1021/bi00295a026.

Warren, W. C., Hillier, L. D. W., Tomlinson, C., Minx, P., Kremitzki, M., Graves, T., et al. (2017). A new chicken genome assembly provides insight into avian genome structure. *G3 Genes, Genomes, Genet.* 7, 109–117. doi:10.1534/g3.116.035923.

Waterland, R. A. (2006). Assessing the Effects of High Methionine Intake on DNA Methylation. *J. Nutr.* 136, 16365–16405. doi:10.1093/jn/136.6.1636s.

Waterland, R. A., Dolinoy, D. C., Lin, J.-R., Smith, C. A., Shi, X., and Tahiliani, K. G. (2006a). Maternal methyl supplements increase offspring DNA methylation at Axin Fused. *Genesis* 44, 401–6. doi:10.1002/dvg.20230.

Waterland, R. A., and Jirtle, R. L. (2003). Transposable elements: targets for early nutritional effects on epigenetic gene regulation. *Mol. Cell. Biol.* 23, 5293–300. doi:10.1128/MCB.23.15.5293.

Waterland, R. A., Kellermayer, R., Laritsky, E., Rayco-Solon, P., Harris, R. A., Travisano, M., et al. (2010). Season of conception in rural gambia affects DNA methylation at putative human metastable epialleles. *PLoS Genet.* 6, 1–10. doi:10.1371/journal.pgen.1001252.

Waterland, R. A., Lin, J. R., Smith, C. A., and Jirtle, R. L. (2006b). Post-weaning diet affects genomic imprinting at the insulin-like growth factor 2 (Igf2) locus. *Hum. Mol. Genet.* 15, 705–716. doi:10.1093/hmg/ddi484.

Waterland, R. A., Travisano, M., Tahiliani, K. G., Rached, M. T., and Mirza, S. (2008). Methyl donor supplementation prevents transgenerational amplification of obesity. *Int. J. Obes.* 32, 1373–1379. doi:10.1038/ijo.2008.100.

Watkins, A. J., and Sinclair, K. D. (2014). Paternal low protein diet affects adult offspring cardiovascular and metabolic function in mice. *Am. J. Physiol. - Hear. Circ. Physiol.* 306, H1444–H1452. doi:10.1152/ajpheart.00981.2013.

Weber, M., Hellmann, I., Stadler, M. B., Ramos, L., Pääbo, S., Rebhan, M., et al. (2007). Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. *Nat. Genet.* 39, 457–466. doi:10.1038/ng1990.

Whitehead, C. C. (1981). The response of egg weight to the inclusion of different amounts of vegetable oil and linoleic acid in the diet of laying hens. *Br. Poult. Sci.* 22, 525–532. doi:10.1080/00071688108447920.

WHO, and FAO (2001). "Folate and folic acid," in *Human Vitamins and Mineral Requirements* Available at: http://www.fao.org/3/y2809e/y2809e00.htm#Contents [Accessed April 3, 2019].

Wicker, T., Robertson, J. S., Schulze, S. R., Feltus, F. A., Magrini, V., Morrison, J. A., et al. (2005). The repetitive landscape of the chicken genome. *Genome Res.* 15, 126–136. doi:10.1101/gr.2438005.

Wilks, A. F., Cozens, P. J., Mattaj, I. W., and Jost, J. P. (1982). Estrogen induces a demethylation at the 5' end region of the chicken vitellogenin gene. *Proc. Natl. Acad. Sci. U. S. A.* 79, 4252–4255. doi:10.1073/pnas.79.14.4252.

Williams, T. D. (2019). The Hormonal and Physiological Control of Egg Production. *Physiol. Adapt. Breed. Birds*, 8–51. doi:10.1515/9781400842797.8.

Wilson, H. R. (1997). Effects of maternal nutrition on hatchability. *Poult. Sci.* 76, 134–143. doi:10.1093/ps/76.1.134.

Wolf, S. F., Jolly, D. J., Lunnen, K. D., Friedmann, T., and Migeon, B. R. (1984). Methylation of the hypoxanthine phosphoribosyltransferase locus on the human X chromosome: implications for X-chromosome inactivation. *Proc. Natl. Acad. Sci.* 81, 2806–2810. doi:10.1073/PNAS.81.9.2806.

Wolff, G. L., Kodell, R. L., Moore, S. R., and Cooney, C. A. (1998). Maternal epigenetics and methyl supplements affect *agouti* gene expression in *A* ^{vy} / *a* mice. *FASEB J.* 12, 949–957. doi:10.1096/fasebj.12.11.949.

Wolffe, A. P., and Hayes, J. J. (1999). Chromatin disruption and modification. *Nucleic Acids Res.* 27, 711–720. doi:10.1093/nar/27.3.711.

Wu, H., and Zhang, Y. (2011). Mechanisms and functions of Tet proteinmediated 5-methylcytosine oxidation. *Genes Dev.* 25, 2436–2452. doi:10.1101/gad.179184.111.

Wu, S., Guo, W., Li, X., Liu, Y., Li, Y., Lei, X., et al. (2019). Paternal chronic folate supplementation induced the transgenerational inheritance of acquired developmental and metabolic changes in chickens. *Proc. R. Soc. B Biol. Sci.* 286, 20191653. doi:10.1098/rspb.2019.1653.

Wu, Z., Huang, K., Yu, J., Le, T., Namihira, M., Liu, Y., et al. (2012). Dnmt3a regulates both proliferation and differentiation of mouse neural stem cells. *J. Neurosci. Res.* 90, 1883–91. doi:10.1002/jnr.23077.

Wynn, T. (2008). Cellular and molecular mechanisms of fibrosis. *J. Pathol.* 214, 199–210. doi:10.1002/path.2277.
Xing, J., Jing, W., Zhang, Y., Liu, L., Xu, J., and Chen, X. (2018). Identification of differentially expressed genes in broiler offspring under maternal folate deficiency. *Physiol. Genomics* 50, 1015–1025. doi:10.1152/physiolgenomics.00086.2018.
Xing, J., Kang, L., and Jiang, Y. (2011). Effect of dietary betaine supplementation on lipogenesis gene expression and CpG methylation of lipoprotein lipase gene in broilers. *Mol. Biol. Rep.* 38, 1975–1981. doi:10.1007/s11033-010-0319-4.
Xu, P., Denbow, C. J., Meiri, N., and Denbow, D. M. (2012). Fasting of 3-day-old chicks leads to changes in histone H3 methylation status. *Physiol. Behav.* 105, 276–282. doi:10.1016/j.physbeh.2011.06.023.
Xu, Q., Zhang, Y., Sun, D., Wang, Y., and Yu, Y. (2007). Analysis on DNA methylation of various tissues in chicken. *Anim. Biotechnol.* 18, 231–241. doi:10.1080/10495390701574838.
Yan, F., Angel, R., Ashwell, C., Mitchell, A., and Christman, M. (2005). Evaluation of the broiler's ability to adapt to an early moderate deficiency of phosphorus and calcium. *Poult. Sci.* 84, 1232–1241. doi:10.1093/ps/84.8.1232.
Yasuoka, H., Yamaguchi, Y., and Feghali-Bostwick, C. A. (2014). The membrane-associated adaptor protein DOK5 is upregulated in systemic sclerosis and associated with IGFBP-5-induced fibrosis. *PLoS One* 9, e87754. doi:10.1371/journal.pone.0087754.
Yen, T. T., Gill, A. M., Frigeri, L. G., Barsh, G. S., and Wolff, G. L. (1994). Obesity, diabetes, and neoplasia in yellow A(vy)/- mice: ectopic expression of the agouti gene. *FASEB J.* 8, 479–488. doi:10.1096/fasebj.8.8.8181666.
Yin, L. J., Zhang, Y., Lv, P. P., He, W. H., Wu, Y. T., Liu, A. X., et al. (2012). Insufficient maintenance DNA methylation is associated with abnormal embryonic development. *BMC Med.* 10, 1–10. doi:10.1186/1741-7015-10-26.
Yokomine, T., Shirohzu, H., Purbowasito, W., Toyoda, A., Iwama, H., Ikeo, K., et al. (2005). Structural and functional analysis of a 0.5-Mb chicken region orthologous to the imprinted mammalian Ascl2/Mash2-Igf2-H19 region. *Genome Res.* 15, 154–165. doi:10.1101/gr.2609605.
Yokoyama, S., and Asahara, H. (2011). The myogenic transcriptional network. *Cell. Mol. Life Sci.* 68, 1843–1849. doi:10.1007/s00018-011-0629-2.
Yossifoff, M., Kisliouk, T., and Meiri, N. (2008). Dynamic changes in DNA methylation during thermal control establishment affect CREB binding to the brain-derived neurotrophic factor promoter. *Eur. J. Neurosci.* 28, 2267–2277. doi:10.1111/j.1460-9568.2008.06532.x.
Yu, X., Liu, R., Zhao, G., Zheng, M., Chen, J., and Wen, J. (2014). Folate supplementation modifies CCAAT/enhancer-binding protein α methylation to mediate differentiation of preadipocytes in chickens. *Poult. Sci.* 93, 2596–2603. doi:10.3382/ps.2014-04027.
Zaefarian, F., Abdollahi, M. R., Cowieson, A., and Ravindran, V. (2019). Avian liver: The forgotten organ. *Animals* 9. doi:10.3390/ani9020063.
Zambonelli, P., Zappaterra, M., Soglia, F., Petracci, M., Sirri, F., Cavani, C., et al. (2016a). Detection of differentially expressed genes in broiler pectoralis major muscle affected by White Striping - Wooden Breast myopathies. *Poult. Sci.* 95, 2771–2785. doi:10.3382/ps/pew268.
Zambonelli, P., Zappaterra, M., Soglia, F., Petracci, M., Sirri, F., Cavani, C., et al. (2016b). Detection of differentially expressed genes in broiler pectoralis major muscle affected by

White Striping – Wooden Breast myopathies. *Poult. Sci.* 95, 2771–2785. doi:10.3382/ps/pew268.
Zambrano, E., Martínez-Samayoa, P. M., Bautista, C. J., Deás, M., Guillén, L., Rodríguez-González, G. L., et al. (2005). Sex differences in transgenerational alterations of growth and metabolism in progeny (F_2) of female offspring (F_1) of rats fed a low protein diet during pregnancy and lactation. *J. Physiol.* 566, 225–236. doi:10.1113/jphysiol.2005.086462.
Zhang, Y., and Kutateladze, T. G. (2018). Diet and the epigenome. *Nat. Commun.* 9, 1–4. doi:10.1038/s41467-018-05778-1.
Zhang, Y., and Reinberg, D. (2001). Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails. *Genes Dev.* 15, 2343–60. doi:10.1101/gad.927301.
Zhu, Y., Liao, X., Lu, L., Li, W., Zhang, L., Ji, C., et al. (2017). Maternal dietary zinc supplementation enhances the epigenetic-activated antioxidant ability of chick embryos from maternal normal and high temperatures. *Oncotarget* 8, 19814–19824. doi:10.18632/oncotarget.15057.
Zimowska, M., Duchesnay, A., Dragun, P., Oberbek, A., Moraczewski, J., and Martelly, I. (2009). Immunoneutralization of TGF β1 Improves Skeletal Muscle Regeneration: Effects on Myoblast Differentiation and Glycosaminoglycan Content. *Int. J. Cell Biol.* 2009, 1–16. doi:10.1155/2009/659372.
Zimowska, M., Kasprzycka, P., Bocian, K., Delaney, K., Jung, P., Kuchcinska, K., et al. (2017). Inflammatory response during slow- and fast-twitch muscle regeneration. *Muscle and Nerve* 55, 400–409. doi:10.1002/mus.25246.