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GLOBAL GENE EXPRESSION AND DNA METHYLATION OF THE HYPOTHALAMUS IN FEEDER CALVES FED A HIGH CONCENTRATE DIET UPON ENTERING FEEDLOT PHASE

BY

JASON E. GRIFFIN

A thesis submitted in partial fulfillment of the requirements for the

Master of Science

Major in Animal Science

South Dakota State University

2020

THESIS ACCEPTANCE PAGE

Jason Griffin

This thesis is approved as a creditable and independent investigation by a candidate for the master's degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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ACKNOWLEDGEMENTS

The biggest thanks I can give go to my family. Kinsey, my partner in this crazy life has put up with as well as picked up so much. She has put up with my late night, early morning squeaky desk chair, my no sleep induced mood swings as well as picking up double parenting duties many nights running 6 kids where they need to go and making sure they were healthy and happy. You are my rock. My kids, Hunter, Ella, Brianna, Reagan, Colter, and Isaac have put up with an invisible father so many nights all while being so supportive. I can't count the number of times they have had to hear me say "when I get done with my thesis". Kids, I am done! The best parents and siblings a person can have, Darrell, Norma, Curtis, Michelle, Scott, Monica, and Melinda. You have always been there for me whether it was playing cards, offering support, or helping with the kids. You are all people to look up to and I strive to make you proud every day. I am a proud product of my upbringing and that, is all thanks to you.   My family in-laws, you have all been so supportive. You have got my wife through tough times and been there for me and my children. You are not just my in-laws you are my family and I thank you all and know those that have left us too early are smiling down on me today.

Huge thank you to my SDSU family – the graduate students who have moved on and are now no longer graduate students that helped collect samples, delivery cattle, and just came down and had a conversation with me in the laboratory. There are too many to name and I will forget a dozen people if I name them. Retired or past staff that I have had the privilege to work with; Denny, Brenda, Carol, Stephanie, and of course the late Deon have all help me in many ways. Current staff; Cheryl, Teresa, Judy, Bev, Veronica, Adam and Paul you have all became good friends and are among the best of SDSU.

To the most patient advisor one could ask for, Dr. Michael Gonda you have been patient and encouraging. It's been a long time completing my thesis and I know things didn't happen when you wanted them to however you remained supportive through it all. I felt like I avoided you for months at a time because I knew I wasn't doing my part. However, when it came time to do your part you treated me like the prodigal son and helped me get to the finish line.

Dr. Keith Underwood you and Dr. Gonda took a chance on me and started me on this path. You have been there for me with answers to all my question and help me collect carcass data and perform all the analysis.

Dr. Zach Smith, your guidance and help through my thesis has been one of the reasons I have succeeded. Thank you for serving on my committee and helping with all my ruminant nutrition questions. Dr. Jeff Clapper, you have helped me throughout my thesis as well and became a friend who is always good for a conversation. Your help with everything hypothalamus and hormones has been invaluable. Dr. Ethan Blom, Dr. Wes Gentry, Wyatt Smith, and Grady Ruble, our conversations as well as all the grief we gave and still give to each other have kept me going. All the great conversations, arguments, nicknames, and your love of what you do has help push me to the end. No Charge says thanks!

Dr. Rachel McDaniel, thank you for serving on my committee and being patience through this long process. Dr. Joe Cassady and Dr. Robbi Pritchard you two have pushed me to finish my thesis up when no one else would. You have both given me great guidance and help set me on a path for success.   Dr. Pritchard you got me here and

encouraged me to pursue my master's degree and allowed me to use your animals and data for my research.

Finally, I would like to thank the South Dakota Beef Industry Council for taking a chance on a laboratory assistant and funding this study.

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ABBREVIATIONS

5-hmC	5-hydroxymethylcytosine modified nucleotide
5-mC	5-methylcytosine modified nucleotide
ADSW	Assay design software
AgRP	Agouti related peptide
ANOVA	Analysis of variance
ARC	Arcuate Nucleus
BHBA	β-hydroxybutyric acid
B/F	Background/finishing
BPA	Bisphenol A
CGI	CpG island
CNS	Central Nervous System
COX7C	Cytochrome C oxidase subunit VIIc
CpG	Cytosine-guanine dinucleotide
CRH	Corticotropin-releasing hormone
DDT	Dichlorodiphenyltrichloroethane
DHA	Docosahexaenoic acid
DFM	Direct-fed microbials
DMC	Differentially methylated cytosine
DNA	Deoxyribonucleic acid
DNMTs	DNA methyltransferases
EPA	Eicosapentaenoic acid
ERK	extracellular signal-regulated kinase
FDR	False discovery rate
FPKM	Fragments per kilobase of transcript per million mapped reads

FT	Fat thickness
GB	Gene body
GLM	General linear model
GHR	Growth hormone receptor
GnRH	Gonadotropin releasing hormone
H2A	Histone 2a
H2B	Histone 2b
H3	Histone 3
H4	Histone 4
HBB	Hemoglobin β
HCW	Hot carcass weight
HDAC	Histone deacetylase
HDACi	Histone deacetylase inhibitor
HG	High Gain
HMGA2	high mobility group AT-hook 2
IFIT3	Interferon-induced protein w/tetratricopeptide repeats 3
IGF2	Insulin growth factor 2
IL-1	Interleukin-1
IR	Intergenic regions
KPH	Kidney, pelvic and heart fat
LG	Low gain
LINE	Long interspersed nuclear elements
LMA	Longissimus muscle area
LTR	Long terminal repeats
MAP	Mitogen-activated protein

MEm	Maintenance energy
mRNA	messenger RNA
MeCP2	Methyl CpG binding protein 2
MX2	MX dynamin-like GTPase 2
NEg	Net energy for gain
NPY	Neuropeptide Y
PMCH	Pro-melanin concentrating hormones
POMC	proopiomelanocortin
PRSS3	Protease, Serine 3
PVN	Paraventricular nucleus
RFI	Residual feed intake
RNA	Ribonucleic acid
RSAD2	Radical S-adenosyl methionine domain containing 2
SAH	S-adenosyl homocysteine
SAM	S-adenosyl-L-methionine
SCFA	Short chain fatty acid
SINE	Short interspersed nuclear elements
TCA	Tricarboxylic acid
TET1	Ten-eleven translocation 1
TRH	Thyrotropin-releasing hormone
TRY2	Trypsin-2
TSS	Transcription start site
qrt-PCR	quantitative reverse transcription polymerase chain reaction
VMH	Ventromedial hypothalamus
WBSF	Warner-Bratzler shear force

WWII	World War 2

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ABSTRACT

GLOBAL GENE EXPRESSION AND DNA METHYLATION OF THE HYPOTHALAMUS IN FEEDER CALVES FED A HIGH CONCENTRATE DIET UPON ENTERING FEEDLOT PHASE

JASON E. GRIFFIN

2020

Feedlot finishing diets contain high energy to allow for rapid growth and fat deposition. Previous studies have shown the use of high concentrate diets changed DNA methylation and expression of genes in the hypothalamus involved with regulating feed intake, metabolism, signaling, and neuronal communication. This study investigates whether feeding a high concentrate diet to calf-feds enter the feedlot could alter DNA methylation in the hypothalamus affecting expression of growth and development genes. Angus-influenced steers (n=12) were randomly allocated to 2 treatment groups: 1) High Energy/High Concentrate (HE/HC, 1.33Mcal/kg net energy gain NEg) for 202 days; or 2) traditional Backgrounding/Finishing (B/F), fed to provide 1.10Mcal/kg NEg for the first 84 days then 1.43Mcal/kg NE gain for the remaining 118 days. Growth from day 28 to 105 ($P<0.04$) was greater for the HE/HC treatment group. At slaughter, hypothalami were dissected and frozen for RNA and DNA. RNA was extracted and expression differences between treatments were measured by RNA-seq. DNA was isolated and cytosine-guanine dinucleotide (CpG) site methylation for 5 genes found to be differentially expressed was determined using targeted, next-generation bisulfite sequencing. Global 5-methylcytosine and 5-hydroxymethylcytosine was analyzed by colorimetry. Carcass characteristics, composition, and Warner-Bratzler shear force

(WBSF) were measured. Kidney, Pelvic, and Heart fat percentage was higher ($P<0.04$) in HE/HC steers; however, no difference in other carcass traits ($P>0.10$) and WBSF ($P>0.37$) were detected. There were 335 loci differentially expressed ($P<0.05$), however only 5 were annotated to genes. Two had higher expression and 3 had lower expression in the HE/HC steers. Gene Ontology terms involved with differentially expressed genes included virus immune response, cell cycle regulation, signaling pathway regulation, and ion transport pathways. CpG methylation analysis found one CpG site in two genes to have differential methylation levels and to be correlated with gene expression of those two genes. However, global 5-mC and 5-hmC methylation was not different between treatments. Feedlot calves fed a high concentrate diet upon entering the feedlot had altered gene expression in the hypothalamus. Although CpG site methylation differences were found, the difference in gene expression could have also been independent of methylation differences.

Chapter 1

LITERATURE REVIEW

Introduction

In many parts of the United States, cattle are raised on range conditions prior to being weaned and shipped to a feedlot facility. One of the most important factors affecting profitability in feedlot animals is the time entering the feedlot. This time is one of the most stressful times in the life of mammals because of several factors including a change in location, long haul transportation, separation from their mothers and a change in diet (Chester-Jones et al., 2012). The change in diet can cause complications including cattle going off feed which can result in a significant loss of body mass (Chester-Jones et al., 1994, Loerch et al., 1999). To mitigate this loss, high energy concentrates are fed in receiving diets at restricted levels to increase the available energy to the animal at this vital stage in growth.

A traditional receiving diet often consists of differing levels of fibrous materials such as hay, silage or fodder with diets containing 75% to close to 85% of these fibrous feedstuffs. The remainder is typically protein, mineral supplement and low levels of high energy concentrates. Ionophores can be added to receiving diets to prevent acidosis, coccidiosis, overeating and bloat; or fed in growing diets to increase feed efficiency and average daily gain (Erickson et al., 2003). These receiving diets do not typically contain large amounts of concentrates such as corn, oats, barley and wheat because these grains can get fermented in the rumen rapidly to propionic acid which contributes to the increased risk of acidosis. With the use of ionophores, it is possible to feed cattle a high concentrate diet, minimizing negative side effects associated with the increased production of propionic acid. The addition of ionophores has also been shown to improve

performance and carcass traits compared to controls with no other changes to the diet (Arp et al., 2014).

Epigenetic controls are alterations to the DNA that do not change DNA sequences. Methylation of DNA is one epigenetic control on transcription that can alter expression of genes. Methylation of DNA in the hypothalamus has been associated with changes in gene expression in many species, including cattle (Alves et al., 2017). One role of the hypothalamus is to receive metabolic signals from hormones that control many aspects of growth and development in the body. The hypothalamus has regions lacking a blood-brain barrier, increasing sensitivity to metabolites and allowing the release of hormones directly to the blood. Methylation of hypothalamus DNA has been implicated in changing growth and development in mice and rats (Samodien et al., 2019, Cisternas et al., 2019). However, epigenetic control of gene expression and effects of DNA methylation on growth and carcass composition of feedlot cattle have not been widely studied.

Feeding a high concentrate diet to heifers can cause early onset of puberty (Gasser et al., 2006) and affect gene expression (Allen et al., 2012). The objective of the study was to determine if feeding a high concentrate diet relative to a background/finishing diet to incoming feedlot steers would change the DNA methylation of the hypothalamus. Changes in DNA methylation can alter expression levels of genes associated with growth and development of muscle and adipose tissue, which in turn alters carcass composition.

Literature which describes nutritional effect on gene expression in cattle will be reviewed and DNA methylation will be described as well as the abundance of methylated cytosines in two forms in several species and their importance in the control of gene

expression in multiple tissues in the body and the brain with an emphasis on the hypothalamus. Literature will be reviewed which describes the effects of dietary additives and other compounds on gene expression in the hypothalamus in several animal species and at several different stages of growth. With corn being a major ingredient in feedlot diets, the effects of feeding corn on the animal will be discussed, including through dietary restrictions, methionine deficiency, and butyric acid and its ketone, β-hydroxybutyrate (BHBA).

Nutritional Effects on Gene Expression

Nutrition and environment can alter gene expression. In cattle, direct-fed microbials (DFM) changed gene expression of 11 immune related genes. One gene was down-regulated while 10 were up-regulated in whole at day 21 and day 42 compared to start of feeding trial. The 11 genes which had differential gene expression ($P \leq 0.05$) have increased immune response toward intracellular and extracellular pathogens in previous studies (Adeyemi et al., 2019). In addition, plasma metabolites were analyzed by mass spectrometry and circulation of 5-mC was found to be increased 1.2-fold ($P < 0.02$) in animal fed DFM. Higher calcium levels were also found ($P = .01$), which has been shown to improve immune responses (Adeyemi et al., 2019). This paper suggested that DFMs added to the diet can not only have an effect on gene expression and potentially improve animal health, but that the increased gene expression may be due to something other than DNA methylation levels in the blood.

While the addition of dietary ingredients, high concentrate and energy diets, and overfeeding can alter gene expression (Adeyemi et al., 2019, Allen et al., 2012, Plagemann et al., 2009), the restriction of diet can also affect gene expression. In a study on effects of diet restriction on the hypothalamus, heifers were fed a 0.4 maintenance energy (MEm) diet versus controls (1.2 MEm) for 18 days and gene expression was measured in hypothalamus tissue. A total of 15,295 genes were mapped to the bovine genome with 169 genes having differential expression levels in diet restricted heifers versus controls. Signaling pathways involved with these genes included many immune response pathways along with pathways involved in neuroprotection, cell motility and energy homeostasis. Specifically Interleukin-1 (IL-1), Trypsin-2 (TRY2) and Protease,

serine 3 (PRSS3) were differentially expressed and all related to neuroprotection (Matthews et al., 2017). Neuroprotection is connected to energy availability and is thought to protect the brain and hypothalamus. The diet restriction also caused the gonadotropin releasing hormone (GnRH) neurons to discontinue ovulation in half of the diet restricted heifers which is also associated with energy homeostasis and provides a link to neuroprotection (Matthews et al., 2017). This study provides further evidence of differential gene expression based on the increase in energy available to the animal and the importance of the hypothalamus in differing stages of energy supply. A study in rats analyzed the overfeeding in rats and its correlation with gene expression. They found that pre- and post-natal overfeeding increased blood glucose ($P < 0.01$), plasma leptin and insulin ($P < 0.001$), and body fat and weight ($P < 0.001$). Furthermore, the authors found these phenotypes were correlated with mRNA expression and DNA methylation of promoter regions control neurons in the hypothalamus (Plagemann et al., 2009). These studies demonstrate the effects of energy availability on gene expression and phenotypes associated with growth.

DNA Methylation

The addition of a methyl group to the cytosine nucleotide is an epigenetic modification that has been studied since the discovery of 5-methylcytosine (5-mC) (Avery et al., 1944). The addition of a methyl group from S-adenyl methionine is facilitated by a family of DNA methyltransferases (DNMTs). The methyl group is added to the fifth carbon of the cytosine in a cytosine-guanine (CpG) dinucleotide of the DNA strand. Because the DNA is a double helix, the CpG is paired with another CpG on the other strand. When one CpG is methylated, the opposing CpG is also methylated (Robertson et al., 2000) which can affect imprinting of selected genes. Imprinted genes are expressed only from, the chromosome of one parent, and can allow for the inheritance of these modifications for generations through replication of the methylation in a parent-of-origin specific manner. Methylation of DNA, in particular 5-mC, has been increasingly recognized to play important roles in the development in all multicellular organisms, especially in mammals (Moore et al., 2013). This modification is a major epigenetic modification of the genome found in most eukaryotes and is essential for normal development and crucial in many biological processes, such as gene expression regulation, genomic imprinting, X-chromosome inactivation, suppression of repetitive elements, and carcinogenesis (Chen et al., 2013, Wu et al 2014). Methylation of DNA in the promoter or the first exon of a gene generally leads to transcriptional silencing of the transcribed product. The extent to which DNA methylation changes throughout the cell cycle is currently unknown and previous studies of methylation during the cell cycle have focused on the maintenance of methylation during DNA replication.

DNMT1 enzymes are methyltransferases that are responsible for the maintenance of methylation through the replication of the methyl group of the new strand of DNA during replication (Chen et al.,2013). During de novo DNA methylation, DNMT3a and DNMT3b are responsible for the addition of the new methyl group to the replicated cytosine (Chen et al., 2013). The enzymes DNMT3a and DNMT3b have an affinity for unmethylated and hemimethylated cytosine, where DNMT1 has a much higher affinity for hemimethylated cytosines (Rhee et al., 2000, Pradhan et al., 1999). During the methylation of the deoxycytidine substrate, DNMTs catalyze the release of a methyl group from S-adenosyl-L-methionine (SAM) to form 5-methycytidine and S-adenosyl homocysteine (SAH) (Parkhitko et al., 2019). The DNMTs also have demethylation activity. In the presence of Ca^{2+} ion and in the absence of SAM, the methylation status of 5-mC can be reversed in cultured porcine embryos by DNMTs (Chen et al., 2013); however, demethylation has not been seen in vivo.

The mechanism for control of gene expression is through blocking a protein-DNA interaction. The presence of 5-mC in a CpG site will cause the inhibition of transcription through the blocking of transcription factors. Methyl CpG binding protein 2 (MeCP2) is important in the control of gene expression by DNA methylation. MeCP2 binds to methylated DNA through a methyl-CpG-binding domain. Once bound it forms a complex with other proteins to interact with the DNA and histone deacetylase to repress gene expression. The exact function of MeCP2 is unknown and is a topic of much recent research for its implications in disorders of the brain such as Rett syndrome and autism and its high levels in the brain compared to other tissue (LaSalle et al., 2009).

Role in Gene Expression

Control of gene expression by DNA methylation has been hypothesized as early as 1975 (Holiday et al., 1975), but not confirmed until 1980 (Razin et al., 1980). Important to the regulation of gene expression is the location of the methylated CpG. Several regions important to the control of gene expression by DNA methylation have been identified including intergenic regions (IR), CpG islands (CGI) and gene bodies (GB). The IRs are DNA sequences within the gene that are not transcribed and are largely methylated to repress the expression of potentially harmful genetic elements but can also contain promoter activity for genes (Schmidt et al., 2015, Moore et al., 2012). The CGI are regions with a high density of CpG sites which are often unmethylated and can contain gene promoters (Moore et al., 2012). The GBs are the region of the gene past the first exon due to methylation of the first exon having a strong correlation to gene silencing and the remainder of the gene having a much looser correlation (Brenet et al., 2011). Methylation of DNA contained in the GBs of dividing cells is associated with higher levels of gene expression; however, in slowly dividing or non-dividing neuronal cells such as the brain, GB methylation is not associated with increased gene expression (Aran et al., 2011)

To look at the interaction between DNA methylation and IR, CPI and GB, two studies will be reviewed. First a meta-analysis of 30 publicly available studies, which all utilized the Illumina Infinium Human Methylation 450 Bead Chip (450K), by Edger et al. (2014) analyzed 485K CpGs of the human genome. They identified 15,224 CpGs referred to as ultrastable because these CpGs had the same methylation status across tissue and developmental stages. The CpGs and their methylation status were mapped to

the genome and gene structure. From these ultrastable regions they further identified areas of unmethylated DNA located at CGI that were located in transcriptionally active housekeeping genes. These areas contained low levels of methylation surrounded by broader flanking levels of high methylation. These unmethylated regions surrounded by high methylation were significantly associated with housekeeping genes ($P < 0.001$) and also associated with areas that showed an increase in methylation in flanking region. These areas had a significantly higher level of gene expression as a group ($P < 0.001$) compared to areas that did not have this same pattern of methylation. Secondly in a study involving 10.2 million avian CpGs and 1.5 million mammalian CpGs in the brain, CpG methylation was negatively correlated with gene expression at transcription start sites (TSS) located in the IR ($P < 1 \times 10^{-15}$) and GB ($P < 1 \times 10^{-15}$) in the frontal lobe (Schachtschneider et al., 2016). These two studies examined the role in DNA methylation on gene expression in other regions of the genome outside of CpG islands and specifically in the brain.

There are an abundance of studies which have shown a link between DNA methylation and gene expression in humans related to many disorders associated with the brain (Valor et al., 2014, Kato et al., 2014, Klengel et al., 2014). A study using 10 different bovine somatic tissues including the brain compared the impact of DNA methylation on gene expression (Zhou et al., 2016). Methylation and expression patterns in ten tissues (skeletal muscle, whole testes, intercaruncle, frontal cortex of the brain, abomasum, ileum, rumen, nucleated blood cells and d90 lactating mammary gland) were used for this study. Reduced representation bisulfite sequencing was used to generate single-base resolution methylation data and RNA-sequencing was used for gene

expression analysis on eight of the samples. Percentages of CpG methylation ranged from 29.87% in the rumen, 30.89% in the frontal cortex of the brain, to 38.06% in the abomasum. Differentially methylated cytosines (DMC) across all tissues were found within 94.34% of CpGs. The authors found 10,794 DMCs and 839 differentially methylated CGIs including 408 DMC methylation levels which were significantly correlated with expression of 117 transcripts (FDR corrected $P<0.05$). A total of 77.5% of DMCs were significantly negatively correlated with gene expression. An analysis of the expression level (FPKM) of microRNA 202, which is known to regulate Sertoli cell proliferation in the testes, showed a strong negative correlation with DNA methylation. Of the 8 tissue types analyzed for the gene, all except testes had very high DNA methylation levels. Only the testes had a high expression level of microRNA 202 as well as methylation levels averaging 52.11 % compared to 88.10 to 95.10 % in other tissue (Zhou et al., 2016). This study showed a clear correlation in many cell types for DNA methylation to gene expression especially in tissue specific genes such as microRNA 202 in testes.

The hypothalamus is an area of the brain that has a direct connection to blood metabolites via the absence of the blood-brain barrier. I have previously described studies where the hypothalamic gene expression was impacted by nutrition (Matthew et al., 2017, Allen et al., 2012, Plagemann et al., 2009). A recent study on the regulation of gene expression by DNA methylation in the hypothalamus in farm animals has been published. Alves et al. (2017) fed post weaned heifers two treatment diets to gain 0.5 kg/d (LG) and 1.0 kg/d (HG) from age 3.5 to 8.5 months of age. The authors evaluated the hypothalamus for gene expression changes by quantitative reverse transcription PCR

(qrt-PCR) and DNA methylation was evaluated by enriching methylated DNA and utilizing CHip-on-chip hybridization containing 99 genes. Thirteen regions that were differentially methylated were identified with 10 mapping to individual genes and 3 regions located within the IGF2-H19 gene cluster. Eight regions were hypermethylated and 5 regions were hypomethylated. Interestingly, the IGF2-H19 gene cluster (n=3) was both hypomethylated and hypermethylated in different regions. This gene cluster is an imprinted region which is known to have a high correlation between DNA methylation and gene expression. It contains a number of promoter regions and the expression levels of H19 and IGF2 are highly linked. As DNA methylation of the promoter region of H19 reaches 100%, the expression of H19 approaches 0 and insulin like growth factor 2 (IGF2) expression increases (Gao et. al., 2002). Three genes, IGF2, growth hormone receptor (GHR) and high mobility group AT-hook 2 (HMGA2), were chosen for qrt-PCR. The IGF2 gene showed no difference in relative mRNA abundance; however, GHR and HMGA2 had a significant ($P \leq 0.05$) down-regulation of relative mRNA abundance in HG heifers compared to LG heifers indicating their hypermethylation status may play a role in transcription (Alves et al., 2017). The study in Chapter Two further examines energy status of the animal and looks at this effect of energy on the hypothalamus and gene expression.

5-hmC

Another modified nucleotide which is the product of oxidation of 5-methylcytosine by the Ten-Eleven translocation (TET) family of enzymes is 5-hydroxymethylcytosine (5-hmC). Research on the conversion of 5-mC to 5-hmC by the TET1 enzyme indicates a putative association with epigenetic control in the hypothalamus due to the increased activity of TET at times of demethylation in embryo development, primordial germ cell development, pluripotency and differentiation, as well as neuronal functions (Tahiliani et al., 2009, Wu et al., 2017). Reports have also indicated that the proportion of 5-hmC is higher in the developing brain than other organs in the body (Wang et al. 2012, Szulwach et al., 2011). Gene promoter methylation alterations in the hypothalamus of overfed rats (Plagemann et al., 2009) and higher 5-hmC levels in the hypothalamus (Münzel et al., 2010) provide evidence for nutritional alteration of the epigenetic status of the hypothalamus. During DNA repair and replication, the distribution pattern of 5-mC is copied to the new DNA by DNA methyltransferase and the methylation status of the DNA is maintained. However, changes in DNA methylation status may occur when responding to developmental cues or to environmental factors where the 5-mc is converted to 5-hmC by TET1, resulting in an unmethylated cytosine that could result in heritable, epigenetic changes (Shen et al., 2012).

The modified nucleotide 5-hmC was 13-fold higher in brain tissue compared to other tissue types (Wang et al., 2012). Modified nucleotide 5-hmC was especially abundant in the hypothalamus (Münzel et al., 2010). To further provide evidence for the role of 5-hmC in the hypothalamus, the impact of 5-hmC on gene expression was

investigated (Mellén et al., 2017). Postmitotic neurons accumulate 5-hmC at 10 times the levels present in peripheral cell types and are a stable epigenetic mark. Although MeCP2 is one of the primary binders of 5-mC and has a high affinity for 5-mC, its affinity for binding 5-hmC was lower in the brain (Mellén et al., 2017). Highly expressed gene bodies contained elevated levels of 5-hmC and were depleted of 5-mC. Conversely, genes that were lowly expressed were depleted of 5-hmC and contained the high-affinity MeCP2 binding site 5-mC. Levels of MeCP2 binding were compared with gene expression and lower gene expression was associated with higher MeCP2 binding, supporting the role of MeCP2 binding in gene regulation. Normally, trimethylation of histone 3-lysine 4 represses gene expression. However, enrichment of 5-hmC near trimethylated histones reversed this effect, resulting in increased gene expression (Mellén et al., 2017). These results demonstrated the importance of the conversion of 5-mC to 5-hmC in highly expressed genes in postmitotic neurons of the brain and associated effects of MeCP2 (Mellén et al., 2012, Mellén et al., 2017).

Active DNA demethylation is one proposed way 5-hmC acts in the brain and regulates transcription. The TET family of enzymes catalyze the oxidation of 5-mC to 5-hmC and regulate gene expression by preventing MeCP2 binding (Mellén et al., 2017) resulting in active demethylation during DNA replication or repair. The DNMTs responsible for methylation of cytosine do not recognize hydroxymethylation and therefore do not replicate methylation on the complimentary strand of DNA, leaving an unmethylated cytosine.

Three TET family member enzymes have been characterized: TET1, TET2, and TET3. These enzymes are highly conserved and contain a double-stranded β-helix region

which contains ferrous (Fe^{2+}) and α-ketoglutarate binding sites that are critical for TET catalytic activity (Yin et al., 2017, Xiao et al., 2012). A cysteine-X-X-cysteine domain in TET binds to cytosine and its modified forms. This domain provides a role of TET enzymes in the control of DNA methylation and demethylation and a putative link to nutritional aspects of modification in the hypothalamus by the binding of available ferrous (Fe^{2+}) and α-ketoglutarate in overfed diets and the restriction of binding in underfed diets. In mouse brain, the expression levels of TET and DNMT where measured at birth (day 1), day 7, day 25, and day 60 and were highest in day 1 hypothalamus and hippocampus. Enzyme activity of TET was higher at birth and lower at weaning. Expression of TET1, TET2 and TET3 was measured in three tissues, two neuron areas of the hypothalamus and the hippocampus. The mRNA expression levels of these enzymes were highest at day 1 ($P < 0.05$) and then significantly decreased at day 7 and again at day 25 ($P < 0.05$). In the hypothalamus expression levels then were significantly different ($P < 0.05$) at day 60 for TET1 in the preoptic area, however for TET2 and TET3 there was no difference between days 25 and 60. Interestingly as TET enzyme activity decreased through puberty, the global amount of 5-hmC increased. This has been hypothesized to be due to condensed chromatin structure in newborns (Cisternas et al., 2019) or by the decreased availability of TET binding recruiters in newborns (Sardina et al., 2018). A significant difference in 5-hmC levels from day 1 to day 7 in the preoptic area of the hypothalamus was not observed; however, a significant increase ($P < 0.05$) in 5-hmC, in two neurons in the hypothalamus and one area of the hippocampus, was found between days 1 and 25 (Cisternas et al., 2019).

This increase in 5-hmC as the animal ages was previously reported in humans (Wen et al., 2014). However, the abundance of 5-hmC was quantified and associated with gene structure. Adult brain tissue had over 10-fold more 5-hmC than fetal brain tissue with 28.4 million and 2.6 million 5-hmC respectively. The adult brain was also examined for abundance of 5-mC compared to 5-hmC. Poised enhancers, which are inactive enhancers bound by transcription factors or coactivators and in communication to target promoters poised to enhance gene expression, had the highest 5-hmC levels (32.6%), followed by active enhancers (28.6%), introns (27.8%), exons (27.7%), and intergenic regions (23.6%; no significance data available). The 5-mC levels followed the reverse order with 42.1%, 37.6% 56.4%, 57.4% and 58.2% 5-hmC levels respectively. The promoter region had lower levels of both 5-hmC and 5-mC (13.1% and 29% respectively). Repetitive sequences such as LINE, SINE, LTR and major satellite repeats had low 5-mC levels and high 5-hmC levels (Wen et al., 2014). Together, these results supported a role in gene expression for 5-hmC.

Abundance in cell types

The distribution of the 5-mC nucleotide has been reported in humans; the abundance of 5-mC was found to be ~1% in several cell types (Ehrlich et al., 1982). High performance liquid chromatography was used to measure the abundance of 5-mC in 9 cell types. Thymus (1.0%) and brain (0.98%) tissue had the highest levels with placenta (0.76%) and sperm (0.84%) having the lowest ($P < 0.01$) (Ehrlich et al., 1982). In mouse this percentage is higher in several cell types. The spinal cord (4.66%) and heart (4.42%) had the highest percentage with kidney, pituitary gland (4.13%), and nasal epithelia (3.73%) having the lowest, however these differences were not significantly different (Globisch et al., 2010). A study comparing the abundance of 5-mC in Purkinje neurons compared to granula cells in the cerebella found that there was no difference in the abundance of the CpG dinucleotides between the two cell types. However there was a significant difference in the levels of 5-mC; Purkinje cells had ~25% less 5-mC than granula cells (1.6 and 2.1% respectively; $P < 0.001$; Kriaucionis et al., 2009). Münzel et al. (2010) further characterized the abundance of 5-mC in mouse brain tissue. Percent of methylated cytosine located at CpG dinucleotide was assessed in 7 regions of the brain. The hypothalamus (3.5%) had the lowest levels of 5-mC and the cerebral cortex, hippocampus, brainstem, olfactory bulb, cerebellum and retina all had ~4.5% 5-mC. Levels of 5-mC in 1 day old mice were ~3.5% where in 90 day old mice 5-mC levels increased to ~4.5% (significance data not available; Münzel et al., 2010). This result would suggest that the developing brain is dynamic and that methylation patterns change with growth of the animal. These changes could allow for nutritional alteration in the hypothalamus due to its dynamic nature.

The other modified nucleotide, 5-hmC, has been shown to be important in epigenetic control of gene expression. To provide evidence that 5-hmC is especially abundant in the brain, Globisch et al. (2010) categorized the abundance of 5-hmC into three categories: low, medium and central nervous system (CNS; high). Low levels (0.03% - 0.06%) of 5-hmC were found in testes, spleen, liver and pituitary gland, and medium levels (0.15% - 0.17%) were found within the lungs, muscle, heart, bladder, nasal epithelia and kidney. The CNS was found to have by far the highest levels (0.3% - 0.7%) of 5-hmC (no significant value reported). Although the pituitary gland is located in the brain and contained low levels of 5-hmC, the authors hypothesized that the highest levels of 5-hmC were located in the area of high neuronal tissue supporting the hypothesis that the hypothalamus would have high levels of 5-hmC. It is of importance to note that 5-hmC levels deviated strongly among tissues indicating a tissue specific attribute when contrasted with 5-mC levels previously reported. Munzel et al. (2010) found that in 7 tissues collected from the brain of mouse, the hypothalamus, which is made up of different neurons, was found to have the highest levels of 5-hmC. In this study the brain was divided into 4 categories, I-III and the hypothalamus. Category III contained the cerebellum and retina and had significantly less 5-hmC levels than the other groups with ~0.3% 5-hmC. The brainstem and olfactory bulb formed category II which contained ~0.55% 5-hmC compared to category I. Category I included the cerebral cortex and the hippocampus, containing ~0.60-0.65% 5-hmC. The hypothalamus, being a part of the endocrine system, contained the highest levels at ~0.7% 5-hmC (no significant value reported; Munzel et al., 2010). The hypothalamus connects the brain to the endocrine system. The hypothalamus contains many groups of

neurons that control many functions in the body including growth, immunity, digestion, sexual maturity and behavior, energy storage and expenditure, reproduction, aging, metabolism and mineral homeostasis. The increased levels of 5-hmC compared to other parts of the body would indicate a role in epigenetic regulation of gene expression and hormone release in the hypothalamus.

Dietary Effects on the Hypothalamus

The hypothalamus is the area of the brain that integrates neuronal and hormonal signals by receiving neuro-hormonal information and sending neural, neurosecretory or autonomic motor outputs to regulate energy homeostasis among other functions. There are many different nuclei contained in the hypothalamus, each controlling distinct aspects of energy metabolism. The hypothalamic paraventricular nucleus (PVN), for example, regulates metabolism through its projections to the median eminence and posterior pituitary gland. Hypophysiotropic hormones, including thyrotropin-releasing hormone (TRH), gonadotropin-releasing hormone (GnRH) and corticotropin-releasing hormone (CRH) are released into the median eminence to control the release of trophic hormones from the anterior pituitary. However, the PVN also projects to many other brain areas, including the brainstem and the spinal cord, to regulate autonomic function. Another important hypothalamic nucleus, adjacent to the third ventricle and the median eminence, is the arcuate nucleus (ARC). The ARC contains two antagonistic groups of neurons, the agouti-related peptide (AgRP) and proopiomelanocortin (POMC) containing neurons. Both neurons are pivotal in the regulation of food intake, but with opposite effects. The AgRP neurons release neuropeptide Y (NPY) to stimulate appetite and in contrast POMC neurons release melanocyte-stimulating hormone to suppress appetite. A third hypothalamic nucleus with a key role in metabolic regulation is the ventromedial hypothalamus (VMH), which controls energy intake and expenditure (Zhang 2017). Because the hypothalamus is a dynamic part of the brain which acts as a neural stimulator, a receptor of stimulus, and is part of the endocrine system the hypothalamus has been the target of research for many years. Nutrition has been shown to significantly

change many aspects of the normal hypothalamic control of growth, homeostasis and reproduction.

Dietary restrictions and chemical additives have long been known to have effects on offspring growth through changes in the hypothalamus. Some of the first studied examples of these effects were the Dutch famine offspring and agouti mice (Painter et al., 2008, Dolinoy 2008). The Dutch famine study was a historical study were men and women born in Amsterdam between November 1943 and February 1947 were interviewed. This period was marked by severe food shortages due to German blockade of supply routes into the Netherlands toward the end of WWII. Women who were pregnant at the time of the famine (F0) were lighter at end of gestation ($P < 0.05$). The authors also found that females exposed to famine conditions prenatally (F1) experienced lower birth weights and gestation lengths ($P < 0.05$). Also offspring of F1 females (F2) also had lower birth lengths and had high body fat index at birth ($P < 0.05$; Painter et al., 2008). This study provides a link to dietary alteration and phenotypic changes in body characteristics. Another study involved a chemical additive and an epigenetic effect was the agouti mouse model. Bisphenol A (BPA) is a chemical contaminant which was used in the production of plastics and epoxy resins used for many common items including food and beverage containers, baby bottles, and dental sealants up until it was banned from use in baby bottles in the United States in 2012. This study took genetically identical mice and included BPA into the diet of one treatment group. The offspring of these mice had a different coat color than control animals due to the epigenetic influence on the production of yellow phaeomelanin. Furthermore, putatively because of the ARC nucleus of the hypothalamus utilizing the agouti neurons to stimulate feeding, the

offspring of the mice fed BPA also suffered from adult-onset obesity, diabetes and tumorigenesis at a higher rate than control mice (significance data not available; Dolinoy et al., 2008). These two studies indicated growth effects on nutritional status as well a nutritional effect on the epigenome. As examined in an earlier section the hypothalamus has a major role in growth as well as the control of feed intake though agouti neurons.

An abundance of research on the effects of maternal nutrition on offspring performance in feedlot or carcass characteristics of ruminant animals has been published (Funston et al., 2010, Mohrhauser et al., 2015). However, research on the effects of maternal nutrition on the hypothalamus is not as numerous. One study in lambs evaluated the effects of supplementing eicosapentaenoic acid (EPA) omega-3 and docosahexaenoic acid (DHA) omega-6 which are known to be beneficial to brain development and function (Kidd et al., 2007). An enriched diet containing EPA and DHA was fed in a 2 x 2 factorial design where finishing lambs born from ewes supplemented with or without EPA and DHA during late gestation were also either supplemented or not supplemented with EPA and DHA. Changes in mRNA abundance for several metabolic neuropeptides in the hypothalamus were subsequently measured. The AgRP and melanocortin receptor 4 genes showed a tendency ($P = 0.09$) for lower expression in supplemented lambs born from supplemented ewes compared to non-supplemented lambs born from ewes with no supplementation. A decrease in melanocortin receptor 3 and cholecystokinin receptor gene expression was observed ($P \leq 0.05$) as well as a tendency for a difference ($P \leq 0.08$) in cocaine and amphetamine regulated protein and cortisol receptor gene expression in lambs supplemented with EPA and DHA and born to ewes supplemented during late gestation with EPA and DHA

(Martin et al., 2018) compared to all other groups. This study reports a link between nutritional control of the AgRP gene in the hypothalamus, providing a possibility for the control of feed intake by the NYP/AgRP neuron in the hypothalamus

A recent study on residual feed intake (RFI) and its association with gene expression in the hypothalamus found a significant effect of feed ingredients and the level of feeding of the animal due to its ability to utilize the feed. Thirty-two genes in the hypothalamus were measured and an efficiency and age effect on gene expression was identified. Significant upregulation of Pro-Melanin Concentrating Hormone (PMCH) gene in the most-efficient heifers and least-efficient steers was observed ($P<0.05$). The PMCH gene was significantly upregulated in the most efficient heifers compared to steers. The AgRP gene was upregulated by 2.1-fold in the least efficient heifers compared to least efficient steers. Many genes had a tendency toward a difference in gene expression ($P < 0.15$; Elolimy et al., 2017). Another study utilizing cattle on the effects of RFI on gene expression in the hypothalamus found NPY, relaxin-3, melanocortin 3 receptor, relaxin/insulin-like family peptide receptor 1, POMC gonadotropin releasing hormone and gonadotropin inhibiting hormone mRNA were differentially expressed ($P < 0.05$) among high efficiency and low efficiency animals. Pituitary expression of follicular stimulating hormone-β and luteinizing hormone-β were correlated ($P < 0.05$) with hypothalamic GnRH levels (Perkins et al., 2014), indicating a functional consequence to differential gene expression in the hypothalamus and further suggesting an epigenetic effect on residual feed intake through functional changes to hypothalamus mRNA expression. These two studies also demonstrate the significance of the hypothalamus on feeding and gene expression.

Chemical contaminants have been used to test the relationship between oxidative stress and DNA methylation in the hypothalamus. The agouti mouse model is one such study (Dolinoy 2008) which tested the effects of the contaminant bisphenyl A (BPA). Low doses of dichlorodiphenyltrichloroethane (DDT) have also been used to test oxidative stressor effects in young male rats. Oxidative stress had a greater impact on gene expression in the hypothalamus compared to gene expression in the hippocampus. Forty genes were differentially expressed in the hypothalamus as indicated by microarray. Of these 40 genes, 10 were validated by qrt-PCR. Out of the ten genes, 7 were significantly associated with oxidative stress ($P < 0.05$). Furthermore, MeD-PCR found DNA methylation of 6 CpG islands was significantly lower compared to control animals with no DDT exposure (Shutoh et al., 2009). This study provides evidence that oxidative stress affects hypothalamic expression and DNA methylation of the hypothalamus more than to the hippocampus providing evidence for the hypothesis that the hypothalamus is a tissue that has higher sensitivity to nutritional and environmental effects.

Methionine levels are often found as the first limiting essential amino acid in high corn diets and have led to impaired growth, effects on the immune system has affected gene expression through decreased methyl group availability for DNA methylation (Fagundes et al., 2019, Ehrlich 2009, Dong et al., 2005). Effects of altered methionine levels on growth, digestibility and gene expression of amino acid transporters were studied in meat type chickens. The birds were divided into a control group fed 0.49% methionine and deficient group fed 0.28% methionine. Dietary methionine restriction increased gene expression levels in 4 genes in the hypothalamus: solute carrier family 38,

member 2; solute carrier family 38, member 7; solute carrier family 7, member 8; and catalase 1 (Fagundes et al., 2019). The increase in mRNA expression may be caused by decreased methylation due to decreased methyl group availability. This result shows the effects on the hypothalamus that a diet high in corn can have through one pathway, methionine methyl group donor deficiency.

Epigenetic Aspects of Butyric Acid

Histones are proteins found in the nuclei of eukaryotes that package DNA into chromatin and control gene expression. Chromatin consists of the histone proteins H2A, H2B, H3 and H4 in duplicate. Around this chromatin core is wrapped the DNA double helix. Histones are highly alkaline with an N-terminal lysine tail that is positively charged. This tail is susceptible to histone acetylation by the enzyme histone acetylase. When an acetyl group is added to the lysine tail the histone tail has a neutral charge, resulting in DNA that is wound loosely around the histone proteins. When this loosening occurs, promoter regions on the DNA are exposed to transcription factors and gene expression is increased. Conversely, with increased histone deacetylase (HDAC) enzyme activity, the acetyl group is removed from the lysine N-terminal tail resulting in a positive charge that attracts the negative charge of the phosphate group of the DNA backbone. This deacetylation results in a tightening of the DNA around the histone proteins and the previously exposed gene promoter regions are no longer accessible by transcription factors leading to decreased gene expression. Strong inhibitors of the enzymes controlling histone modifications exist, such as butyric acid (Candido et al., 1978).

Butyric Acid is usually produced in the rumen by microorganisms such as the bacteria *Butyrivibrio fibrisolvens, Eubacterium cellosolvens, Selenomonas ruminantium, Succinomonas amylolytica, Eubacterium limosum, Megasphaera elsdenii*, and several proteolytic species as well as most protozoa. Butyric Acid is metabolized mostly in the rumen epithelium into the ketone BHBA and can account for up to 70% of energy in ruminants (Wu et al., 2012). What does escape the rumen is quickly metabolized in the liver. After oxidation, butyric acid is metabolized by the TCA cycle and is used as

energy by cardiac and skeletal muscles. An intermediate of BHBA to the TCA cycle is acetoacetyl CoA which is converted to 3-methylglutaryl CoA by the enzyme acetyl CoA acetyl transferase and 3-hydroxy, 3-methyglutaryl synthase (Penner et al., 2011). It is thought that this reaction is the rate limiting step in ketogenesis (Lane et al., 2002). Thus, the increased short chain fatty acid (SCFA) present in a high concentrate diet would result in increased activity of these enzymes (Penner et al., 2011, Connor et al., 2013, Baldwin et al. 2013). Increased expression levels of these key enzymes suggest a control mechanism for butyric acid.

Another factor affecting increased gene expression is histone deacetylase inhibitors (HDACi). The HDACi act by downregulating DNMT1 levels and repressing MAP kinase 1 (ERK) activation (Sarkar et al., 2011) leading to cytosine demethylation or unmethylation of the cytosine nucleotide. BHBA is a HDACi that has been shown to affect gene expression in the hypothalamus of sheep (Cope et al., 2017). This mechanism was explored by Cope et al (2017) by injecting BHBA into sheep and measuring effects of gene expression in the hypothalamus. Animals were injected with 1 ml of BHBA sodium salt solution at 12,800 μmol/L or saline solution at 0.9% NaCl. Two hours after injection the hypothalamus was harvested and gene expression was measured by RNA-sequencing. Forty-four genes were found to be altered in the hypothalamus of sheep injected with BHBA. Functional enrichment analysis revealed genes in pathways related to stimulus perception, inflammation, and cell cycle control had altered expression levels ($P < 0.05$; Cope et al., 2017). The actions of BHBA on gene expression in the hypothalamus (Cope et al., 2017) together with butyric acid alteration of gene expression

in cultured bovine cells (Candido et al., 1978) provide another method for modification gene expression in high corn diets.

Conclusion

Hypothalamus control of growth, development and homeostasis is well documented. The hypothalamus senses metabolites and hormones in the body and creates a rapid response in protein production and hormone release. Thus, changes in gene expression and DNA methylation in the hypothalamus and their susceptibility to compounds in the diet are of great interest.

Altering gene expression in the hypothalamus by use of several common feedstuffs or additives fed in feedlot diets has been examined in this review. Corn is a common in feedlot diets and is fed in varying amounts and is responsible for the production of propionic acid in the rumen. This SCFA is accompanied by butyric acid, another SCFA, in corn-based diets. With increased levels of corn in the diet, more butyric acid is produced. Butyric acid is converted to β-hydroxybutyric acid in the rumen epithelial cells and liver and enters the blood stem making it an available metabolite that can affect gene expression. Methionine is an essential amino acid that is known to be lower in corn-based diets. Increased methionine has also been associated with decreased DNA methylation and increased expression of genes.

We have explored two methods to modified DNA methylation: 1. the inhibition of histone deacetylation by BHBA leading to lowered methylation levels and 2. the activity of TET enzymes on 5-mc to produce 5-hmC. Both methods appear to be active in the hypothalamus in regulating growth of muscle and bone and by regulating inflammation and immune response in the body. The side effects of increased acid produced in the rumen is acidosis. The increase of inflammation and immune response gene expression

in the hypothalamus could be a response to acidosis. The influence of corn in large quantities in the feedlot receiving diet on the concentrations of BHBA in the blood stream and in the hypothalamus has yet to be researched.

The effects of 5-hmC on the methylation status is of great interest due to the large abundance of this modified nucleotide in the hypothalamus. Its abundance in the gene body may be associated with gene expression leading to increased expression of genes responsible for feeding behavior, bone development, blood sugar levels, and inflammation and immune response. Low methionine, ketone inhibition of DNMT1 activity and ERK, cell signaling, and increased binding agents for TET activity are all factors that could have an impact on DNA methylation and gene expression in the hypothalamus. Because corn is a large part of cattle diets in the Midwest and has been shown to affect DNA methylation and gene expression related to growth and immune response in the hypothalamus of heifers. Entering the feedlot stage is a major economic and health challenge to the producer. If hypothalamic gene expression controlled by DNA methylation can be altered in post weaned steers through the increase of corn in the receiving diet, improved performance and health to animal would help the producer. Currently studies on the effects of the feedlot diets on epigenetic regulation of growth and health are lacking. To merge ruminant nutrition and genomics in the feedlot stage could open the door to an area of agriculture that could provide valuable information to feedlot nutritionists and the producers.

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Chapter 2

GLOBAL GENE EXPRESSION AND DNA METHYLATION OF THE HYPOTHALAMUS IN FEEDER CALVES FED A HIGH CONCENTRATE DIET UPON ENTERING FEEDLOT PHASE

Introduction

Gene expression is biologically important for all aspects of cattle including growth, development and immune response; gene expression is controlled transcriptionally, translationally and post-translationally. Transcription can be controlled by several methods such as histone modifications, expression of transcription factors and repressor proteins, as well as methylation of cytosine nucleotides of DNA. Methylation status of these CpG dinucleotides of the DNA strand are important for control of gene expression. The modification 5-hmC is formed by the oxidation of 5-mC and catalyzed by the TET enzyme (Tahiliani et al., 2009). Interestingly 5-hmC has been discovered to be abundant in the brain when compared to other organs (Kriaucionis et al., 2009, Li & Liu, 2011, Wang et al., 2012, Szulwach et al., 2011). Although the functions are not fully known, it is known that 5-hmC is important in development. In the brain, 5-hmC is enriched in DNA associated with promoters, gene bodies and intergenic areas near genes. This correlates with increased expression of the enriched genes (Pastor et al., 2011, Song et al., 2011). The formation of 5-hmC from 5-mC and its role in the methylation status of promoters is supported by the knockdown of TET1 in mouse embryonic stem cells and its correlation with increased expression of genes important to embryonic development (Ito et al., 2010). Knockdown of TET1 and TET2 caused a downregulation of a group of genes that included many pluripotent related genes and an increased methylation of their promoters (Ficz et al., 2011). These studies indicated that not only is 5-hmC important in active DNA demethylation but could be influenced by the activity of TET enzymes, possibly through their dependence on iron and α-ketoglutarate (Tahiliani et al., 2009).

The hypothalamus is an area of the vertebrate brain that controls many metabolic, immunological and growth processes. Regions of the hypothalamus that control these processes include the arcuate nucleus, paraventricular nucleus, dorsomedial hypothalamus, and lateral hypothalamus area (Coupe et al., 2010). In post-wean heifers, a high concentrate diet changed expression in the hypothalamus of approximately 345 genes versus heifers fed a high forage diet (Allen et al., 2012). Among these genes that were differentially expressed were those involved in feeding behavior, hormone metabolic process, cellular response to insulin stimulus and regulation of phosphorylation. Researchers analyzed 3 genes from differentially methylated regions in the hypothalamus of Charolais-crossbred heifers restricted to 0.4X Maintenance energy (MEm) requirements and found 2 of the 3 were differentially expressed compared to control (1.2X MEm) (Matthew et al 2017). Further, heifers fed to gain weight at a low rate of 0.5 kg/day or at a high rate of 1.0 kg/day between 4.5 months and 8.5 months of age had altered methylation patterns in the arcuate nucleus of the hypothalamus (Alves et al., 2017). In another study the methylation status of the promoter regions of genes in the hypothalamus was shown to be altered by early overfeeding of rats. These genes encoded for hormones that lead to rapid early weight gain and other aberrant metabolic phenotypes. Among these phenotypes are obesity, hyperleptinaemia, hyperglycaemia, hyperinsulinaemia, and an increased insulin/glucose ratio compared to rats not overfed (Plagemann et al., 2009; Plagemann et al., 2010).

Backgrounding diets contain lower amounts of concentrate than finishing diets. It is unclear if these diets change DNA methylation status and gene expression in the hypothalamus. The brain is extremely susceptible to major metabolic changes, especially

in the hypothalamus (Cone et al., 2001). Our hypothesis was that a high concentrate diet early in the feedlot phase of immediately post-weaned steer calves will change DNA methylation of the promoter regions of genes in the hypothalamus and therefore hypothalamic gene expression. To test this hypothesis, post-weaned steers entering the feedlot were fed a high energy/high concentrate ration designed for rapid growth and compared to control background/finisher ration designed for normal gain. After the 202 days on feed, animals were harvested, and the hypothalamus was analyzed for differences in gene expression and DNA methylation.

MATERIALS AND METHODS

Receiving and Treatment Allotment

Steer calves (n=56) were received at the South Dakota State University Ruminant Nutrition Unit (Brookings, SD) where they were individually tagged and processed. Newly-weaned steer calves from spring calving cows with less than a 90 day calving window were from cow-calf operations in western South Dakota with predominately Angus breeding (≥75%). These cattle were part of a feeding trial to evaluate the economics of feeding a high corn diet to steers at an earlier stage in development in the feedlot. The two treatment diets were a normal background/finishing (B/F) diet and a diet high in concentrate and energy (HE/HC) (Table 1). Animals were fed on a pen basis, where 14 steers were included in each pen and two pens were randomly allocated to each treatment for a total of 4 pens. The B/F diet was fed in two stages: d0 to d84 was fed at 1.10 Mcal/kg predicted net energy gain (NEg) and d84 to d202 was fed a higher concentrate at 1.43 Mcal/kg predicted NEg. The HE/HC diet was fed at 1.33 Mcal/kg predicted NEg for the entire 202 days on feed.

Harvest and Sample Collection

A total of twelve steers, three steers from each pen closest to the average daily weight of each pen, were harvested at the South Dakota State University's Meat Laboratory using accepted slaughter methods. Immediately following exsanguination and head removal, the top portion of the skull and brain was removed using a reciprocating saw with a 12-inch blade. The cut was made through the cerebrum close to the thickest part of the brain. The bottom portion of the brain was then carefully removed, and the

hypothalamus was identified by locating the pituitary stalk. The hypothalamus was then divided into two equal pieces and snap frozen in liquid nitrogen and maintained at -80°C. The hypothalamus has bilateral symmetry, therefore both halves should have identical attributes (Yu, 1994).

Carcass Data and Sample Collection

Carcasses were weighed to determine Hot Carcass Weight (HCW) prior to chilling. After cooling for 48 h postmortem at 4°C, the right side of each carcass was ribbed between the 12th and 13th rib. Each carcass was evaluated for longissimus muscle area (LMA), 12th rib subcutaneous fat (FT), kidney, pelvic and heart fat (KPH), marbling scores, lean maturity, and skeletal maturity by trained university personnel as previously described (Mohrhauser et al., 2015). USDA Yield Grades were calculated from the HCW, LMA, FT and % KPH values (USDA, 1996). Additionally, the 9-10-11 rib section was fabricated from the right side of each carcass as described by Hankins and Howe (1946). Soft tissue was separated from bone and both were weighed.

Warner-Bratzler Shear Force

Strip loins (IMPS 180) were excised from the right side of each carcass and the anterior side was squared off to allow for three even steaks (2.54cm thick) to be removed from the anterior portion of each strip loin. The steaks were allotted sequentially for Warner-Bratzler shear force (WBSF) at 7, 14, at 21 d postmortem. Steaks were stored at -20ºC after their predetermined postmortem aging period until subsequent WBSF analysis.

Steaks aged from 7, 14, and 21 d were thawed for 24 h at 4ºC and cooked to a mean endpoint internal temperature of 74.6 ± 3.54°C on an electric clam shell grill (George Foreman, Indoor/Outdoor Grill, Model GGR62, Lake Forest, IL). Peak internal temperatures were recorded for each steak using a hand-held digital thermometer (RT600C-N, ThermoWorks, Lindon, UT) inserted into the geometric center of the steak. Steaks were then cooled overnight at 4ºC before removing 6 to 8 cores (1.27 cm diameter) parallel to the muscle fiber orientation (AMSA, 1995). Each core was sheared perpendicular to the muscle fiber orientation using a Warner-Bratzler shear machine (BFG 500N, G-R Electric Manufacturing Company, Manhattan, KS). Peak force (kg) required to shear each core was recorded and an average was calculated using the values from the corresponding cores for each steak.

RNA-Seq Analysis

The RNA was extracted from one half of the hypothalamus using Direct-zol RNA Mini-Prep (Zymo Research, Irvine, CA). Samples were homogenized using ZR BashingBead™ (Zymo Research) and the PoweLyzer® 24 (MoBio Laboratories, Inc., Carlsbad, CA) prior to RNA extraction. Briefly, the frozen hypothalamus was separated into 50mg pieces and each 50mg piece was added to 500µl of TRI Reagent® (Molecular Research Center, Inc. Cincinnati, OH) until all the tissue was homogenized. The mixture was centrifuged at 12,000 x g for 1 minute and then supernatant from each piece of the hypothalamus was combined. Combined supernatant (350µl) was mixed with 350µl of 100% ethanol. The mixture was then loaded into a Zymo-Spin™ Column and RNA purified following manufacturer's instructions. To obtain sufficient quantity of RNA for analysis, five separate columns were used for each combined supernatant. The purified

RNA from each column was combined and the RNA concentration and quality were determined by spectrophotometry (Nanodrop 1000, Thermo Scientific, Wilmington, DE). All RNA samples were considered of good quality with a 260/280 ratio of approximately 2.0. Samples were then sent to Zymo Research for RNA-seq library preparation and sequencing (Illumina HiSeq 2 x 50 bp paired-end reads).

Sequence reads were first adaptor trimmed and then analyzed using TopHat and Cufflinks software (Trapnell et al. 2012) with default parameters. TopHat (v2.0.9) was utilized for alignment of short reads to the cow genome UCSC BosTau7, Cufflinks (v2.2.0) for transcript assembly and differential expression analysis, and CommeRbund (v2.0.0) for visualization of differential analysis. Normalization was completed using cuffdiff after assembling transcripts (cufflinks software) and the final transcriptome was assembled with cuffmerge. The normalization was to fragments per kilobase of transcript per million mapped fragments (FPKM), consisted of two normalization steps: first, normalizing to each transcript's length, and second, to the total mapped fragments of a run (Trapnell et al. 2012). Differential expression values were reported in fold change of the FPKM values of treatment group to controls and considered significant when FDR (false discovery rate) adjusted p-value <0.05 (Trapnell et al. 2012).

DNA Isolation

The DNA was isolated from the second half of the hypothalamus using a DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany). Hypothalamus was weighed and chopped using a small razor. Lysis buffer (180µl) and 20µl proteinase K was premixed with 25mg of tissue. The mixture was vortexed periodically and incubated at 56ºC until tissue was completely lysed. The lysis mixture (200µl per column) was then

used for DNA isolation. A total of 5 columns were used per sample to obtain sufficient amounts of DNA for analysis. The DNA concentration was determined using spectrophotometry (Nanodrop 1000, Thermo Scientific, Wilmington, DE). Samples lacking sufficient concentration of DNA were concentrated using a DNA Concentrator Kit (EpigenTek, Farmingdale, NY).

Global 5-mC and 5-hmC analysis

Quantification of 5-mC and 5-hmC DNA was performed using the MethylFlash™ Methylated DNA quantification kit and the Hydroxymethylated DNA quantification kit (EpigenTek), Farmingdale, NY) respectively. Methylation analysis was completed with 100µg DNA, while 200µg of DNA was used for hydroxymethylation as per manufacturer instructions. The DNA and binding solution were added to plates provided with the kit and incubated for 60 minutes. Plates were washed with 150µl washing buffer then incubated with 50µl detection solution for 50 minutes followed by another 5 washes with washing buffer. For detection, 50µl of detection solution, containing optimized antibodies, was added and mixed for 5 minutes followed by addition of stop solution. After 2 minutes optical densities were measured by spectophotometry (Spectra Max 190, Molecular Devices, Sunnyvale, CA).

Targeted, Next-Generation Bisulfite Sequencing Analysis

Extracted DNA (500ng) was bisulfite modified using the EZ DNA Methylation Kit (Zymo Research) and eluted in 40µl. Bisulfite treatment of DNA will allow detection of methylated cytosines by converting unmethylated cytosines into uracil and leaving methylated cytosines alone. PCR amplification of the DNA can detect uracil vs cytosine

by designing primers with uracil to detect unmethylated CpGs and another primer for cytosine to detect methylated CpGs. Genes analyzed were Hemoglobin β (HBB), Cytochrome C oxidase subunit VIIc (COX7C), MX dynamin-like GTPase 2 (MX2), Radical S-adenosyl methionine domain containing 2 (RSAD2) and Interferon-induced protein with tetratricopeptide repeats 3 (IFIT3). These genes were chosen from the RNA Seq analysis from the loci that were differentially expressed. A total of 79 assays were designed and grouped into multiplex PCRs based on average melting temperature. Flanking primers were designed using Pyrosequencing ADSW (Assay Design Software) 1.0 software (Qiagen) to the CpG site for which a β-value was reported by Illumina. Reported β-values are an estimate of methylation level using ratio of intensities between methylated and unmethylated alleles and are reported between 0 and 1; with 0 being unmethylated and 1 being methylated. On average, about 9 CpG sites per amplicon were interrogated. Amplification was carried out using 1 to 2µl of bisulfite treated DNA using Hot Start *Taq* DNA polymerase (Qiagen) with the following PCR protocol: 95ºC 15 min; 45 x (95ºC 30s; 56 - 60ºC 30 s; 72ºC 30 s); 72ºC 5 min; 4°C ∞. Seventy assays, grouped into new multiplex PCRs, were successful and approved for next generation sequencing. Bisulfite treated DNA was amplified with methylated and unmethylated primers using the Ion PGM (Personal Genome Machine) Template OT2 (One Touch 2) 200 kit. These amplicons were sequenced using an Ion PGM™ Sequencing HiQ Kit with an Ion 318™ v2 Chip (Life Technologies) on the Ion Torrent PGM platform.

The FASTQ files from the Ion Torrent PGM were aligned with a local FASTA program using open source Bismark Bisulfite Read Mapper with the Bowtie2 alignment algorithm. Methylation levels were calculated in Bismark by dividing the number of

methylated reads at a CpG site by the number of total reads at a CpG site, considering all CpG sites covered by a minimum of 30 total reads.

Statistical Analysis

Growth date was analyzed using a Student's t-test to compare the means of both treatments. The WBSF data were analyzed using a Mixed Model (SAS Inst. Inc., Cary, NC) with diet, aging, and diet x ageing as the main effect. Remaining carcass measurement data were analyzed with R open source software using ANOVA. The cut-off for statistical significance was $P < 0.05$ and trends were identified when $P < 0.10$. Differential expression and methylation of genes was determined as previously described above. Pearson's Correlation was used to estimate a correlation between methylation and gene expression. Global methylation differences of 5-hmC and 5-mC between treatment groups were compared using a Student's t-test to compare the means of both treatments.

Results and Discussion

Steers in B/F and HE/HC treatments started with no significant difference in body weight (BW, 287.8 and 289.4 kg respectively) and ended treatment with no significant difference in BW (570.6 and 577.6 kg respectively). There were four weigh days with a significant weight difference between treatment groups; 28, 63, 84, 105 ($P < 0.05$, Figure 1). The growth rates for the HE/HC treatment were higher at each weigh day. With the exception of %KPH ($P < 0.04$, Table 2), no differences in any carcass measurements were observed. Increased %KPH fat in the HE/HC treatment (2.0%) compared to the B/F treatment (1.4%) is likely due to the order of fat deposition in the growth of the animal. Internal fat has a higher priority earlier in the growth phase. Therefore when the animal is in a high caloric intake state early in the feedlot phase, more of these calories can go to internal fat deposition (Camfield et al. 1999). The goal of the initial feeding trial these steers belonged to was to have altered growth patterns and harvest when steers appeared to have the same back fat thickness of ~ 1.27 cm^2, however, this study wanted to test whether an altered growth pattern between treatments would have an impact of carcass traits. Our results of only %KPH fat having a significant difference could be due to the cattle never being in a restricted diet and negative energy balance. The growth rate for these cattle from initial weight to 202 days on feed was 1.41 and 1.43 kg/day in B/F and HE/HC respectively.

Tenderness, as measured by WBSF, improved between seven and 14 days postmortem ($P < 0.05$; Table 3) with no significant difference at d7, d14 or d21 between treatment groups. These results suggest that much of the postmortem proteolysis relevant to tenderness improvement was complete after seven days of aging. The mean

WBSF of steaks from every aging group would be considered “tender” [98% consumer tenderness acceptability of 4.1 kg (Huffman et al., 1996, Hruska, 1999)], therefore any difference in WBSF would likely not be detected by the consumer.

A total of 335 loci were differentially expressed of which 131 were only expressed in the B/F diet and 195 only expressed in the HE/HC diet. Five genes were annotated within the 9 loci expressed in calves from both treatment groups. The other 4 were not annotated to a gene. The genes that were annotated were HBB, COX7C, MX2, RSAD2 and IFIT3 (Table 4). Differences in gene expression were discovered between treatments for 335 loci which were not annotated to specific gene.

The genes HBB and COX7C had higher expression levels in the HE/HC treatment. The gene HBB encodes for one of the proteins which make up blood hemoglobin which is responsible for carrying oxygen from the lungs to the cells and carbon dioxide from the cell to the lungs. The hemoglobin molecule is capable of limiting the potential for the production of reactive oxygen species caused by its association with iron and free oxygen (Thom et al., 2013). The enzyme encoded my COX7C is found in the inner mitochondrial membrane, catalyzes electron transfer and drives synthesis of ATP (Seelan et al., 1997). Both genes have Gene Ontology terms that are important in metabolic processes involving the flow of nutrients to and from cells. Genes MX2, RSAD2 and IFIT3 all had lower expression in the HE/HC treatment. The protein IFIT3 is associated with apoptosis in different cell types because of its anti-proliferative and pro-apoptotic role (Kazezian et al., 2017). Along with lower expression levels, MX2 (r = -0.994) and RSAD2 (r = -0.878) each had one CpG with decreased methylation which had a high correlation to increased gene expression (Table 5).

In MX2 this CpG site was 5 base pairs downstream (3') of the translation start codon (ATG) and 6569 base pairs 3' of the transcription start site (TSS) and could play a role in controlling the expression levels of MX2. In humans, MX2 is known to inhibit the HIV-1 virus post-entry through interferon control of replication (Goujon et al., 2013). In bovine MX2 has been shown to have antiviral activity (Babiker et al., 2007) as well as being a useful biomarker (Kizaki et al., 2013), detecting gestation within three weeks of insemination in granulocyte fractions. In RSAD2 this position is 6330 base pairs 3' of ATG and 6378 3' of the TSS. RSAD2 is also associated with the immune response to viruses. This gene has a role in metal ion binding and alpha-beta T cell activation and differentiation. The gene RSAD2 is also a pregnancy-regulated and could have a role in the uterus response to conceptus during implantation and development of the caruncle in sheep (Mansouri-Attia, et al., 2009). Thus, differential expression of these two genes may affect immune response and fertility. However, these phenotypes were not collected in this experiment.

Two CpGs from the bisulfite sequencing showed low methylation levels across treatments (0.0 to 3.3%), 25 CpGs had high methylation levels across treatments (90.0 to 94.8%) and 39 CpGs had differential methylation levels between treatment groups with a percent methylation range of 9.1 to 98.7%. However, as stated above, increased DNA methylation at only two CpG sites on genes MX2 and RSAD2 were negatively correlated ($r = -0.994$ and -0.878 respectively) with lower gene expression (Table 5). The two lowly methylated CpGs were located in COX7C and incorporate 17 CpG sites 155 to 501 bp 5′ of the TSS. Only 2 of the HE/HC treatments animals had high COX7C expression levels and the remaining 10 animals had very low or no detectible expression. However, there

was no significant difference between groups. No difference was observed in global 5-hmC and 5-mC between treatments (Table 6). This data measures the entire genome for quantity of 5-mC and 5-hmC; however it does not give us a locus specific map of methylated cytosines.

Methylated CpGs were similarly methylated between treatment groups for each gene. Bubble plots for methylation levels showed one CpG having a different methylation level, less than 50% is unmethylated and greater than 50% is methylated, for genes MX2 and RSAD2 (Figure 2). However, this difference in methylation may not be a functional regulator of gene expression. Gene expression and DNA methylation of the CpG that was correlated with gene expression can be found in Table 5. With the exception of one animal, the gene expression and DNA methylation values appear to be very similar. Similarly, the difference in methylation in the bubble plot (Figure 2) values for methylation appear very similar (49.89 and 53.89 for MX2, 53.19 and 49.68 for RSAD2). Moreover, the CpG from the bubble plot shown to be different and the CpG from the correlation data were not the same.

Conclusion

The objective of the study was to determine if feeding a high concentrate diet relative to controls to incoming feedlot steers would change the DNA methylation of the hypothalamus. Changes in DNA methylation can alter expression levels of genes associated with growth and development of muscle and adipose tissue, which in turn alters carcass composition.

Growth of animals in both treatment groups was significantly different on day 28, 63, 84, and 105 on trial with growth of HE/HC treatment steer having larger mean body weights at each weigh day. Steers fed a high concentrate diet had greater %KPH but no difference in other carcass traits. A total of 335 reads were differentially expressed in this study, however, only five reads annotated to genes. It was interesting that all five of these genes were expressed in both treatment groups. MX2, RSAD2, and IFIT3 were down regulated and COX7C and HBB were upregulated in the HE/HC group. The increased mRNA levels of metabolic associated genes COX7C and HBB may be linked to increased available substrate for ATP production and oxygen demands. The decrease in MX2, RSAD2, and IFIT3 could have a connection to improved health of animals fed a higher energy diet. The increased energy available, much like the increased energy available in more efficient cattle or overfed cattle has been shown to affect gene expression. Targeted next generation bisulfite sequencing identified 107 CpG sites within these five genes which had differential methylation between treatment groups. Although different, the actual methylation differences between treatment groups can only be a few percent methylation. Although likely not a functional correlation do to the possibility of one animal providing the difference between treatment in both genes, 1

CpG site in genes MX2 and RSAD2 had higher methylation levels and were correlated to lower gene expression in the HE/HC. No difference in hypothalamic 5-mC and 5-hmC was observed.

Implications

The increased KPH fat accumulation may indicate excessive fat deposition resulting from increased caloric intake in the early stage of the feedlot phase. The smaller size of animals entering the feedlot could have made it possible in the HE/HC treatment for more calories to be available for internal and intramuscular fat deposits leading to early increases in KPH fat and marbling, however increased marbling was not observed in this study. The animals in the HE/HC treatment were never in negative energy balance due to a adaption phase so the need for mobilizing this fat deposit was not necessary. Although average weight was not statistically different between treatments throughout most of the feeding phase and ultimately only a difference in % KPH fat was observed, the potential for a larger difference in carcass measurements could be realized with a larger difference between growth curves.

The difference in expression in immune response genes, such as defense response to virus and immune signaling pathways, could further indicate a connection between nutrition and morbidity in animals that are in subprime condition entering the feedlot. This finding could also lead to custom diets for animals entering the feedlot phase in a stress or immune challenged condition. To help understand these findings and provide further information for producers and nutritionists, more research into the effects of high concentrate diets on the immune system, including pathogen challenge by diet interactions, could be conducted. Final yield grade is affected by the % KPH through an ultimate adjustment of the carcass weight. Regulation of % KPH fat deposition could differ from other fat depots early in the feedlot phase, and manipulation of % KPH fat

deposition changes yield grades because of the use of % KPH in the yield grade calculations.

Lower gene expression has been correlated with increased DNA methylation (Razin et al., 1991). Decreased methylation in MX2 and RSAD2 were correlated with increased gene expression of these two genes in this study. These genes are associated with immune response to antigen stimulus in the bovine and to metal ion binding respectively. The upregulation of immune response genes may be beneficial to cattle entering the feedlot phase in a stressed or immuno-compromised condition. This increase in immune gene expression could result in decreased morbidity and mortality rates in animals fed a higher concentrate diet. Metal ion binding, in particular iron, may play a role in increased activity of TET enzymes associated with 5-hmC through dependence on iron and alpha-Ketoglutaric acid (Ito et al., 2011), which is a Krebs cycle intermediate. While only two significant correlations between gene expression and DNA methylation were found, further research into the relationship between 5-hmC and deposition of fat could elucidate effects of increased fat levels in the HE/HC treatment. Although this study did not find a difference in global 5-hmC abundance between treatments, nucleotide specific 5-hmC detection may be a more sensitive indicator of local gene expression changes in response to diet manipulation.

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Table 1. Diet formulations for B/F treatment and HE/HC treatment

	B/F[c]		HE/HC[c]
Ingredients	**Background**	**Finishing**	
Corn silage[a]		10.00	35.00
Corn fodder[a]	82.70		
High moisture corn[a]		30.00	30.00
Dry rolled corn[a]		37.00	18.35
DDGS[ac]	10.00		
MDGS[ac]		20.00	12.00
Dry supplement 1[ab]	7.30		
Dry supplement 2[ab]		3	
Dry supplement 3[ab]			4.65
Crude Protein[a]	12.03	13.31	12.21
Net energy maintanence[d]	1.76	2.17	2.04
Net energy gain[d]	1.14	1.47	1.38
Days fed	84	118	202

[a] Percent inclusion; dry matter basis

[b] Supplements fortified to meet or exceed NRC (1996) requirements for vitamins and minerals.

[c] B/F = background/finishing treatment, HE/HC = High Energy/High Concentrate treatment, DDGS = Dry distillers grain with solubles, MDGS = Modified distillers grain with solubles

[d] Mcal/kg

Table 2. Effect of B/F and HE/HC treatment diets on carcass performance and composition

	Treatment			
Measurement	**B/F[a]**	**HE/HC[a]**	**SEM[a]**	***P-value***
Hot carcass weight, kg	352	362	8.4	0.10
DP, %[b]	63.7	64.2	0.56	0.56
Longissimus muscle area, cm^2	81.2	79.1	3.36	0.69
FT, cm^2, [c]	1.37	1.30	0.086	0.60
KPH, %[d]	1.4	2.0	0.203	0.04
Yield Grade[e]	3.08	3.26	0.234	0.62
Marbling score[f]	460	480	19.83	0.49
Lean color score	160	167	6.11	0.47
Skeletal Maturity	143	135	4.07	0.16
Bone weight, %	16.18	16.02	0.112	0.68
Soft tissue weight, %	83.82	83.98	0.295	0.62

[a] B/F = Background/Finishing Treatment, HE/HC = High Energy/High Concentrate Treatment, SEM = standard error of means

[b] Dressing percent (DP) calculated by (Hot Carcass Weight/Live Weight).

[c] 12th rib fat thickness (FT).

[d] Percent kidney, pelvic, and heart fat (%KPH).

[e] Calculated according to USDA (1996).

[f] Marbling score: 200=$Traces^0$, 300=$Slight^0$, 400=$Small^0$, 500=$Modest^0$.

Table 3. Warner Bratzler shear force analysis of strip loins harvested from steers fed B/F and HE/HC treatment diets

Aging	B/F[a], kg	HE/HC[a], kg	SEM[a]	*P-value*
7 Days	3.93[b]	3.57[b]	0.422	0.376
14 Days	3.06[c]	3.18[bc]	0.422	0.791
21 Days	2.80[c]	2.74[c]	0.340	0.848
Mean	3.16	3.26	0.225	0.644

[a] B/F = background/finishing treatment, HE/HC = High Energy/High Concentrate treatment, SEM = standard error of means.

[b, c] Column means with different superscripts differ $P<0.05$.

Table 4. Differential gene expression and Gene Ontology terms for Biological Process of differentially regulated genes in High Energy/High Concentrate treatment when compared to Background/Finishing treatment

Gene[b]	**Gene Name**	**Fold Change**[a]	**Biological Process Terms**
HBB	Hemoglobin β	+3.2	Oxygen, nitric oxide and bicarbonate transport
COX7C	Cytochrome C oxidase subunit VII c	+6.0	Cellular metabolic process, generation of precursor metabolites and energy, hydrogen ion transport
MX2	X dynamin-like GTPase 2	-3.6	Cytokinase-mediated signaling, defense response to virus, regulation of cell cycle
RSAD2	Radical S-adenosyl methionine domain-containing 2	-3.6	α-β t-cell activation, cytokine mediated signaling pathway, defense response to virus
IFIT3	Interferon-induced protein w/tetratricopeptide repeats 3	-2.2	Cellular response to interferon α, cytokine-mediated signaling, defense response to virus, negative regulation of apoptotic process and cellular proliferation

[a] Fold change is the $\log_2$ of the value of High Energy/High Concentrate treatment divided by value of Background/Finishing treatment

[b] Genes annotated and expressed in both treatment groups

Table 5. RNA expression and DNA methylation of two CpG sites having a high correlation in animals fed a Background/Finishing and High Energy/High Concentrate diet

		Bovine MX2 - ADS4972 CpG#1		**Bovine RSAD2 - ADS4993 CpG#87**	
Cell ID	**Treatment**	**RNA expression[b]**	**DNA methylation[c]**	**RNA expression[b]**	**DNA methylation[c]**
381	HE/HC	0.34	94.83	0.53	92.35
382	HE/HC	0.47	92.83	0.37	91.87
384	HE/HC	0.14	91.90	0.24	93.88
446	HE/HC	0.22	93.12	0.35	93.61
515	HE/HC	1.21	92.89	1.55	93.20
534	HE/HC	0.23	91.67	0.66	92.99
Treatment Mean[a]		0.43 ± 0.36	92.87 ± 1.02	0.62 ± 0.44	92.98 ± 0.69
309	B/F	0.39	93.08	0.82	92.42
315	B/F	0.60	92.16	0.64	91.33
316	B/F	0.53	91.72	0.95	92.69
453	B/F	0.30	89.69	0.62	92.04
507	B/F	0.32	90.96	0.46	93.02
561	B/F	29.63	45.95	40.52	88.05
Treatment Mean[a]		5.30 ± 10.88	83.93 ± 17.02	7.34 ± 14.84	91.59 ± 1.67

[a] Treatment mean of 6 animals in each treatment ± standard deviation.

[b] FPKM

[c] Percent methylation

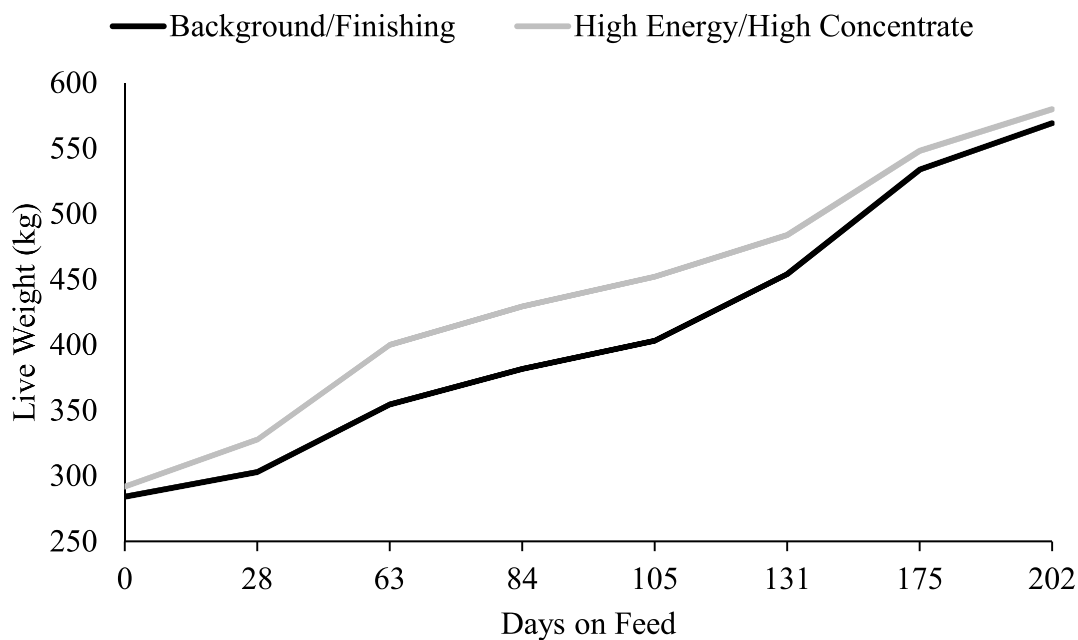
Table 6. Effect of B/F and HE/HC diets on global methylation of hypothalamus as determined by colorimetric assay

Modification	**B/F[a]**	**HE/HC[a]**	**SEM[a]**	***P*-value**
% 5-hmC[b]	0.131	0.126	0.031	0.80
% 5-mC[b]	0.590	0.690	0.199	0.53

[a] B/F = background/finishing treatment, HE/HC = High Energy/High Concentrate treatment, SEM = standard error of means.

[b] 5-hmC = 5-hydroxymethylcytosine, 5-mC = 5-methylcytosine

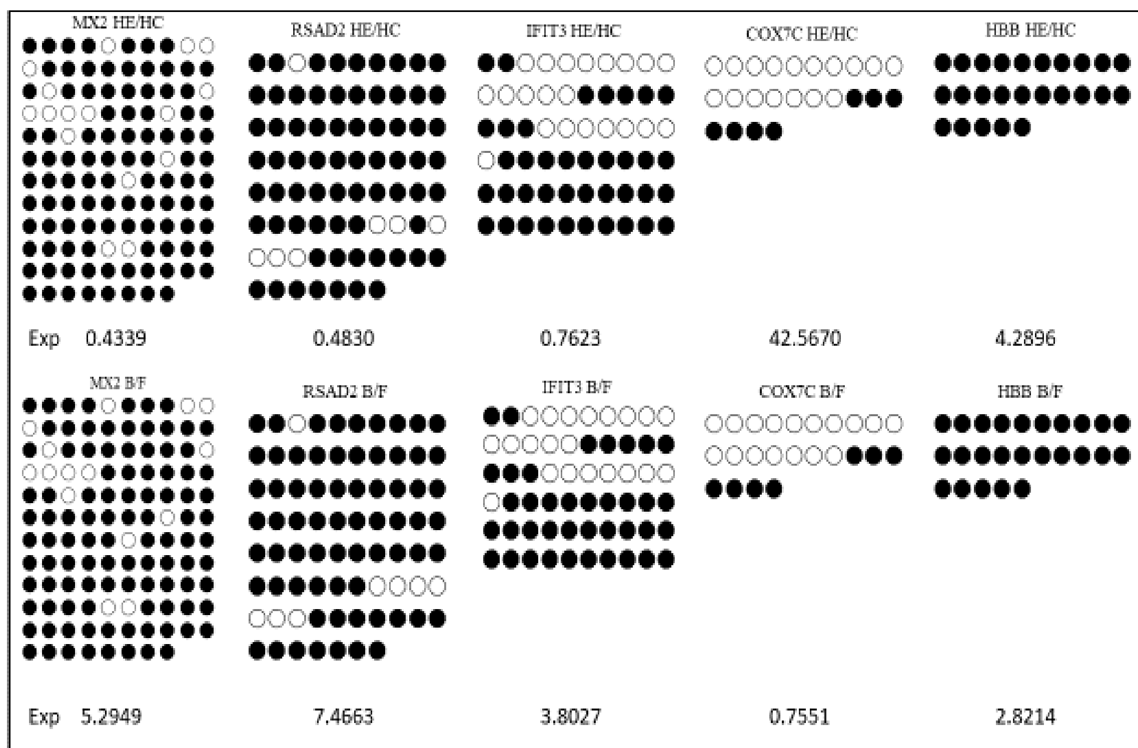
Figure 1. Feeding a Background/Finishing diet and High Energy/High Concentrate and their effects on average weights of steers through duration of study[a]



[a] Live weight measured on d 0, 28[b], 63[b], 84[b], 105[b], 131, 175 and 202

[b] $P < 0.05$

Figure 2. Bubble plots of the methylation of CpG sites in differentially expressed genes and mean gene expression values in Background/Finishing and High Energy/High Concentrate diets



Black dots represent methylation levels at CpG sites 50% or greater.

White dots represent methylation levels at CpG sites less than 50%.

Exp = Average gene expression levels of treatment from RNA sequencing data in FPKM.