DNA METHYLATION PATTERNS IN PRENATALLY STRESSED BRAHMAN FEMALES

A Dissertation

by

EMILIE CLARE BAKER

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Chair of Committee, David G. Riley
Committee Members, Thomas Welsh Jr
Ron D.Randel
Noushin Ghaffari
Head of Department, Andy Herring

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ABSTRACT

Stress-induced epigenetic modifications and the accompanying shifts in gene expression could be a potential mechanism behind the performance differences seen in prenatally stressed offspring. Two groups of cows were used to investigate the impact of prenatal transportation stress on DNA methylation patterns in Brahman females. One group was transported 5 times for 2-hour periods every 20-day starting from day 60 of gestation, while the other was maintained as a non-transport group. At 28 days old, blood was collected from heifer calves born from the two groups. At 5 years of age, cows were slaughtered, and the adrenal cortex, adrenal medulla, anterior pituitary, paraventricular nucleus of the hypothalamus, and amygdala were harvested from each cow. Ultimately 6 females born from the transport group (PNS) and 8 females born from the non-transport group (Control) were used to obtain methylation data.

In the DNA from leukocytes, 16,377 cytosine-guanine sites were differentially methylated in PNS females compared to the Control cows. Some differentially methylated sites were located within promoter regions of genes involved in important biological pathways. In amygdala tissue harvested at 5 years, there were only 29 differentially methylated sites between the PNS and the Control. Analysis of the overall methylation of specific genomic features (including genes) revealed 134 differentially methylated promoter regions, 202 gene bodies, and 133 cytosine-phosphate-guanine islands. Only 2 genes were differentially expressed between the two groups: *The solute carrier family 28 member 3* and *Fc fragment of IgG receptor IIa.* Neither gene had any differentially methylated regions associated with them. Inter-individual variability of DNA methylation was the greatest in the anterior pituitary gland for both groups, followed by the leukocytes and the amygdala with the least. There was minimal overlap of variably methylated genomic regions between tissues and treatment groups.

There were extensive differences in DNA methylation patterns from leukocytes harvested at 28 days in the prenatally stressed heifer calves relative to the control. However, at 5 years of age, the two groups had minimal differences in methylation patterns. The interindividual variation observed appears to be tissue specific. An interaction between the prenatal environment and cow genotype could be responsible for the differences in locations of variably methylated regions between the PNS and Control animals.

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This work was supervised by a dissertation committee consisting of Professors David G. Riley (advisor), Thomas Welsh Jr, Ronald Randel of the Department of Animal Science and Texas A&M AgriLife Research, Overton, TX, USA and Noushin Ghaffari from the Department of Computer Science, Prairie View A&M University, Prairie View, TX, USA.

Dr. Ronald Randel, Dr. Charles Long and Dr. Thomas Welsh Jr. designed the animal methodologies and provided the tissues for this research.

All other work conducted for the dissertation was completed by the student independently.

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1. INTRODUCTION

Prenatal stress has resulted in lasting phenotypic differences in livestock offspring, potentially influencing performance and overall profitability. In previous studies, prenatally stressed offspring have exhibited altered immune response (Merlot et al., 2013), behavioral differences (Littlejohn et al., 2016), and altered physiological response to stress (Lay et al., 1997). Stress-induced epigenetic modifications are a potential biological mechanism responsible for these alterations. Epigenetic modifications influence gene expression without changing the underlying DNA sequence and shift and respond to environmental stimuli, including the prenatal environment (Cao-Lei et al., 2020).

Prenatal stress has induced changes in the most common epigenetic modifications, DNA methylation. Methylation is the addition of a methyl group to the 5' carbon of the nitrogenous base cytosine. The addition or loss of DNA methylation throughout the genome can influence the binding of transcription factors, activity or retrotransposable elements, and even alternative splicing (Razin and Cedar, 1991). Prenatal stressors have altered DNA methylation patterns of the genes nuclear receptor subfamily 3 group c member 1 and corticotropin releasing hormone, both of which are important to the hypothalamic-pituitary-adrenal and, therefore, stress response (Sosnowski et al., 2018). Severe nutrient restriction throughout gestation has resulted in persisting differences in DNA methylation patterns of the Insulin Growth Factor 2 gene in humans (Heijmans et al., 2008). Offspring of mice exposed to a variable stress paradigm (restraint, swimming, and social situations) showed an increase in DNA methylation of the gene brain-derived neurotrophic factor and a decrease in gene expression within the amygdala (Boersma et al., 2014). The stress-induced changes in DNA methylation patterns and the gene expression shifts of genes such as these could be responsible for the differences observed in prenatally stressed livestock species.

Epigenetic modifications also have a role in promoting phenotypic variation (Triantaphyllopoulos et al., 2016). Interindividual variation of DNA methylation patterns have been observed within different human populations and in numerous tissue types, including blood mononuclear cells, neurons, and brain tissues (Flanagan et al., 2006, Shen et al., 2013). Genetics, tissue and the environment can contribute to inter-individual variation of DNA methylation patterns (Illingworth et al., 2015, Hannon et al., 2018). The interaction between genotype and prenatal environment can also explain portions of inter-individual variation (Teh et al., 2014). Regions with high inter-individual variation of DNA methylation patterns within Holstein bull spermatozoa overlap with numerous quantitative trait loci, including some important to health, reproduction, and meat and milk production in cattle (Liu et al., 2019). This suggests that variable DNA methylation patterns influence phenotypic differences of complex production traits in cattle.

Genome-wide analysis of various somatic tissues and the limbic system in cattle revealed tissue-specific differences in DNA methylation patterns (Zhou et al., 2016, Cantrell et al., 2019). However, the effect prenatal stress has on tissues such as those involved in the limbic system has not been investigated. Nor has the inter-individual variation of DNA methylation patterns of specific tissues of female cattle and the effect that prenatal stress might have on that variation been investigated. It has been documented that prenatal transportation stress altered genome wide DNA methylation patterns in Brahman bull calves (Littlejohn et al., 2018). This research aimed to continue the investigation of the impact of prenatal transportation stress on DNA methylation patterns of Brahman females across numerous tissues as well as the inter-individual variation of DNA methylation patterns.

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2. EFFECT OF PRENATAL TRANSPORTATION STRESS ON DNA METHYLATION IN BRAHMAN HEIFERS*

2.1. Introduction

Exposure to prenatal stressors can cause lasting phenotypic differences in offspring (Lay et al., 1997; Markham and Koenig, 2011; Tamashiro et al., 2009). Epigenetic modifications in an individual, such as DNA methylation, could induce phenotypic changes. Methylation is the addition of a methyl group to the 5' carbon of the cytosine base in the DNA sequence (Moore et al., 2013); this process has the potential to alter gene expression without changing that sequence. The methylome plays an important role in cellular development and differentiation (Suelves et al., 2016). The process of DNA methylation is dynamic and can change dramatically throughout bovine embryo and fetal development (Jingyue et al., 2019). Stressors experienced by the dam during gestation when development is occurring may change DNA methylation patterns of the offspring (Tachibana et al., 2015; Karlsson et al., 2018). Increased methylation in promoter regions corresponds with decreased gene expression (Razin and Riggs, 1980; Tate and Bird, 1993). Alternatively, gene body methylation may be involved in gene activation and stability (Aran et al., 2011; Hellman and Chess, 2007). Altered patterns of methylation caused by prenatal stress could alter biological pathways, resulting in differences in performance traits that are important to the beef cattle industry such as reproduction, stress response, and temperament. The effect of prenatal transportation stress on the methylome has been quantified and analyzed in Brahman bull calves (Littlejohn et al., 2018).

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*Reprinted with permission from "Effect of prenatal transportation stress on DNA methylation in Brahman heifers" by Baker, E. C., K. Z. Cilkiz, P. K. Riggs, B. P. Littlejohn, C. R. Long, T. H. Welsh, R. D. Randel, and D. G. Riley. 2020. *Livestock Science* 240:104116. doi: 10.1016/j.livsci.2020.104116.

Considering the female's importance in the cattle production industry, it is imperative that stress effects are characterized among heifer calves. Thus, the objective of this study was to investigate the effects of a prenatal environmental stressor on the DNA methylation patterns of Brahman heifers.

2.2 Methods and Materials

All procedures were in compliance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010) and approved by the Texas A&M University Animal Care and Use Committee.

2.2.1 Animal procedures

Experimental design and animal handling were described by Littlejohn et al. (2018). Cows were randomly assigned to groups with respect to age, parity and temperament score. The treatment group (n = 48) was transported for a duration of 2 h every 20 d ($\pm$ 5 d) from 60 to 140 d of gestation (Price et al., 2015). A group of non-transported cows was maintained as controls (n = 48). Both groups were managed together in the same pasture under identical nutrient conditions at the Texas A&M AgriLife Research and Extension Center at Overton (Littlejohn et. al 2018). The prenatal stress (**PNS**) treatment group consisted of 20 male and 21 female calves born to cows exposed to transportation stress during gestation, and 26 male and 18 female calves born to control cows (**Control**). At 28-d of age, heifer calves were restrained and 10 mL of blood was collected via vacuum tube (BD, Franklin Lakes, NJ) by venipuncture. For this study, 6 PNS heifers and 8 Control heifers that were used to obtain methylation data. The dams of heifers used in this study had no significant difference in age, parity or temperament.

2.2.3 Sample analysis

Extraction of DNA from leukocytes was accomplished as described by Littlejohn et al. (2018). In brief, purified DNA was suspended in 150-200 uL TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), and stored at -80 ºC until shipped on dry ice to Zymo Research Corp (Zymo Research; Irvine, CA) for DNA methylation analysis.

2.2.4 DNA methylation library

Reduced representation bisulfite sequencing was done through the Methyl-MiniSeq (Zymo Research; Irvine, CA) assay to assess DNA methylation in DNA from 6 PNS and 8 Control females. The bisulfite sequencing assay was directed by Zymo Research; this assay and associated laboratory procedures were detailed by Littlejohn et al. (2018).

2.2.5 Methylation analysis

Illumina base-calling software was used to sequence the bisulfite-treated libraries (Illumina, San Diego, California) . Sequence reads were analyzed via a Zymo Research analysis pipeline written in Python. The Bismark analysis program (http://www.bioinformatics.babraham.ac.uk/projects/bismark/) was used to align fragments to the UMD 3.1 bovine assembly (Zimin et al., 2009). Methylation occurs at Cytosine-phosphate-Guanine (**CpG)** sites, CHG, and CHH (C = cytosine; G = guanine; H = adenine, cytosine or thymine) sites. Methylation ratios were calculated by dividing the number of mapped cytosines by the total number of cytosines (either a C or a Thymine) unique to sites. Methylation differences were calculated as the average Control methylation ratio at a site from the average PNS methylation ratio at a site. Fisher's Exact Tests were performed for methylation differences for sites with minimum coverage of 5 reads. Sites were classified as hypermethylated (PNS 0 to 33% more methylated than Control), hypomethylated (0 to 33% less methylated than the control),

strongly hypermethylated (33 to 100% more methylated than the control) and strongly hypomethylated (33 to 100% less methylated than the control).

Annotation of significant sites was conducted as follows: Genomic intervals of introns, exons and promoter regions from the UMD 3.1 *Bos taurus* genome were exported from the University of California at Santa Cruz Main Table Browser into Texas A&M High Performance Research Computing's Maroon Galaxy (https://hprcgalaxy.tamu.edu/maroon/). The Operate on Genomic Intervals tool "Intersect" within Galaxy was utilized to identify the intervals in which the differentially methylated sites were located. Promoter regions were classified as 1,000 base pairs upstream of the transcription start site of a gene.

2.2.6 Pathway analysis

Differentially methylated sites *(*$P \leq .05$) located in promoter regions were evaluated with Ingenuity Pathway Analysis software (**IPA**; Redwood City, CA) to identify potentially affected canonical pathways and biological functions. Two analyses were performed; one with hypermethylated (at least 10% more methylated than the Control) sites and the other evaluated hypomethylated (at least 10% less methylated than the Control) sites. Significance of pathways and functions were probabilities from right-tailed Fisher's Exact tests. This probability, often referred to as the *P* value of overlap, represents the correspondence between the set of the given molecules (differentially methylated sites) and the pathways and biological functions.

2.3. Results and Discussion

2.3.1 Genome wide DNA methylation in PNS and control heifer calves

Prenatally stressed heifers had 16,378 differentially methylated CpG sites ($P \leq .05$) compared with Control heifers. Overall, 6,369 sites were identified as either being located within introns, exons, or promoter regions. Our findings are consistent with prior reports that large

fractions of methylated sites are located on CpG sites (Bird, 1980; Deaton and Bird, 2011). The remaining 10,009 sites were located in intergenic regions. Details of the distribution of classifications are presented in Table 2.1. Methylation at CHG and CHH sites can also have a biological impact (Gou et al., 2014; Ziller et al., 2011). Therefore, we assessed the distributions of differentially methylated CHG and CHH sites in leukocyte DNA from the heifers of this study (Table 2.1). In the present study, 309 CHG and 612 CHH sites were considered significant.

Zhou et al. (2016) reported results from reduced representation bisulfite sequencing of 10 bovine somatic tissues. In that work, global methylation levels within CG-enriched regions were distributed as 33.5% CpG, 1.1% CHG and 1.5% CHH. The 10 tissues were compared against each other to identify differentially methylated sites. The overwhelming majority (94.3%) of the differentially methylated cytosines reported by Zhou et al. (2016) were within CpG sites. Results of the present study were consistent: 94.7% of the differentially methylated regions were in CpG sites and the remainder in CHG or CHH sites.

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Table 2.1. Summary of genome wide distribution of differential DNA methylation across CpG, CHG and CHH sites in prenatally stressed (PNS compared to Control heifer calves).

			Hypomethylated[3]		Hypermethylated	
Site	**N[1]**	**%**	**Number**	**%**	**Number**	**%**
CpG[2]						
Promoter	1,137	16.7	450	39.5	687	60.4
Intron	3,639	53.8	1976	54.3	1663	45.7
Exon	1,596	23.6	643	40.2	953	59.7
Total	6,369		3,066		3,303	
CHG						
Promoter	9	9.68	0	0.0	9	100.0
Intron	73	78.50	18	24.7	55	75.3
Exon	11	11.83	1	9.01	10	90.0
Total	93		19		74	100.0
CHH						
Promoter	21	11.9	2	9.1	19	90.0
Intron	128	24.2	31	24.7	97	75.8
Exon	26	14.1	5	19.2	21	80.8
Total	175		38		137	

[1] Number of affected regions $P \leq 0.05$
[2] CpG = Cytosine-phosphate-Guanine, C= cytosine; G= guanine; H = adenine, cytosine or thymine
[3]PNS calves were hypomethylated (hypermethylated) when methylation ratios were ≤ 10% less (greater) than Controls

2.3.2 Differential DNA methylation in CpG sites

2.3.2.1 Promoter regions.

Methylation in promoter regions can lead to repressed transcription of genes (Tate and Bird, 1993), either through direct interference with DNA binding proteins or indirectly by binding with proteins that in turn block transcription (Razin and Riggs, 1980; Cedar, 1988; Boyes and Bird, 1991). This means that hypermethylation of promoter regions in PNS heifers could lead to downregulated genes compared to Controls. Alternatively, hypomethylated sites located in promoter regions could lead to upregulated genes in the PNS heifers. In promoter regions of genes, 1,137 differentially methylated CpG sites were identified. Of the differentially methylated sites in promoter regions, 681 were hypermethylated and 6 were strongly hypermethylated (33-100% more methylated than the control). The top 30 hypermethylated (lowest *P* values) and the 6 most strongly hypermethylated sites are presented in Tables 2.2 and 2.3, respectively. Two CpG sites

are located within promoter regions of the *Solute Carrier Family 1 Member 1* ***(SLC1A1)*** gene, and the *Solute Carrier Family 6 Member 2* ***(SLC6A2)*** gene. Both genes have been associated with neurological disorders such as schizophrenia, major depression and panic disorders (Buttenschon et al., 2013; Afshari et al., 2015).

A total of 441 hypomethylated sites and 7 strongly hypomethylated sites were identified in the PNS calves. The top hypomethylated sites (lowest *P* values) are listed in Table 2.4. The *Ephrin B1 (**EFNB1***) gene had a hypomethylated site within its promoter region. Prenatal stress has been shown to alter DNA methylation of *EFNB1* in other species, as well. Mouse pups whose dams were Serotonin1A receptor **(5-HT1AR**) deficient (considered as an adverse maternal environment) exhibited differential methylation of the ephrin genes (Oh et al., 2013).

The 6 strongly hypomethylated CpG sites that were located in promoter regions are listed in Table 2.5. Of these 6 sites, 2 were located in the promoter region of the *Zinc Finger DHHC Domain-Containing Protein 9* (***ZDHHC9)*** gene. Research in mice suggests that loss of *ZDHHC9* has the potential to disturb hippocampus function (Kouskou et al., 2018). While DNA methylation of *ZDHHC9* has not been extensively studied, the hypomethylation of the gene's promoter region in the PNS heifers could potentially alter *ZDHHC9* expression therefore impacting stress behavior and brain development.

2.3.2.2 Gene body regions. In the treatment group 5,325 differentially methylated CpG sites were located in gene body regions; 3,639 sites were located in introns and 1,596 located in exons, with a slight majority hypermethylated (56%). Methylation differences in these regions are important because changes in the patterns has the potential to influence alternate splicing, transcription silencing, and gene stability (Brenet et al., 2011; Jones, 2012; Maor et al., 2015). The top 30 (lowest P values) of the hypermethylated and hypomethylated regions located in gene body regions

are listed in AA-1 and AA-2, respectively. One site was located within the gene body of the Solute Carrier Family 30 Member 3 (SLC30A). While there has been no real focus on DNA methylation of SLC30A3 and schizophrenia, some studies have found a link between prenatal or early life stress and increased risk of schizophrenia in the offspring (Ellenbroek and Riva, 2003; Khashan et al., 2008). Prenatal transportation stress led to worse temperament in calves as compared to the non-transported group (Littlejohn et al., 2016). Methylation of SLC30A3 due to prenatal stress could contribute to the behavioral and temperament changes observed.

Table 2.2. Top 30 (P < 0.002; listed from smallest to largest P values) hypermethylated cytosine-guanine sites located within promoter regions of genes in prenatally stressed (PNS) compared with Control heifer calves.

		Mean methylation ratio		
BTA	**Mb**	**PNS[1]**	**Control**	**Gene Name**
18	34.6	0.17	0.00	*CKLF Like MARVEL Transmembrane Domain Containing; CMTM4*
1	83.5	0.15	0.10	*ALG3 Alpha-1,3- Mannosyltransferase; ALG3*
16	33.2	0.15	0.02	*Heterogeneous Nuclear Ribonucleoprotein U; HNRNPU*
15	24.7	0.34	0.11	*Ubiquitin Specific Peptidase 28; USP28*
10	46.2	0.22	0.02	*Ubiquitin Protein Ligase E3A; UBE3A*
6	108.7	0.21	0.03	*Phosphatidylinositol Glycan Anchor Biosynthesis Class G; PIGG*
1	81.7	0.19	0.02	*ETS Variant 5; ETV5*
23	3.1	0.16	0.02	*Zinc Finger Protein 451; ZNF451*
25	42.0	0.24	0.04	*Integrator Complex Subunit 1; INTS1*
15	59.0	0.2	0.03	*Leucine-Rich Repeat-Containing G Protein-Coupled; LGR4*
22	51.8	0.24	0.03	*SAC1 Like Phosphatidylinositide Phosphatase; SACM1L*
16	28.1	0.37	0.18	*Delta(4)-Desaturase, Sphingolipid; DEGS1*
18	62.5	0.20	0.03	*Ubiquitin Conjugating Enzyme E2 S; UBE2S*
11	21.6	0.18	0.02	*Cyclin Dependent Kinase Like 4; CDKL4*
2	107.9	0.24	0.04	*Autophagy Related 9A; ATG9A*
5	27.1	0.11	0.00	*SPRY Domain Containing 3; SPRYD3*
18	24.0	0.15	0.00	*Solute Carrier Family 6 Member 2; SLC6A2*
8	112.9	0.23	0.04	*Ribosomal Protein S7; RPS7*
1	110.3	0.11	0.00	*Short Stature Homeobox 2; SHOX2*
8	40.3	0.11	0.00	*Solute Carrier Family 1 Member 1; SLC1A1*
10	8.8	0.20	0.06	*Tubulin Folding Cofactor A; TBCA*
17	66.1	0.11	0.00	*Forkhead Box N4; FOXN4*
7	19.7	0.23	0.02	*Vimentin Type Intermediate Filament Associated Coiled-Coil Protein; VMAC*
14	36.5	0.16	0.02	*Lactamase Beta 2; LACTB2*
6	46.7	0.20	0.02	*Solute Carrier Family 34 Member 2; SLC34A2*
10	19.4	0.13	0.00	*ADP Dependent Glucokinase; ADPGK*
3	30.9	0.18	0.05	*Capping Actin Protein Of Muscle Z-Line Subunit Alpha 1; CAPZA1*
12	87.4	0.29	0.08	*Family With Sequence Similarity 155 Member A; FAM 155A*
14	3.1	0.19	0.05	*T-SNARE Domain Containing 1; TSNARE1*
9	91.4	0.36	0.10	*Microrna Mir-2480; MIR2480*

[1]PNS = Prenatally stressed

Table 2.3. Strongly hypermethylated (methylation difference ≥ 0.33 ratio) cytosine-guanine sites located within promoter regions of genes in prenatally stressed (PNS) compared with Control heifer calves.

		Mean methylation ratio		
Chr[1]	**Mb**	**PNS**[2]	**Control**	**Gene Name**
3	48.4	0.43	0.08	*RWD Domain Containing 3; RWDD3*
4	114.4	0.68	0.31	*Solute Carrier Family 4 Member 1; SLC4AS*
5	94.5	0.88	0.53	*Serine/Threonine Kinase Receptor Associated Protein; STRAP*
25	1.5	0.45	0.10	*Methionine Sulfoxide Reductase B1; MSRB1*
25	2.6	0.77	0.33	*Zinc Finger Protein 205; ZNF205*
X	83.2	0.61	0.23	*Histone Deacetylase 8; HDAC8*

[1]Chr = Chromosome
[2]PNS = Prenatally stressed

Table 2.4. Top (P < 0.002; listed from smallest to largest P values) hypomethylated cytosine-guanine sites located within promoter regions of genes in prenatally stressed (PNS) compared with Control heifer calves

		Mean methylation ratio		
Chr[1]	**Mb**	**PNS**[2]	**Control**	**Gene Name**
X	13.7	0.11	0.50	*Zinc Finger DHHC-Type Containing 9; ZDHHC9*
X	13.7	0.14	0.49	*Zinc Finger DHHC-Type Containing 9; ZDHHC9*
X	39.9	0.04	0.39	*B Cell Receptor Associated Protein 31; BCAP31*
X	39.9	0.04	0.39	*ATP Binding Cassette Subfamily D Member 1; ABCD1*
18	62.7	0.34	0.68	*Microrna Mir-7865**;** MIR7865*
2	107.7	0.19	0.53	*Crystallin Beta A2; CRYBA2*
21	33.6	0.44	0.80	*Outer Dense Fiber Of Sperm Tails 3 Like 1; ODF3L1*

[1]Chr = Chromosome
[2]PNS = Prenatally stressed

2.3.3 Differential DNA methylation in CHG sites

The majority of the 309 differentially methylated ($P \leq .05$) CHG sites (70%) in the treatment group were hypermethylated. Among those, 93 were annotated leaving 216 located in intergenic regions. The annotated regions included 73 sites within introns, 11 with exons, and 9 sites located in promoter regions. The 9 CHG sites that were located in promoter regions of genes were strongly hypermethylated (AA-3). One of these sites was within the *Heat Shock Protein 90 Alpha Family Class A Member* 1 (***HSP90AA1***). DNA methylation of *HSP90AA1* has been

identified in other livestock species. Salces-Ortiz et al. (2015) identified methylated regions of *HSP90AA1* promoter and gene body regions in DNA from leukocytes in adult sheep.

Table 2.5. Strongly hypomethylated (methylation difference ≤ − 0.33 ratio) cytosine-guanine sites located within promoter regions of genes in prenatally stressed (PNS) compared with Control heifer calves.

		Mean methylation ratio		
Chr[2]	**Mb**	**PNS[3]**	**Control**	**Gene Name**
8	11.2	0.00	0.26	*Transmembrane Protein 215; TMEM215*
12	32.9	0.05	0.32	*Ubiquitin Specific Peptidase 12; USP12*
1	66.2	0.02	0.20	*Syntaxin Binding Protein 5 Like; STXBP5L*
11	106.2	0.00	0.14	*Ucosyltransferase 7; FUT7*
12	32.9	0.05	0.28	*Ubiquitin Specific Peptidase 12; USP12*
20	70.9	0.00	0.23	*Iroquois Homeobox 4; IRX4*
12	32.9	0.05	0.28	*Ubiquitin Specific Peptidase 12; USP12*
25	0.2	0.00	0.14	*Hemoglobin Subunit Theta 1; HBQ1*
25	17.4	0.00	0.11	*Lysine Rich Nucleolar Protein 1; KNOP1*
23	39.8	0.02	0.21	*RNA Binding Motif Protein 24; RBM24*
12	32.9	0.06	0.31	*Ubiquitin Specific Peptidase 12; USP12*
6	64.8	0.00	0.25	*Potassium Channel Tetramerization Domain Containing 8; KCTD8*
5	44.8	0.03	0.18	*Cleavage And Polyadenylation Specific Factor 6; CPSF6*
X	126.4	0.10	0.27	*Bos taurus chromosome X CXorf58 homolog; CXCHORF58*
X	39.9	0.23	0.47	*ATP Binding Cassette Subfamily D Member 1; ABCD1*
X	39.9	0.18	0.47	*ATP Binding Cassette Subfamily D Member 1; ABCD1*
X	40.1	0.03	0.18	*Interleukin 1 Receptor Associated Kinase 1; IRAK1*
X	3.2	0.07	0.38	*LON Peptidase N-Terminal Domain And Ring Finger 3; LONRF3*
13	54.5	0.04	0.19	*Regulator Of Telomere Elongation Helicase 1; RTEL1*
X	87.0	0.28	0.48	*Ephrin B1; EFNB1*
12	32.9	0.07	0.32	*Ubiquitin Specific Peptidase 12; USP12*
X	39.9	0.24	0.51	*B Cell Receptor Associated Protein 31; BCAP31*
X	39.9	0.24	0.51	*ATP Binding Cassette Subfamily D Member 1; ABCD1*

[1]Chr = Chromosome
[2]PNS = Prenatally stressed

Eighty-four differentially methylated CHG sites were located within gene body regions (11 in exons and 73 within introns). Hypomethylated sites are shown in AA-4. The most significant differentially methylated site (lowest *P* value) was located in the *Iodotyrosine Deiodinase* (***IYD***) gene which has active role in thyroid hormone biosynthesis (Iglesias et al., 2014). Prenatal stress in other species has affected the *IYD* gene. Lambs whose dams were exposed to nutritional stress

(fed 50% of their daily energy requirements) showed increased expression of *IYD* in the thyroid gland at both 6 months of age and 2 years of age (Johnsen et al., 2013). The top 30 (lowest *P* values) hypermethylated CHG sites are listed in AA-5. One site is located within the *Homer Scaffold Protein 1 (**HOMER1**)* which has vast involvement in neurodevelopment (Foa and Gasperini, 2009). *Homer Scaffold Protein 1* expression has been affected by prenatal stress in rats. Rat pups whose dams were repeatedly restrained had altered expression of the *HOMER1* gene as compared to the control group (Ary et al., 2007).

2.3.4 Differential DNA methylation in CHH sites

In the treatment group, 612 differentially methylated CHH sites were detected ($P \leq 0.05$). Of those, 21 CHH sites were located in promoter regions; 19 were hypermethylated (AA-6). A hypomethylated site was also found within the *tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (**YWHAZ**)* gene on BTA 20, and one hypomethylated site within *Zyxin (**ZYX**)* on BTA 4. One hundred fifty-three sites were classified as gene body regions; 104 of those were classified as hypermethylated, 28 as hypomethylated, 14 as strongly hypermethylated, and 8 strongly hypomethylated. The top 30 (lowest *P* values) hypomethylated CHH sites located in gene body regions are shown in AA-7. The top 30 (lowest *P* values) hypermethylated CHH sites that were located in gene body regions are shown in AA-8. Among the genes with hypermethylated CHH sites was the *Neurexin 2* gene (***NRXN2***). Research in mice support the changes in methylation seen in the PNS heifers. Mice whose dams were immune-challenged (viral challenge) during gestation showed differential methylation of *NRXN2* (Richetto et al., 2017).

2.3.5 Biological functions and canonical pathways

All significant sites located in promoter regions of genes were input into IPA for analysis. The analyses were conducted in two groups, hypermethylated (at least 10% more methylated than the controls) and hypomethylated (at least 10% less methylated than the controls).

2.3.5.1 Endocrine system disorders. Four of the genes that contained hypomethylated sites within their promoter regions can contribute to abnormal function of the hypothalamus: *Dopamine Receptor D1* (*DRD1), Nuclear Receptor Subfamily 1 Group H Member 2* (*NR1H2*), *Orthodenticle Homeobox 1* (*OTX1*), and *Single-minded homolog 1 (SIM1*). The D(1A) is involved in dopamine-mediated stimulation of the hypothalamic-pituitary-adrenal axis (Borowsky and Kuhn, 1992). Prenatal stress has influenced gene function of *DRD1* in other species, such as mice and the Rhesus monkey (Converse et al., 2014; Sasagawa et al., 2017). The 11 genes that had hypermethylated sites in their promoter regions that are involved in the IPA group "pituitary dysfunction" are presented in Figure 2.1. Two of the genes involved are the *progesterone receptor gene* (*PGR*) and the *LIM-homeobox 3 (LHX3)*. Proper expression of both is important for pituitary function.

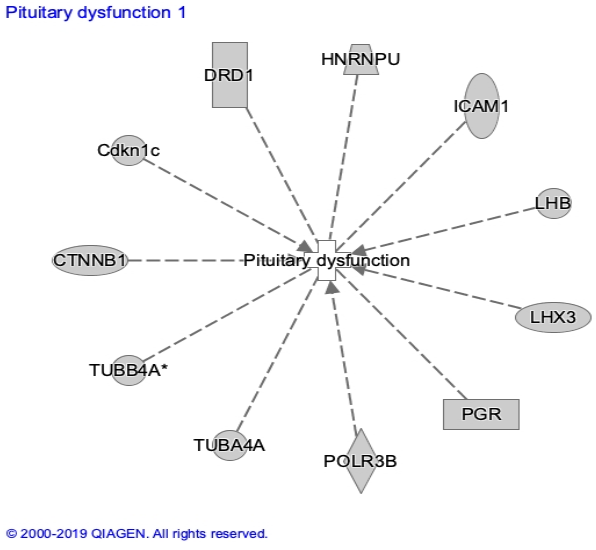

Figure 2.1. The 11 genes with hypermethylated (at least 10% more methylated than the control) genes within the promoter regions that contribute to pituitary dysfunction.

2.3.5.2 Psychological disorders. Hypomethylated and hypermethylated sites in promoter regions of genes have been identified to have roles in anxiety disorders, schizophrenia, major depression, and mood disorders (AA-9a&b). Prenatal stress may influence expression and function of neurotransmitters and neurohormones such as serotonin and dopamine, as well as stress-related genes and alleles therefore contributing to different psychological disorders. Epigenetic modification may affect gene expression after prenatal stress (Kuehner et al., 2019; McEwen, 2019). The results of the current study supported this hypothesis, as the PNS group DNA was differentially methylated at promoter regions sites in genes that contribute to psychological and behavioral disorders.

2.3.5.3 Neurological function and development. Many genes that had differential methylation located in promoter regions are involved in signaling pathways related to neurological function and development (AA-10a). The "GABA Receptor Signaling" pathway and the "Axonal Guidance Signaling" pathway are two of the significantly pathways in PNS calves as compared to the control group; this was consistent with results in Brahman bull calves of this study (Littlejohn et al., 2018). Gamma-aminobutyric acid (GABA) receptors play a key role in neuron development and function in the brain. Prenatal stress in mice has caused changes in the GABA receptors in the frontal cortex of the brain leading to increased anxiety-like behavior in the adult mice (Lussier and Stevens, 2016). There are 27 genes that had hypermethylated sites in the promoter regions that supported the pathway "Axonal Guidance Signaling" (AA-11). Previous studies have revealed that DNA methylation can also influence this pathway. Analysis in individuals with post-traumatic stress order showed reduced methylation of genes involved in axon signaling (Martin et al., 2017).

Another supported canonical pathway relative to neurological function is the "Corticotrophin Releasing Hormone Signaling" pathway. Nine hypermethylated sites were

identified in promoter regions of genes included in the "Corticotrophin Releasing Hormone Signaling pathway" (Figure 2.2). Integral regulation of corticotrophin release in conjunction with stress related behaviors and stress response of the hypothalamic-pituitary-adrenal axis occurs via this pathway (Stengel and Tachè 2010). Prenatal and early life stress (separation from dams) altered signaling and methylation patterns (both hypermethylation and hypomethylation) in this gene network in mice (Chen et al., 2017 Wang et al., 2014). The results of the current study provide support that prenatal stress can affect DNA methylation of genes involved in stress response pathways such as the "Corticotrophin Releasing Hormone Signaling" pathway.

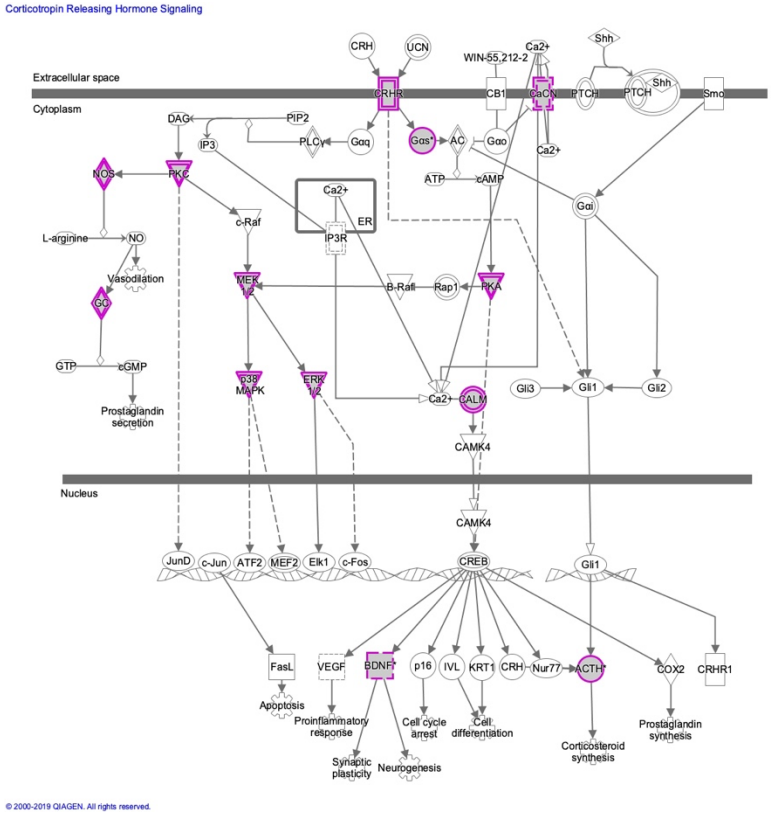

Figure 2.2. Corticotrophin Releasing Hormone Signaling: Genes highlighted pink had differentially methylated cytosine-guanine sites located within the promoter regions.

2.3.5.4 Cellular development and communication. Many of the genes with differentially methylated sites in the promoter regions are involved in biological pathways that are important for cellular development and communication, the PCP (planar cell polarity) pathway being one.

Prenatal stress in mice also caused multiple genes involved in the PCP pathway to be hypomethylated in the folate deficient group (prenatally stressed) compared to a control group (Geng et al., 2018). The "Neurotrophin/TRK Signaling" and the "CDK signaling pathway" were both among the significant pathways related to cellular development and communication. Prenatal and early life stress in mice lead to altered expression of genes in both pathways. Early life stress by way of iron deficiency in mice resulted in altered gene expression in the hippocampus of genes that are involved in neurotrophin/TRK Signaling (Barks et al., 2018). Prenatal exposure to alcohol led to decreased concentrations of CDK5 (Cyclin Dependent Kinase 5) in the frontal cortex of the mouse offspring (Goggin et al., 2014)

Lastly, "Gap Junction Signaling" is among the top 5 canonical pathways with respect to hypomethylated genes. Mice exposed to long-term stress also showed altered methylation patterns and gene expression in the medial prefrontal cortex genes involved with gap junction signaling (Mychasiuk et al., 2016). The "Gap Junction Signaling" pathway was also among the significant pathways found in the PNS bull calf comparison (Littlejohn et. al 2018). It is clear that prenatal and early life stress has an immense impact on gap junction signaling.

2.3.5.5 Immune response. Prenatal stress is known to alter immune function in the offspring (Veru et al., 2014). Children of mothers that were in Quebec during the 1998 ice storm showed different methylation patterns in T-cells. Pathway analysis revealed that the many (6 out of the top 10) of the altered pathways were involved in immune function (Cao-Lei et al., 2014). Brahman bull calves showed altered immune response to an endotoxin challenge compared to a non-transported group (Littlejohn et al., 2019). Pathway analysis in the PNS heifer calves revealed genes involved in the "IL-8 Signaling pathway" and "NF-kB Signaling pathway" had sites that were differentially methylated when compared to the Control group.

2.3.6 Genome wide methylation in prenatally stressed Brahman heifers and bulls

Genome-wide methylation comparisons were performed in Brahman bull calves that were from the same treatment groups as the present study (Littlejohn et al., 2018). Prenatal stress has caused sex specific responses in immune function, metabolism and behavior (Jerrels, 1991; Brunton et al., 2013; Iturra-Mena et al., 2018). Changes in methylation patterns after prenatal stressors can be also be sex specific/dependent. (Tobi et al., 2009; Bale 2017).

The overall distribution of differentially methylated sites was similar in heifer calves and bull calves. In heifer calves, 17,298 sites were differentially methylated (CpG, CHG, and CHH) with 94.67% being CpG sites, 1.79 % being CHG sites, and 3.54% being CHH sites. In the bull calves, a total of 16,745 sites were differentially methylated, with 96.32% being CpG sites, 1.31% CHG sites and 2.34% being CHH sites (Littlejohn et al., 2018). Both distributions are very similar to the average DNA methylation distribution of 10 bovine somatic cells (Zhou et al., 2016).

2.3.6.1 CpG site comparison. The number of CpG sites that were differentially methylated in heifer calves is close to the number observed in results from the bull calves (Littlejohn et al., 2018). The gene annotation distribution was also similar; 61.11% of the CpG sites in the heifer calves were located in intergenic regions vs 65.5% in bull calves. The distribution of hypermethylated CpG sites (47.2%) and hypomethylated sites (52.8%) in heifer calves was close to the distribution that was seen in bull calves: 45.93% hypermethylated, 54.07% hypomethylated. The greater amount of hypomethylated sites in males exposed to prenatal stress as compared to females is consistent with sex differences reported in humans (Broberg et al., 2014). In that work, males with prenatal arsenic exposure had more CpG sites with lower DNA methylation compared to the females that underwent the same treatment.

The distribution of significant CpG sites by chromosome of the PNS males and females are shown in Figure 2.3. The number of sites is fairly even across the 30 chromosomes except in BTA 11 where the males had 184 more sites than the females, and the X chromosome; the females had 756 more significant CpG sites than the males. Sex differences in DNA methylation on the X chromosome have been characterized in humans and are thought to play a role in X-inactivation (Cotton et al., 2014; Martin et al., 2017). Among all significant CpG sites in the PNS males (16,128) and PNS females (16,377) 267 of the sites were differentially methylated at the exact (same chromosome and coordinate) site in both males and females. While the site of methylation was shared the direction (hypermethylated or hypomethylated) was not always the same. Out of the 267 sites, 155 were methylated in the same direction. There were 51 sites that were hypomethylated (at least 10% less methylated than the Controls) in the PNS heifers that were hypermethylated (at least 10% more methylated than the Controls) in the PNS bulls. Alternatively, there were 61 sites that were hypermethylated in the PNS heifers and were hypomethylated in the PNS bulls.

No similarity was observed in genes that had strongly hypermethylated or strongly hypomethylated CpG sites in promoter regions between the bull and heifer calves. None of the most strongly supported 30 genes (CpG sites in promoters or in gene body regions) either hypermethylated or hypomethylated of the categories for bull and heifer calves overlapped.

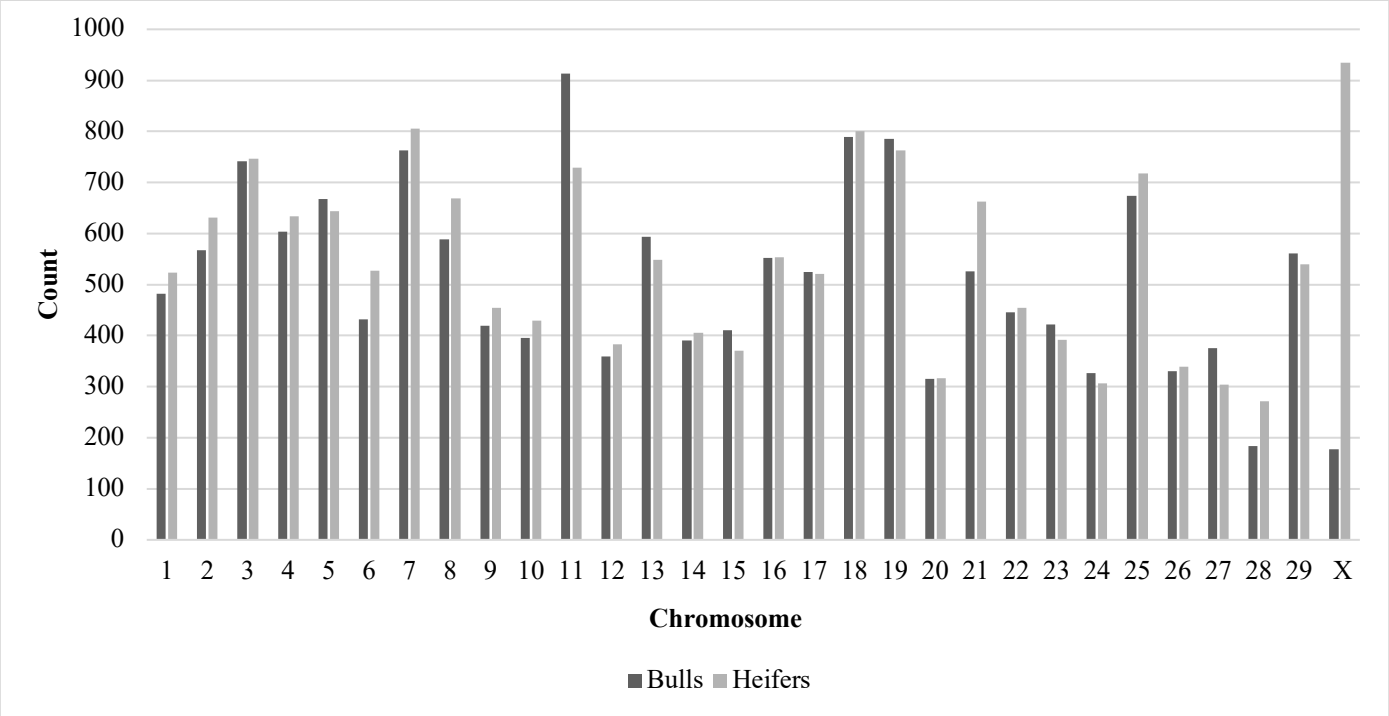

Figure 2.3. Distribution of significant cytosine-guanine sites by chromosome for prenatally stressed bulls and heifers..

2.3.6.2 CHG site comparison. The number of differentially methylated CHG sites in heifer calves was larger than bulls (309 versus 226, respectively), as well as percentage hypermethylated (68.9% and 47.8%, respectively). Comparison of site distribution by chromosome is presented in AA-12. More variation between the CHG site distribution was observed in PNS heifers vs PNS bulls as compared to the CpG sites; this may be because of the smaller number of significant sites. Out of all significant sites, only 2 CHG sites were located in the same exact position. Those two sites were hypomethylated in both PNS heifers and PNS bulls.

No overlap was observed in the genes that contained differentially methylated CHG sites in the promoter regions (hypermethylated or hypomethylated) between the heifer calves and the bull calves. In the top 30 (lowest *P* values) hypermethylated CHG sites located in gene body regions, three genes were identified in both groups: *Regulator Of G Protein Signaling 7* (RGS7),

Integrator Complex Subunit 1, and *GDP-Mannos 4,6-Dehydratase* (*GMDS*), although the location of the significant CHG site was not the same. Out of the 30 most strongly supported hypomethylated CHG sites in gene bodies, a single gene (but not the same site) was common for heifers and bull calves: *Ring Finger Protein 122* (RNF122).

2.3.6.3 CHH site comparison. In heifers, 612 CHH sites were differentially methylated in comparison to 309 in the bull calves. The majority of the sites in the heifers were hypermethylated (68.3%), which was higher than that observed from the bull calves (53.54%). Comparison of CHG site distribution by chromosome is presented in AA-13. The PNS heifers had almost twice the amount of significant CHH sites compared to the PNS bulls and that was seen throughout the chromosomal distribution comparison. Out of all significant CHH sites, there were only 6 sites that were located in the same location in both the PNS heifers and PNS bulls. Three of those sites were hypermethylated in the PNS heifers but hypomethylated in the PNS bulls, two of the sites were methylated in the same direction, and one site was hypomethylated in the PNS heifers that was hypermethylated in the PNS bulls.

The Zyxin gene contained a CHH site located in a promoter region in both the heifer and bull comparison, however the *ZYX* chromosomal coordinate was not the same between the sexes. No overlap was observed in genes that were associated with the top 30 (lowest *P* values) hypermethylated CHH sites within gene bodies of the bulls and heifers. One gene, *Tumor Necrosis Factor Receptor Superfamily 10D* (*TNRSF10D*) was identified in the top 30 of both comparisons. The location of the hypomethylated CHH site within *TNRSF10D* is not the same coordinate in the heifer calves as the bull calves.

2.3.6.4 IPA comparison Prenatal transportation stress altered the methylome pattern of both bull and heifer calves. Several of the significant canonical pathways identified through IPA analysis were shared by the bull and heifer calves, however, not all of the genes involved in the shared pathways were the same. The similarities between the two sexes suggests that prenatal stressors affect both sexes in similar ways. However, multiple pathways and genes with differentially methylated sites were unique with respect to sex. These differences may be responsible for the sex specific responses that have been reported in previous prenatal stress experiments. Further analysis using the same statistical methods as this experiment between the PNS heifers and PNS bulls could reveal sites that are differentially methylated between the two treatment groups. Those results could provide more information on the sex specific nature of the impact of prenatal stress on the epigenome. Although the pathways had genes that were differentially methylated in both PNS bulls and PNS heifers, the severity of response may differ between the two. Many of those pathways are involved in neurological and cellular development as well as cellular communication.

2.4. Conclusion

Results presented are one of the first genome wide assessments of DNA methylation in leukocytes of prenatally stressed heifer calves. The genes in which the differentially methylated sites were located are involved in canonical pathways that relate to behavior, cellular communication and development, neurological signaling and development, metabolism and immune response. It is likely that prenatal stress perturbs tissue specificity. A combination of methylation quantification and gene expression may highlight fundamental combinations of effects of the DNA methylation changes caused by prenatal stress. Similarities are found between the sexes in the distribution of differentially methylated sites and the significant canonical

pathways. In regard to the location of the differentially methylated sites, there was little in common between the PNS vs Control comparison in bull calves and the PNS vs Control in heifer calves. (Littlejohn et al., 2018). Direct comparison of methylation profiles of the PNS bulls with PNS heifers may clarify sex differences; thereby, providing further insight to the mechanisms governing alterations and responses to prenatal stress.

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3. DNA METHYLATION PATTERNS AND GENE EXPRESSION FROM AMYGDALA TISSUE OF MATURE BRAHMAN COWS EXPOSED TO PRENATAL STRESS*

3.1 Introduction

The amygdala is a cell mass composed of nuclei that are classified into three cell groups located in the temporal cortex of the brain: 1) the basolateral amygdala; 2) cortical like cells; and 3) centromedial cells (Yang and Jian-Zhi, 2017). The cell groups have neural connections that receive stimuli from areas of the brain including the sensory cortex, the prefrontal cortex, and the hippocampus. It is through those connections the amygdala processes and influences emotions including fear, anxiety, and stress response (Rasia-Filho et al., 2000; Davis and Whalen, 2001). Loss of amygdala function causes emotional based memory loss and aberrant social behavior (Fine and Blair, 2000). In contrast, increased amygdala activity has been linked to various disorders including schizophrenia and bipolar disorder (Lawrie et al., 2003; Kalmar et al., 2009). Increased activation of amygdala neurons can increase vigilance, anxiety, and stress.

The amygdala is a part of the body's system for detecting stressful and frightening stimuli and then initiating the body's coping response (LeDoux, 1994). Chronic stress can cause increased anxiety and behavior changes potentially due to hyperexcitability of the amygdala (Rosenkranz et al., 2010). Prenatal stress influences how the amygdala functions by shaping the development and connectivity within it and the tissues it communicates with (Kraszpulski et al., 2006; Scheinost et al., 2016). Shifts in gene expression in the amygdala of prenatally stressed offspring have been observed in mice and sheep (Ward et al., 2000; Petit et al., 2015).

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The shifts of gene expression in the amygdala may be responsible for the behavioral differences observed in prenatally stressed offspring. Prenatally stressed rhesus monkeys showed altered social behavior including a decrease in play and an increase in clinging to others. When alone the prenatally stressed monkeys showed more inactively relative to those who did not experience prenatal stress (Park et al.,1996). Calves subjected to prenatal transportation stress showed an increase in exit velocity from a restraining chute as well as an increase in temperament score (Littlejohn et al. 2016).

Gene expression shifts in the amygdala of prenatally stressed animals could result from stress-induced DNA methylation alterations. Prenatal stress in Brahman cattle resulted in changes of DNA methylation patterns of leukocytes from 28-day old bull and heifer calves, with differences persisting through 5 years of age (Littlejohn et al., 2018; Baker et al., 2020; Cilkiz et al., 2021). Shifts in DNA methylation patterns have been linked to prenatal stress and changes in temperament of the offspring (Littlejohn et al., 2016, 2018; Gartstein and Skinner, 2018). Methylation of DNA is the addition of a methyl group to the nitrogenous bases in the DNA sequence. In mammals, the addition of the methyl group often occurs to the 5' carbon of the nitrogenous base cytosine (Razin and Riggs, 1980). Methylation is primarily found within Cytosine-Phosphate-Guanine (CpG) dinucleotides. Methylated cytosines can lead to inhibition of gene expression, while demethylation can promote gene expression. (Tate and Bird, 1993). The methylome changes drastically throughout fetal development and therefore can be influenced by maternal environment. Methylation patterns continue to change postnatally (Salpea et al., 2012). These stress-induced epigenetic modifications can be transgenerational and have the potential to affect many generations in the production system (Feeney et al., 2014; Thompson et al., 2020).

In cattle, the amygdala tissue had the highest percent genome wide DNA methylation relative to other tissues in the limbic system (Cantrell et al., 2019). Considering the amygdala's important role in behavioral and stress response, modifications to the DNA methylation patterns and gene expression within the amygdala could cause phenotypic differences in the offspring. The long-term phenotypic changes observed in prenatally stressed livestock, including temperament changes, can impact production, animal welfare and profitable traits (Lay et al., 1997; Cooke, 2014; Serviento et al., 2020). Suckling calves that were exposed to prenatal stress were more temperamental and have a greater serum cortisol concentration than control calves. The early life difference in serum cortisol concentration appears to have been sustained cows selected for harvest at 5 years of age (Control: 29.5 +/- 9.8 ng/mL; Prenatally Stressed: 40.34 +/-5.2 ng/mL).

Early life alterations in DNA methylation patterns in humans has had measurable effects on behavior and is associated with depression and anxiety (Vonderwalde, 2019). The effects of prenatal stress on methylation and gene expression patterns in the amygdala has been well studied in mice, but less so in our livestock species (Kundakovic and Jaric, 2017). Thus, the objective of this study was to investigate whether prenatal stress alters DNA methylation and gene expression in the amygdala of 5-year-old prenatally stressed Brahman cows relative to Control cows.

3.2 Methods and Materials

All procedures were done in compliance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2020), and its earlier versions, and approved by the Texas A&M AgriLife Research Animal Care and Use Committee.

3.2.1 Animal Procedures

Details of the experimental design and animal handling were described in detail in Littlejohn et al. (2016; 2018) and Cilkiz et al. (2021). Briefly, 96 cows were determined pregnant

by rectal palpation 45 days after the breeding date. Cows were then assigned randomly to groups with respect to age, parity, and temperament assessment. The treatment group (n = 48) was transported for a duration of 2 hours on 60 ± 5, 80 ± 5, 100 ± 5, 120 ± 5, and 140 ± 5 d of gestation (Price et al., 2015). The physiological and metabolic variables measured in the PNS cows were: vaginal temperature (recorded by use of an indwelling vaginal temperature monitoring device), the percentage of weight lost (shrink), and serum concentrations of cortisol and glucose. The dams of the cows used in the present study experienced significantly increased vaginal temperature, shrink, and serum concentrations of cortisol and glucose in response to the transportation events. The findings of Price et al. (2015) reaffirmed our prior reports that transportation constitutes a stressor for pregnant cattle and thereby could influence post-natal development and physiology (Lay et al., 1996; Chen et al., 2015). A group of non-transported cows (n = 48) was maintained as a control. Both groups were managed together under the same nutrient and environmental conditions at the Texas A&M AgriLife Research and Extension Center at Overton.

Twenty bull calves and 21 heifer calves were born from the transported cows (PNS), and 26 bull and 18 heifer calves were born to cows that had not been transported (Control). The 39 calves entered a development regimen typical of cows in the herd, which included exposure to bulls for mating at 1 year of age and annually thereafter. Of those females remaining when the group was 5 years old, 8 Control and 6 PNS nonpregnant cows were slaughtered and the whole amygdala from each was harvested and stored at –80°C.

3.2.2 RNA and DNA Extraction

Frozen amygdala tissue samples were submitted to the Texas A&M Institute for Genome Sciences and Society (TIGSS) Experimental Genomics Core Laboratory for RNA sequence analysis. The TRIzol Plus RNA Purification Kit (Thermo Scientific, Waltham, MA) was utilized

to extract purified RNA from each amygdala sample (approximately 20 mg per sample). Quantification of purified RNA was performed with the Qubit RNA Fluorometric Assay Kit (Thermo Scientific) and the quality was assessed using the RNA ScreenTape Assay (Agilent Technologies, Santa Clara, California, USA). The RNA was prepped and sequenced with the HS protocol of the Illumina TruSeq Stranded mRNA library preparation kit and mRNA isolated with globin and ribosomal RNA depletion. Paired-end sequencing by the NovaSeq 6000 Sequencing System Illumina Inc.) produced raw RNA FASTQ files as the final output. The FASTQ file format is a text-based format use to represent both a biological sequence (DNA or RNA) and its corresponding quality scores (Cock et al., 2010)

Approximately 20 mg of each amygdala tissue sample were digested to extract DNA for methylation analysis. First, 150 µL of sodium chloride-Tris-EDTA buffer, 25 µL of Proteinase K (20mg/mL) and 25 µL 20% sodium dodecyl sulfate were added to the microcentrifuge tube containing the tissue and gently mixed. Samples were then incubated in a 56°C water bath for 2 hours, then 20 µL of RNAse A (10 mg/mL) were added to the sample tubes and the mixture was incubated at 37˚C for 30 minutes. Purified DNA was isolated from the digested amygdala tissue using the protocol for the GeneJET Genomic DNA Purification Kit (Thermo Scientific). Once purified, DNA was quantified with a NanoDrop Spectrophotometer (NanoDrop Technologies, Rockland, DE) and stored at –80˚C until further analysis.

3.2.3 DNA Methylation Library Prep and Sequence Alignments

Isolated DNA was submitted to Zymo Research (Irvine, CA) for reduced representation bisulfite sequencing methylation analysis. Input DNA was digested with 60 units of TaqαI followed by 30 units of MspI, and then purified with DNA Clean & ConcentratorTM-5. Purified DNA was then ligated to adapters containing 5'-methyl-cytosine. Adapter-ligated fragments of

150 to 250 bp and 250 to 350 bp were recovered using the ZymocleanTM Gel DNA Recovery Kit. Fragments were then bisulfite-treated using the EZ DNA Methylation-LightningTM Kit followed by preparative-scale PCR and purification.

Standard Illumina base calling was used to identify sequence reads from bisulfite-treated libraries and the raw FASTQ files were trimmed with the TrimGalore 0.6.4 software based upon adapter content and quality. The trimmed sequences were then aligned to the *Bos taurus* genome (ARS-UCD1.2) using Bismark 0.19.0 (Babrahman Bioinformatics, Cambridge, United Kingdom). Methylated and unmethylated read totals at each Cytosine-Phosphate-Guanine (CpG) site were quantified from the aligned binary alignment map (BAM) files using MethylDackel 0.5.0 (Zymo Research).

3.2.4 DNA Methylation Analysis

3.2.4.1 Feature specific. Overall methylation of defined features was compared between the PNS and Control groups. The features analyzed included gene bodies, promoter regions (defined as 1000 bp upstream to 500 bp downstream of the transcription start site), and CpG islands. These features are CpG rich areas of the genome that are vital to epigenetic regulation (Papin et al., 2021). Binary alignment map files that were produced by Zymo Research were read into the SeqMonk program (Babrahman Bioinformatics, Cambridge, United Kingdom). Each feature type was defined, and a bisulfite feature methylation pipeline (SeqMonk) was applied with the requirement of the sites within the feature to have at least 5x coverage, a threshold utilized in other livestock methylation studies (Livernois et al., 2021). Reduced representation bisulfite sequencing can provide accurate analysis at lower coverage, allowing for more biological replicates (Ziller at al., 2015, Crary-Dooley et al., 2017). The pipeline calculates a percentage methylation for each cytosine within the feature and averages these to give an overall value. After

the quantification pipeline was applied, a logistic regression was fit, and chi square tests for each feature was performed with the contrast of Control minus PNS. Because this experiment was a very early investigation of methylation in this tissue and species, the false discovery rate (Benjamini and Hochberg, 1995) was controlled at 0.15.

3.2.4.2 Genome wide methylation. Individual CpG sites across the genome, that is, without regard to predefined features, were also tested. Using the information provided by the methylation call tables the total coverage count, percent methylation, methylated counts, and unmethylated counts were calculated. Sites were filtered in edgeR (Robinson et al., 2010) by requiring 5x coverage at the site in all 14 samples as well as removing sites that were always methylated or unmethylated. A negative binomial generalized linear model was fit to the methylation counts for each site, and a likelihood ratio test was performed at each site using the contrast of Control minus PNS. The false discovery rate (Benjamini and Hochberg, 1995) was controlled at 0.15. Locations in the genome of the significant site were identified using Ensembl BioMart tool, Ensembl Release 104 (Howe et al., 2021). Multi-Dimensional Scaling (MDS) analysis and plotting were conducted utilizing the M values. The M values are the base 2 logit transformation of the proportion of methylated to unmethylated signal at each locus.

3.2.5 RNA Sequence Analysis and Differential Gene Expression

Raw RNA FASTQ files were subjected to a 3-step pipeline to generate gene counts. The Trim Galore program (Babrahman Bioinformatics) was used to remove any remaining adapter content. The Spliced Transcripts Alignment to a Reference (STAR) (Dobin et al., 2013) program was used to first create an index file using the ARS-UCD1.2 genome assembly. The trimmed reads were then aligned to the index using the default STAR parameters which had been optimized for alignment of mammalian genomes (Dobin et al., 2013). The BAM files produced by STAR were

subjected to procedures of the HTSeq program (Anders et al., 2015) to generate gene counts for each sample.

Differential gene expression analysis was performed in edgeR using a matrix consisting of gene counts from each sample. Genes with no counts were filtered and the remaining counts were normalized using the trimmed mean of M-values method. Tagwise dispersion was calculated, and a negative binomial generalized log-linear model was fit to the read counts for each gene. Finally, a likelihood ratio test corresponding to each gene was calculated with a contrast of Control minus PNS. The false discovery rate was controlled at 0.15 (Benjamini and Hochberg, 1995). Multi-dimensional scaling analysis and plotting were calculated utilizing the normalized read counts.

3.2.6 Cell Processes and Pathway Identification

Further analysis of the significant features and differentially expressed genes was conducted with the PANTHER Classification System 16.0 (Thomas et al., 2003) to identify cellular processes and biological pathways corresponding to identified genes.

3.3 Results and Discussion

3.3.1 DNA Methylation

3.3.1.1 Feature Specific-Bodies. Gene bodies of 26,900 genes were assessed for methylation status. Of those, 202 were differentially methylated between the PNS and Control (FDR ≤ 0.15), with 104 having increased methylation in the PNS group and 98 having decreased methylation (AB-1). The top 10 differentially methylated genes in amygdala tissue of prenatally stressed mature Brahman cows relative to Control cows are presented in Table 3.1. A heatmap of the mean methylation levels of the 202 differentially methylated genes in each sample is presented in Figure 3.1. Gene body methylation can lead to a decrease in gene expression which can then impact cellular processes (Klose and Bird, 2006). Through use of the Panther Classification System,

numerous cell processes and biological pathways, including response to stimulus, growth, and metabolic processes, were associated with the differentially methylated genes (AB-2). *Dual Specificity Phosphatase 26* (*DUSP26*) is active in the oxidative stress response biological pathway, which, in the amygdala contributes to pain response and pain related behavior (Sagalajev et al., 2018). Another highlighted pathway is the ubiquitin proteasome pathway, which is involved in the formation of fear memory within the amygdala (Jarome et al., 2011). Deviations in methylation patterns of genes involved in these pathways could result in altered response to fear and pain in animals.

Table 3.1. Top 10 differentially methylated genes in amygdala tissue of prenatally stressed mature to Control cows.

Gene Name	Chr	FDR	Difference[1]
Eukaryotic translation initiation factor 5A2	1	0.044	15.86
Homeobox D1	2	0.061	11.45
Centrosomal protein 41	4	0.044	25.61
Salvador family WW domain containing protein 1	10	0.003	-17.05
Brain expressed associated with NEDD4 1	18	0.003	15.87
Translocase of outer mitochondrial membrane 40	18	0.045	17.81
5S ribosomal RNA	21	5.68E-18	-3.34
Ornithine aminotransferase	26	0.047	12.57
5.8S ribosomal RNA	27	0.003	-1.00
Mitochondrial ribosomal protein L21	29	0.044	29.03

[1]A positive (negative) difference indicates the prenatally stressed cows had decreased (increased) methylation of the gene relative to the Control cows.

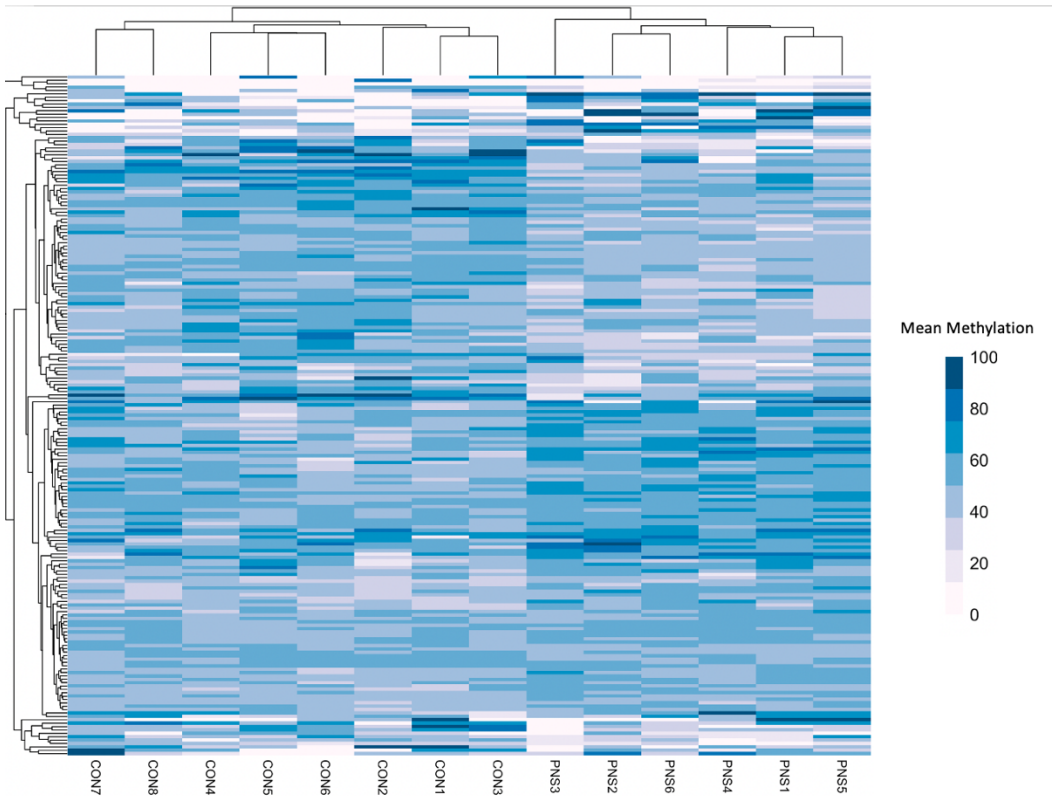

Figure 3.1. Heatmap showing the mean methylation of the differentially methylated (n = 202) genes in the Prenatally Stressed (PNS) cows and the Control (CON).

3.3.1.2 Feature Specific--Promoter Regions. A total of 134 gene promoters were identified as differentially methylated (FDR ≤ 0.15) in the amygdala tissue of PNS cows when compared to the Control group. Seventy promoter regions had increased methylation in the PNS group, and 64 had decreased methylation (AB-3**)**. The top 10 (lowest FDR value) differentially methylated promoter regions are presented in Table 3.2. Methylation shifts in promoter regions of genes impact gene expression mainly by influencing the accessibility of the promoter region to transcription factors (Klose and Bird, 2006). One stress-related gene that had increased methylation in its promoter region was *brain derived neurotrophic factor* (*BDNF*). This gene is critical for neural development and function of the amygdala. Alterations of methylation patterns of *BDNF* have been associated with increased anxiety behavior in rats and psychiatric disorders in humans (Redlich et al., 2019).

Increased methylation of *BDNF* was observed in individuals that experienced early life stress (Doherty et al., 2016; Blaze et al., 2017). Because of the relationship between *BDNF*, aberrant behavior, and changes in DNA methylation, the methylation of *BDNF* is considered a potential biomarker for early life stress in mammals (Kundakovic et al., 2014). Changes in methylation of this gene could be responsible for the temperament differences that have been observed in prenatally stressed livestock. The stressed group also had decreased methylation of the promoter region of *synapse 1* (*SYN1*). This gene has a role in synaptic function in the amygdala. Male mice who were exposed to early life stress showed an increase in synapse formation and altered synaptic responses (Guadagno et al., 2020). Shifts in gene expression *SYN1* because of methylation changes could result in altered brain plasticity in the prenatally stress cows. Rats subjected to early maternal separation exhibited increased methylation of *SYN1* (Park et al., 2014) which is contrary to our results where decreased methylation was reported.

Table 3.2. Top 10 differentially methylated promoter[1] regions of genes in amygdala of prenatally stressed mature Brahman cows relative to Control cows.

Gene Name	Chr	FDR	Difference[1]
Oxysterol binding protein like 8	5	0.005	9.88
RNA terminal phosphate cyclase like 1	8	0.001	-13.90
Maspardin	10	0.0002	23.28
WD repeat domain 34	11	0.005	10.08
Crumbs cell polarity complex component 1	16	0.005	-6.87
5S ribosomal RNA	21	0.003	-2.24
Dual Specificity Phosphatase 26	27	0.006	13.11
N-deacetylase and N-sulfotransferase 2	28	0.002	-14.98
Annexin A8 like 1	28	0.003	23.37
Hepatic and glial cell adhesion molecule	29	0.002	14.75

[1]Promoter regions were defined as 1000 base pairs upstream and 500 base pairs downstream from the transcription start site of the gene.
[2] A positive (negative) difference indicates the prenatally stressed cows had decreased (increased) methylation of the promoter region relative to control cow

3.3.1.3 Feature Specific--CpG Islands. Islands of CpG are often located in promoters of genes, are typically resistant to DNA methylation, and are rarely found in tissue specific genes (Bird, 1986). Because of this it is hypothesized that these regions are in genes that are regularly used in cell function and do not need to be repressed (Bird, 1986). In total 22,188 CpG islands were tested and 133 (FDR ≤ 0.15) were differentially methylated; 77 had increased methylation in the PNS cows and 56 had decreased methylation (AB-4). Table 3.3 has the locations of the top 10 (lowest FDR values) differentially methylated CpG islands identified. A CpG island located within *BDNF* also had increased methylation in the PNS while a CpG island located within the defined promoter region of *SYN1* had decreased methylation. The decrease in methylation of the CpG island within *SYN1* is consistent with what has been reported in aging mice, where decreased methylation of CpG islands within the promoter region coincides with an increase in expression of this gene (Haberman et al., 2012). A CpG island with decreased methylation was located within *nuclear receptor corepressor 2* (*NCOR2*), which is involved in amygdala development and anxiety behavior (Jessen et al., 2010). The influence of DNA methylation on gene expression of *NCOR2* is relatively unknown, but the location of a CpG island in the regulatory region of the gene suggests the possibility of epigenetic control.

Table 3.3. Top 10 differentially methylated CpG Islands1 in amygdala tissue of prenatally stressed mature Brahman cows relative to Control cows.

Chr	Start	End	FDR	Difference[2]
4	94192085	94192593	0.040	26.19
7	106955324	106955725	0.058	-35.11
10	43554822	43555817	0.018	-18.35
16	47324909	47326146	0.058	-16.63
18	34194169	34195605	0.003	15.87
19	49916310	49917178	0.058	20.85
20	71009252	71009654	0.058	30.51
21	33001944	33003266	0.000	-3.34
21	33023989	33026059	0.002	2.36
29	42549665	42551050	0.002	14.03

[1]Cytosine-Phosphate-Guanine rich locations within the genome
[2] A positive (negative) difference indicates the prenatally stressed cows had decreased (increased) methylation of the promoter region relative to control cows.

3.3.3.4 Genome wide methylation. Minimal methylation differences of gene bodies, promoter regions and CpG islands were observed in amygdala tissue between PNS and Control cows at 5 years of age when methylation across the genome is considered in its entirety. Of the genome wide CpG sites, 63,255 sites passed filtering. Only 29 of those sites (AB-5) were differentially methylated between the Control and PNS (FDR ≤ 0.15). The significant sites were only 0.046% of the sites tested, indicating that substantial differences in global CpG methylation were not observed between the PNS and Control groups. Visualization of the lack of distinction between treatments is shown in the MDS plot (Figure 3.2). No distinct grouping of PNS and Control samples occurred and many of the samples from the two treatments were closely positioned. The proximity of the samples to each other in the MDS plot reflects the minimal differences in methylation between groups when evaluated globally. These results differ from

analysis of DNA methylation of leukocytes in prenatally stressed Brahman bulls and heifers at 28 days of age which revealed vast differences relative to the Control, some of which were identified in the heifer calves (the cows in this study) and were found in leukocytes 5 years later (Littlejohn et al., 2018; Baker et al., 2020; Cilkiz et al., 2021).

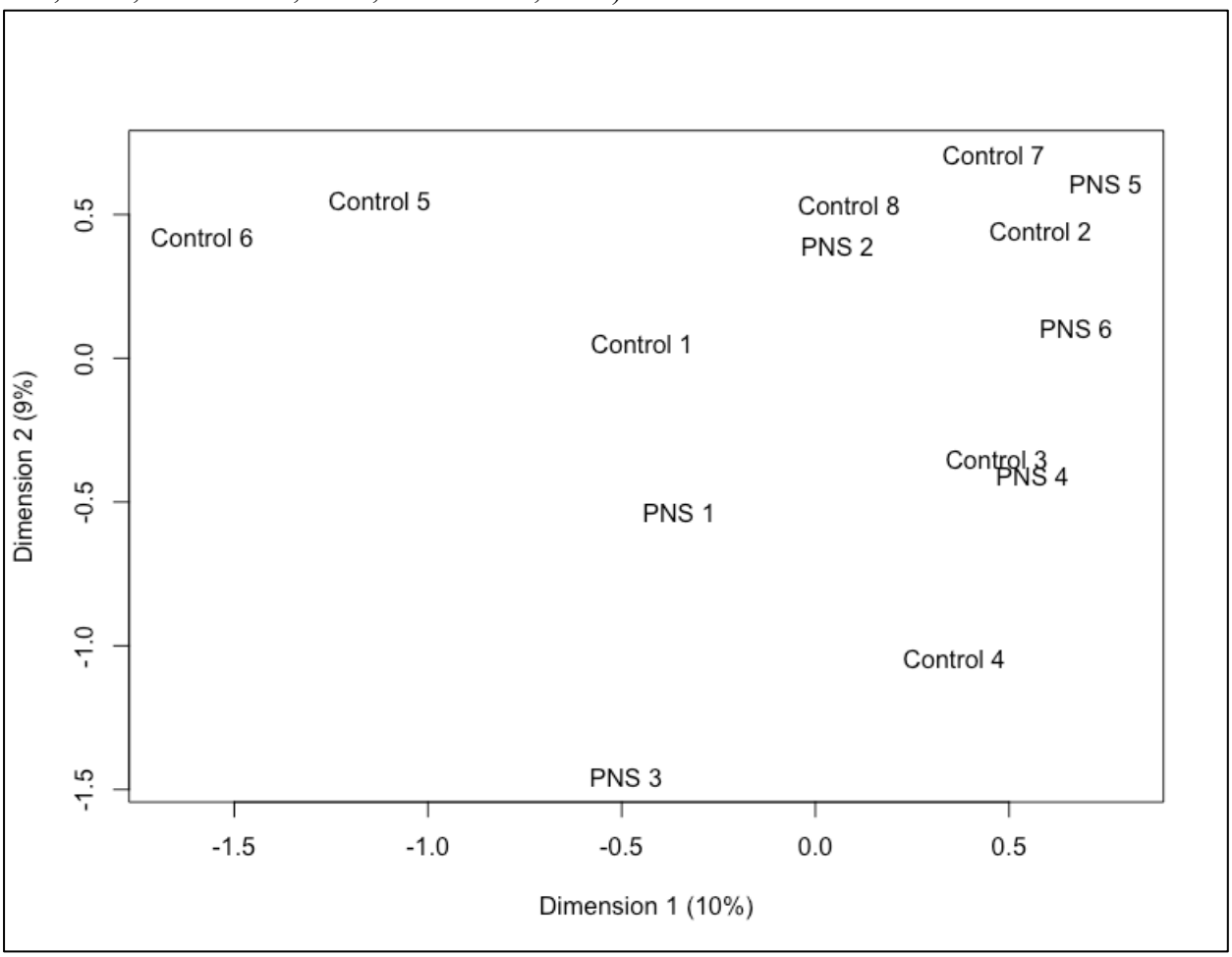

Figure 3.2. Multidimensional scaling plot utlizing M-Values the base 2 logit transformation of the proportion of methylated signal at each locus) to plot distances between methylation profiles of amygdala tissue of 5-year-old prenatally stressed (PNS)

Changes in the epigenetic landscape continue postnatally, with evident differences observed in saliva samples from infants from 6 to 52 weeks of age (Wikenius et al., 2019). In humans, a general trend of demethylation is observed with aging, but some sites that have low methylation at a young age do increase in methylation over time (Wilson et al., 1987; Jones et al.,

2014). Differences in methylation caused by the prenatal stress could be present at an early age in cattle but diminish over time. However, severe prenatal stress (i.e., famine and extreme weather) led to lasting DNA methylation changes that were transgenerational (Heijman et al., 2008; Cao-Lei et al., 2014). The severity of prenatal stress can result in very different outcomes of changes in methylation patterns in the brain (Mychasiuk et al., 2011). Transportation stress during mid to late gestation might not be a severe enough stress to cause enduring epigenetic changes in amygdala tissue in cattle that persist throughout life.

3.3.2 Gene Expression

From expression analyses, 22,867 genes remained after filtering. Even in the context of a permissive FDR (< 0.15), only 2 genes were differentially expressed in the amygdala of the PNS cows compared to the Controls. *The solute carrier family 28 member 3* (*SLC28A3*) had decreased expression in the PNS cows relative to the Control, while the *Fc fragment of IgG receptor IIa* (*FCGR2A*) had increased expression. *Fc fragment of IgG receptor IIa* has an essential role in protecting the body from foreign antigens (Hibbs et al., 1988). Deletion of *FCGR2A* inhibited the invasion of glimoa cells into the brain suggesting the gene product is important for transportation across the blood brain barrier. Members of the solute carrier family are active in the brain, aiding in the transport of hormones, sugars, and amino acids; however, the role SLC28A3 in the brain and stress response has not been documented (Hu et al., 2020). The lack of differences is illustrated by the MDS plot (Figure 3.3) which shows no distinct clustering and some overlap of individual samples from the two groups. There were no methylation differences within the promoter region or gene body of these two differentially expressed genes.

Prenatally stressed Brahman cows had only slight differences in gene expression relative to Controls at 5 years of age. In contrast, in rats, prenatal stress has caused gene expression

disturbances in the brain that persisted into adulthood (Fumagalli et al., 2004; Baier et al., 2015). Similar to the DNA methylation results, the timing and severity of a prenatal stressor can dictate the effect on gene expression. Maternal nutrient restriction in cattle has resulted in varying gene expression changes in the offspring depending on timing of restriction during gestation and the tissue analyzed (Mohrhauser et al., 2015; Sanglard et al., 2018). The stress caused by transportation during mid to late gestation may be insufficient to influence gene expression in the offspring. Expression of genes at the proper level is complex, regulated by many different factors, and varies with aging (Berchtold et al., 2008). Corrections may have occurred over time to compensate for aberrant gene expression caused by prenatal stress.

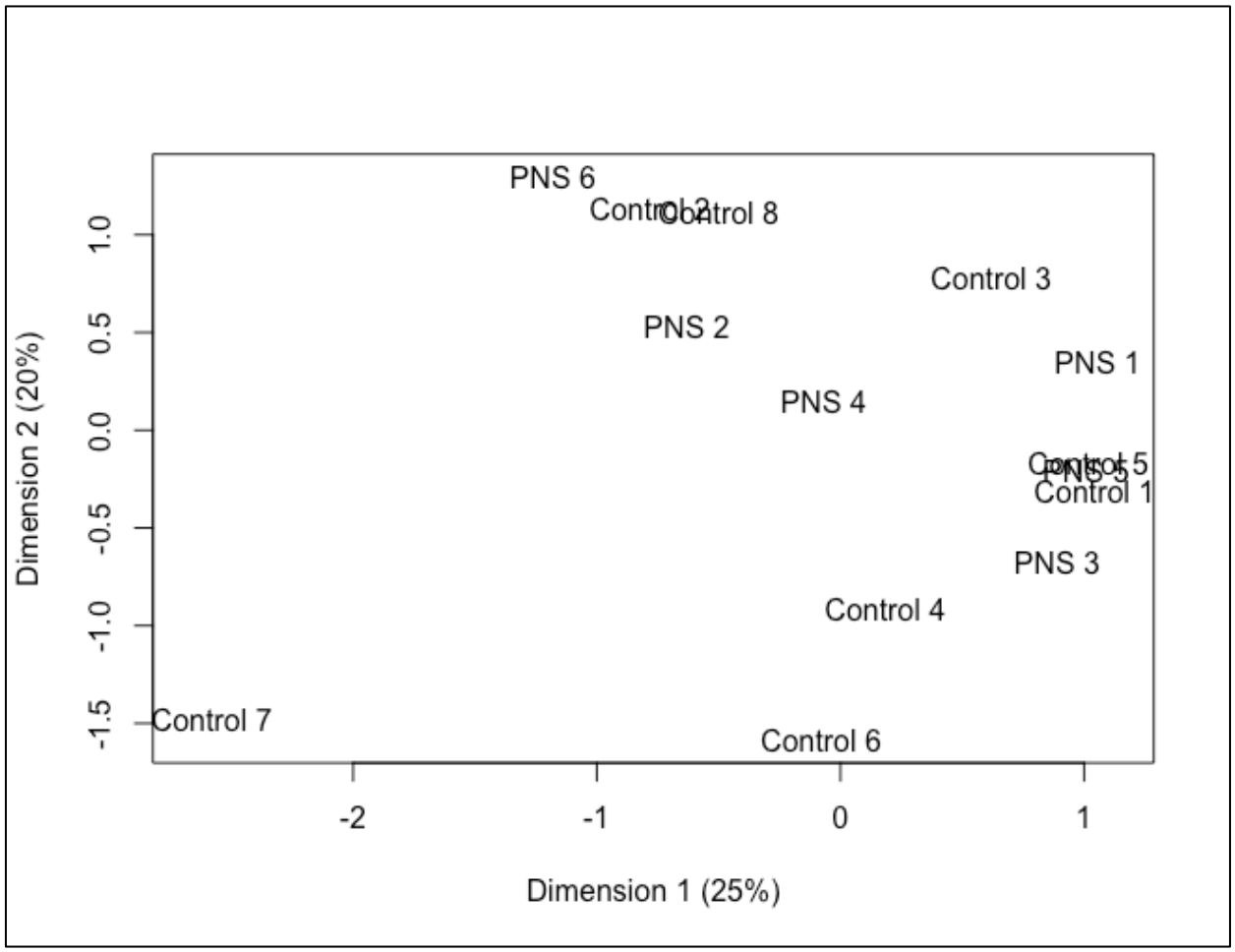

Figure 3.3. Multidimensional scaling plot utlizing normalized read counts to plot distances between expression profiles of amygdala tissue of 5-year-old prenatally stressed (PNS) Brahman cows relative to Control cows.

This is this first study to incorporate the effect of prenatal stress on DNA methylation and gene expression in the amygdala of cattle. Overall methylation of important genes and promoter regions were significantly different between the PNS and Control groups. While gene expression analysis resulted in two significant genes, the two genes are involved in essential biological functions. These novel results provide a foundation for future research on how prenatal stress effects the amygdala in cattle.

3.4 Conclusion

Gene expression and DNA methylation comparison of amygdala tissue from mature Brahman cows that were prenatally stressed relative to non-stressed mature Control cows revealed minimal differences between the groups. A small number of individual CpG sites and a low proportion of genes, promoters and CpG islands were differentially methylated. Two genes were differentially expressed in amygdala tissue when PNS and Control groups were compared. Methylation controls gene expression of many genes; however, no overlap between differentially methylated genes and differentially expressed genes was observed. Since both DNA methylation and gene expression are complex mechanisms that shift and adapt over time, it is feasible that any differences that were caused by the prenatal stress are no longer present at 5 years of age. The timing and severity of the stressor may also be a major influence on the extent of the alterations. Therefore, prenatal transportation stress during mid- to late gestation may not be significant enough to cause lasting effects. Increasing the severity of the transportation stress, such as transport for an extended period and over poorer quality of roadways could potentially result in lasting effects. Also, further investigation is needed to determine if there are differences present at younger ages, which could cause expression changes during important postnatal developmental periods. However, much of the current knowledge of the effects of prenatal stress on methylation

is from model organisms; thus, the novel information and candidate regions and genes reported are valuable for understanding the effects stress induced epigenetic modifications have on livestock.

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4. INTER-INDIVIDUAL VARIATION OF DNA METHYLATION PATTERNS ACROSS TISSUES IN MATURE BRAHMAN CATTLE

4.1 Introduction

Epigenetic mechanisms influence gene expression without changing the underlying DNA sequence. One epigenetic modification is DNA methylation, which in mammals is the addition of a methyl group to the 5' carbon of the nitrogenous base cytosine (Moore et al., 2013). Most DNA methylation occurs at cytosine-phosphate-guanine (CpG) sites and clusters of CpG sites known as CpG islands (Bird, 1986). Methylation can influence gene expression by changing the accessibility of the gene to the needed transcription factors and influencing the splicing of transcripts. Considering the influence epigenetic modifications have on gene expression, variation among individuals in DNA methylation patterns can contribute to phenotypic variation (Issa, 2002). Inter-individual variation of DNA methylation has been observed in different human populations and as early as the germ cell stage (Flanagan et al., 2006; Heyn et al., 2013). Comparison of methylation patterns of neutrophils from a group of healthy individuals identified over 12,000 inter-individual variable fragments throughout the autosomes (Chatterjee et al., 2015). Similar patterns were observed in peripheral blood monocytes in humans (Shen et al., 2013). Variation of DNA methylation patterns throughout the genome could contribute to the variation in behavior, immune response, growth, and response to the environment and drug treatments. Epigenetic variability was a driving factor in behavioral differences in genetically identical honeybee castes (Herb et al., 2012). Variable methylation patterns within *pro-opiomelanocortin* in humans may affect body weight regulation (Kühnen et al., 2016).

There is a strong genetic component to inter-individual DNA methylation variation (Bell et al., 2011; Illingworth et al., 2015). Single nucleotide polymorphisms at CpG sites can directly

lead to variation in methylation patterns due to the cytosine being a different nitrogenous base (Gonseth et al., 2016). Varying expression levels of the transcription factors and DNA methyltransferases due to genetic differences can also lead to variation in DNA methylation patterns (Gutierrez Arcelus et al., 2013). The amount of inter-individual variation is often tissue dependent. Human neuron cells had higher inter-individual variation in DNA methylation patterns relative to non-neuron cells (Iwamoto et al., 2011). Buccal epithelial cells had a more extensive variable range and contained more highly variable CpG sites relative to the peripheral blood mononuclear cells (Jiang et al., 2015). Time and environment also influence the inter-individual variation of DNA methylation. Inter-individual variation of DNA methylation tends to increase over time (Martino et al., 2011; Mulder et al., 2021). In twins, it was found that after genetics, the largest contributor to inter-individual variation of DNA methylation patterns was environmental factors (Hannon et al., 2018). The interaction between genetics and different uterine environments (maternal smoking, maternal depression, maternal body mass index) was the best explanation for 75% of the variably methylated regions found in neonates (Teh et al., 2014).

Prenatal and early life stress caused alterations in DNA methylation patterns of the offspring in cattle, some of those differences persisted later in life (Littlejohn et al., 2018; Baker et al., 2020; Cilkiz et al., 2021). Prenatal environment and stressors can explain a portion of the inter-individual variability of methylation patterns. However, less is known about the effect of the prenatal environment on the magnitude of inter-individual variation. In humans the amount of inter-individual variation of methylation levels in the gene nuclear receptor subfamily 3 group C member 1 did not differ between those who had experienced traumatic events relative to those who had not (Vukojevic et al., 2014).

Quantifying the variation in natural populations has aided in determining the relevance of variation in DNA methylation patterns and the effect it has on promoting phenotypic variation. Understanding the variation between healthy individuals can aid in understanding how a treatment or stressor affects normal methylation patterns (Heyn et al., 2013). The variation between individuals is also important for statistical analysis, such as identifying differentially methylated sites between groups. For example, inter-individual variation is analogous to the mean squared error of an analysis of variance, and high values correspondingly make it difficult to detect true differences between groups. Variability can also make it difficult to identify significant associations with expression data. The variation must be considered when selecting the proper sample size and analysis methodology (Teschendorff and Relton, 2018).

There has been a single study of the inter-individual variation of DNA methylation in cattle. Liu and colleagues (2019) investigated the inter-individual variation of 28 semen samples from Holstein bulls. Highly variable regions were determined by comparing the standard deviation of the methylation levels of each region to the median standard deviation using the chi-square test for variance. There were 1,681 highly variable methylated regions identified. Many highly variable methylated regions between individuals were associated with key regulatory areas of gene expression. Numerous methylated regions were associated with reproduction traits and genomic regions (Liu et al., 2019). These results provide novel insights into the contribution of natural DNA methylation variation to complex traits that are important to cattle productivity.

The inter-individual variation of DNA methylation patterns has yet to be investigated in mature female cattle. With that, little is known about how inter-individual variation of DNA methylation differs from tissue to tissue or how a stressor affects the variation in mature cows. Thus, this project aims to classify the inter-individual DNA methylation variation in tissues of

mature Brahman cows through 1) visualization of the range of methylation at sites across the genome, 2) identification of genomic features with high variability in methylation, and 3) comparison of those results across tissues.

4.2 Methods

4.2.1 Animal Procedures

An in-depth description of the experimental design is described in Littlejohn et al. (2018). In brief, a group of pregnant Brahman cows was transported for 2-hour durations at days 60 ± 5, 80 ± 5, 100 ± 5, 120 ± 5, and 140 ± 5 of gestation. A non-transport group was maintained as a control. Both groups were managed under the same environmental and nutritional conditions at the Texas A&M AgriLife Research & Extension Center at Overton. Twenty-one heifer calves were born from the transported cows (PNS), and 18 heifer calves were born to cows that had not been transported (Control). The heifer calves were exposed to bulls for mating at 1 year of age and annually thereafter. From those females that remained at 5 years of age, 6 PNS and 8 Control cows were slaughtered, and the amygdala and the anterior pituitary gland were collected. Ten mL of blood was collected via a vacuum tube venipuncture.

4.2.2 Sample Preparation & DNA Extraction

The anterior pituitary and amygdala tissues were cut and weighed to 20 mg. All samples were stored at −80˚C until analysis. Before DNA isolation, tissues were digested in a water bath at 56˚C. The GeneJET Genomic DNA Purification Kit (Thermo Scientific, Waltham, MA) DNA purification protocol was used to isolate DNA from anterior pituitary and amygdala tissues. Purified DNA samples were quantified with a NanoDrop Spectrophotometer (NanoDrop Technologies, Rockland, DE) and stored at −80˚C until further analysis.

Blood samples were centrifuged at 2,671 × *g* for 30 min at 6 °C. The white blood cell layer was then isolated and placed into 2-mL nuclease-free microcentrifuge tubes. The white blood cell layer was washed repeatedly until a clean cell pellet was produced. A phenol-chloroform extraction procedure was used to extract DNA from the isolated white blood cell pellet. An in-depth description of the procedure is presented in Littlejohn et al. (2018). In brief, the white blood cell pellets were placed in an extraction buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.5), and mg/mL proteinase K and 20% SDS was added for proteinase K digestion. Samples were then incubated and extracted twice with an equal volume of phenol:chloroform: isoamyl alcohol (25:24:1) and twice with an equal volume of 1-bromo-3-chloropropane (substituted for chloroform). DNA was precipitated by the addition of 10% 3 M sodium acetate (pH 5.2) and 1 volume of isopropanol to the solution. The isolated DNA purified DNA was suspended in 150-200 µL TE buffer (10 mM Tris, one mM EDTA, pH 8.0), and stored at –80 ºC.

4.2.3 DNA Methylation Analysis

The isolated DNA from each tissue was submitted to Zymo Research (Irvine, CA) for reduced representation bisulfite sequencing analysis. First, the DNA was digested with 60 units of TaqαI followed by 30 units of MspI and then purified with DNA Clean & ConcentratorTM. Adapters containing 5'-methyl-cytosine were then ligated to the fragments. Adapter-ligated fragments of 150 to 250 bp and 250 to 350 bp were recovered using the ZymocleanTM Gel DNA Recovery KitRe and then ligated to the purified DNA fragments. Recovered fragments were then bisulfite-treated using the EZ DNA Methylation-LightningTM Kit. Illumina base calling was used to identify and sequence reads from the bisulfite-treated libraries. After the raw FASTQ files were trimmed and quality checked, they were aligned to the *Bos taurus* genome (ARS-UCD1.2; Rosen et al., 2020) using Bismark 0.19.0 (Babrahman Bioinformatics, Cambridge, United Kingdom).

Alignment produced binary alignment map (BAM) files. Methylated and unmethylated read totals for each site were called using MethylDackel 0.5.0 (Zymo Research).

4.2.4 Statistical Analysis

Two different approaches were used to analyze the variation of DNA methylation patterns within each group and each tissue.

4.2.4.1 Genome-wide Inter-Individual Methylation Variation. Methylated and unmethylated read counts for all samples in each tissue were imported into the program edgeR (Robinson et al., 2010). Sites were then filtered using the criteria of having at least 5x coverage across all samples within a group. Beta values, which estimate methylation level using the ratio of reads mapped between methylated and unmethylated alleles plus a scaling factor of 1000, were calculated in each sample at the sites that passed filtering (Du et al., 2010). To visualize the variability of methylation at each site within each group, the beta values were used to calculate each site's inter-individual beta value range. The inter-individual beta value range was calculated by subtracting the smallest beta value from the largest beta value at each site in the groups. As in Jiang et al. (2015), two filters were then applied to identify sites with high standard deviations: SD $\geq$ 0.1 and SD $\geq$ 0.3. Sites that passed the filters were input into the UCSC Data Integrator tool (Navarro Gonzalez et al., 2021) to identify the genomic regions the sites are located in.

4.2.4.2 Genomic Feature Inter-Individual Methylation Variation. The BAM files provided from the Zymo analysis were read into the analysis program SeqMonk (Babrahman Bioinformatics, Cambridge, United Kingdom). Genomic features were defined for variation analysis. Four different genomic features were defined: Gene Bodies, CpG Islands, CpG Shores (2000 bp upstream and 2000 bp downstream of CpG islands), and promoter regions (1000 bp upstream of transcription start site and 500 bp downstream TSS). After each feature type was defined, a

bisulfite feature methylation pipeline (SeqMonk) was applied with the requirement of the sites within the feature to have at least 5x coverage. Estimates of percentage methylation for each cytosine within a features are averaged to that feature give an overall methylation value.

The standard deviation of each feature within a group was calculating using the overall methylation value. The features were then filtered using the variance intensity difference statistical test (SeqMonk), which is used to identify high or low variance values within a replicate set. From the standard deviations of all features, a subset was selected at random to construct a distribution. For these analyses the number of standard deviations selected was equal to 1% of the total number of features. Then each feature's standard deviation was tested to identify the probability of its standard deviation falling outside of the constructed distribution. False discovery rate methodology (Benjamini and Hochberg, 1995) was then applied to adjust for multiple comparisons. The features with an $FDR \leq 0.05$ were considered to have a high standard deviation relative to other features and therefor highly variable in their group. The highly variable features were then input into PANTHER (Thomas et al., 2003) to identify biological pathways and functions the features were involved in.

4.3 Results

4.3.1 Genome-Wide Inter-Individual Methylation Variation

After filtering for adequate coverage, 63,255 CpG sites remained for analysis in the amygdala. The mean inter-individual beta value range was 0.0356 for the Control group (Figure 4.1A). The PNS group had a similar mean inter-individual beta value range of 0.0354 (Figure 4.1B). The distribution and mean of ranges of the CpG sites within the PNS group and the Control group were visually similar. Considering the bimodality of the data the Kruskal-Wallis test was used to test for differences in the overall distributions of inter-individual beta value ranges of the

two groups. The PNS and Control group median ranges did differ ($P = 0.0002646$) despite their similarities. A similar trend is observed in the standard deviations of beta values for each CpG site (Figure 4.2). Neither group had CpG sites that had a standard deviation greater than 0.3. The PNS group (*n = 154)* had slightly more CpG sites than the Control group (*n = 128)* with SD ≥ 0.1. No sites that passed filtering with SD ≥ 0.1 in the PNS group were found in the sites that passed in the Control.

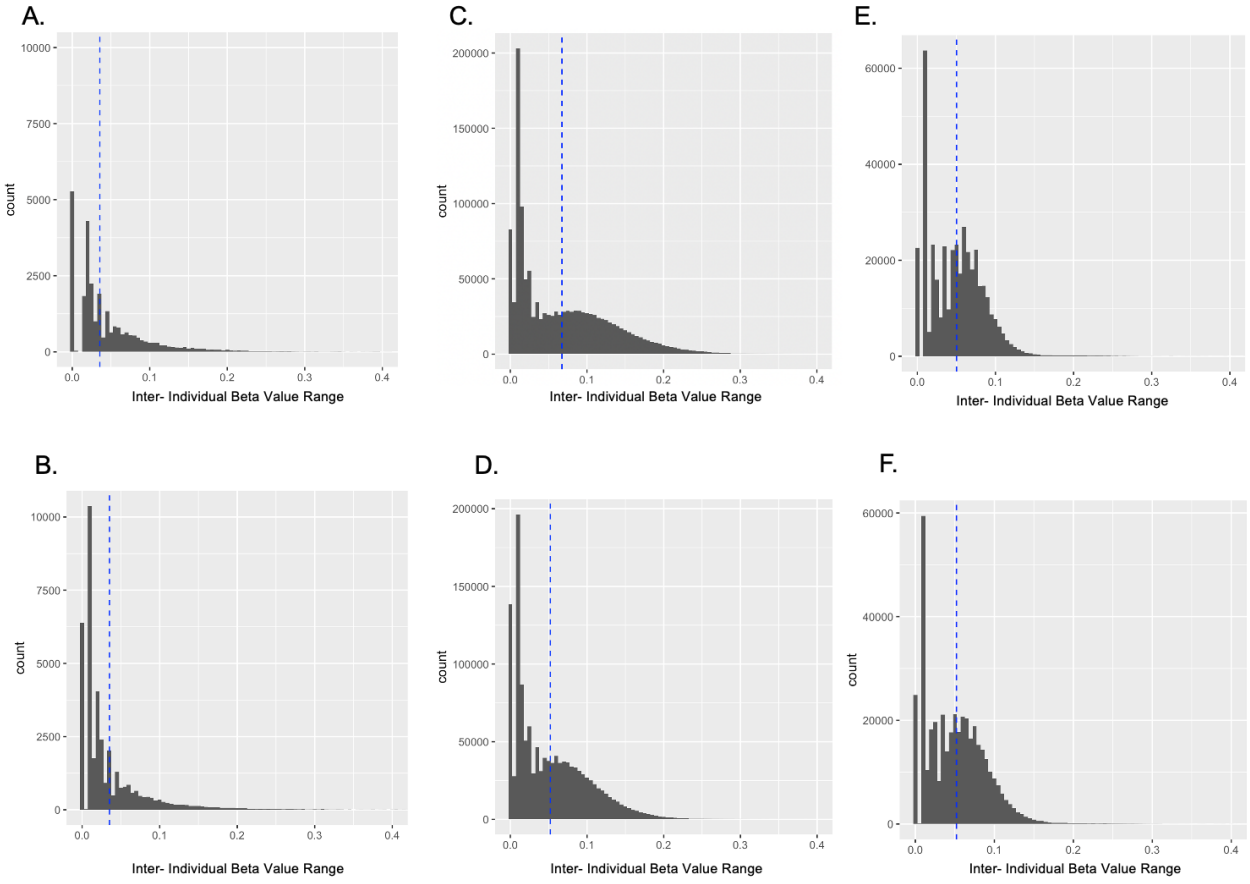

Figure 4.1. Histograms showing the distribution of the inter-individual beta value ranges for the Cytosine-phosphate-Guanine sites in the A). Control Amygdala, B). Prenatally Stressed (PNS) Amygdala, C). Control Anterior Pituitary D). PNS Anterior Pituitary sam

The pituitary gland had a much larger number of CpG sites that passed filtering for analysis (*n = 1,662,183*). The sites within the pituitary gland had the largest mean inter-individual beta

value range, with the Control having a mean range of 0.067 and the PNS having a mean of 0.052 (Figures 4.1C and 4.1D). The PNS group had a single CpG site with a beta value SD ≥ 0.3, and the Control group had 3 (Figure 4.2). The single CpG site with an SD ≥ 0.3 in the PNS group was also a site that had SD ≥ 0.3 in the Control group (Chromosome 21:33002643). The Control group had 2,063 sites, the PNS 1,053, that passed with a filter of SD ≥ 0.1. Of those sites, 550 had a standard deviation of 0.1 or greater in both the PNS and Control groups.

There were 526,816 CpG sites that passed filtering for analysis in the leukocytes. The media of beta values of the Control group and the PNS group differ (P = 4.579e-15) (Figures 4.1 E and 4.1F). Neither the PNS nor the Control group had a CpG site with an SD ≥ 0.3. The PNS group had more sites with SD ≥ 0.1 relative to the Control group (Control *n =173; PNS n =359*). Of the sites identified to have a SD ≥ 0.1, 152 were common to both groups.

The pituitary gland in both the PNS and Control groups had the highest portion of sites with a standard deviation of greater than 0.3 and 0.1. The amygdala had the lowest mean range of beta values in both the PNS and the Control. It is important to note that the amygdala had significantly fewer CpG sites included for analysis. The three tissues from the Control group showed minimal overlap with the sites identified with beta values SD ≥ 0.1, with just 3 CpG sites shared (Figure 4.3A). The three tissues from the PNS group shared more sites than the Control, 21 CpG sites (Figure 4.3B**).** In all three tissues, many of the CpG sites with a beta value SD ≥ 0.1 were located within or near short and long interspersed retrotransposable elements and other highly repetitive regions of the genome.

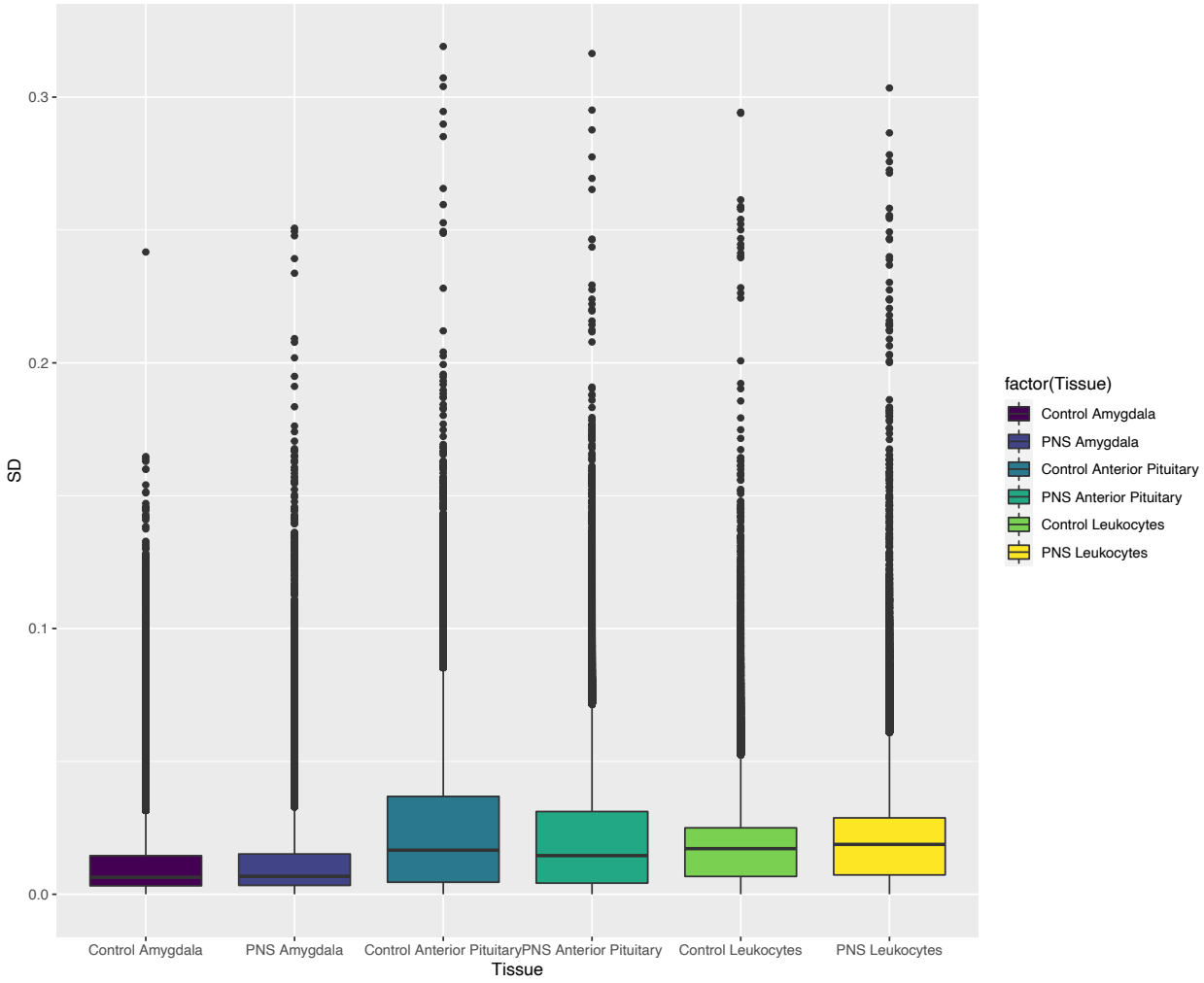

Figure 4.2. Box plots of standard deviations (SD) for the cytosine-phosphate-guanine sites analyzed in each tissue from the prenatally stressed (PNS) and Control groups

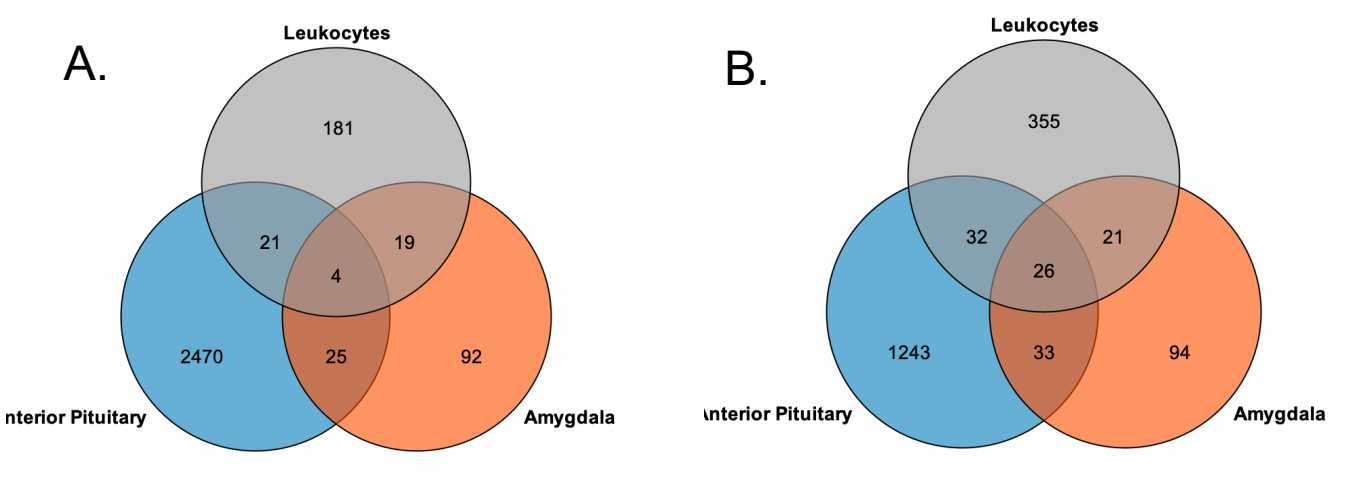

Figure 4.3. Venn Diagrams showing the overlap of cytosine-phosphate-guanine sites with a beta value standard deviation ≥ 0.1 across the three tissues in the A) Control and B) Prenatally Stressed group.

4.3.2 Genomic Feature Inter-Individual Methylation Variation

The number of features analyzed varied for each feature type: 26,863 genes and promoter regions, 22,188 CpG islands, and 44,376 CpG shores.

When testing for highly variable features, the amygdala had few features that were significant in both the PNS group and the Control group (Tables 4.1 and 4.2). Both groups had more variable methylated features in the leukocytes and anterior pituitary relative to the amygdala. Still, all were a small fraction (0.02% to 1.05 %) of the total number of features tested. In the PNS and Control groups, the anterior pituitary gland had the most features that had statistically significant, highly variable genes. While the magnitude of variable methylated features identified in the PNS and Control groups was relatively similar, there was minimal overlap between those features in each tissue (Table 4.3).

Table 4.1. The numbers of genomic features with significant variation in DNA methylation between cows in the Control group.

Tissue	Promoter[1]	Gene	[2]CpG Islands	CpG Shores[3]
Anterior Pituitary	226	314	307	446
Amygdala	52	151	84	81
Leukocytes	146	281	172	106

[1]Promoter regions were defined as 1,000 bp upstream of the transcription start site of a gene and 500 bp downstream of the transcription start site.
[2]Cytonsine-phosphate-Guanine
[3]CpG Shores were defined as 2,000 bp upstream and 2,000 bp downstream of CpG islands

Table 4.2. The numbers of genomic features with significant variation in DNA methylation between cows in the Prenatally Stressed (PNS) group.

Tissue	Promoter[1]	Gene	CpG[2] Islands	CpG Shores[3]
Anterior Pituitary	372	313	313	399
Amygdala	52	178	51	7
Leukocytes	168	298	216	149

Table 4.3.The numbers of genomic features that had significant variation in DNA methylation in both the Prenatally Stressed and Control groups.

Tissue	Promoter[1]	Gene	CpG Islands[2]	CpG Shores[3]
Anterior Pituitary	92	81	73	96
Amygdala	1	6	0	0
Leukocytes	14	54	26	3

[1]Promoter regions were defined as 1,000 bp upstream of the transcription start site of a gene and 500 bp downstream of the transcription start site.
[2]Cytonsine-phosphate-Guanine
[3]CpG Shores were defined as 2,000 bp upstream and 2,000 bp downstream of CpG islands

In the Control group, only two genes had high variability in all three tissues: Guanylate Cyclase Activator 2B and U6 spliceosomal RNA. There was minimal overlap in features that were

variably methylated between each tissue (Figure 4.4A). No promoter had variable methylation in all three tissues; the anterior pituitary shared 15 with the leukocytes and 4 with the amygdala. The leukocytes only shared three variable methylated promoter regions with the amygdala. A similar trend was observed in the CpG islands, with no features shared across all three tissues and a minimal amount shared between two tissues. No CpG shores were common to all three tissues or pairs of tissues.

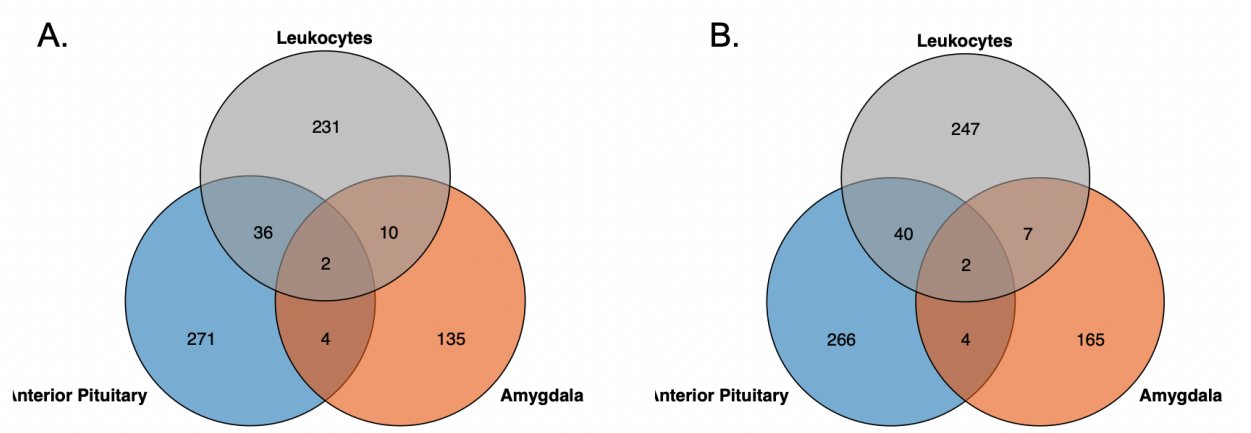

Figure 4.4. Venn-diagrams showing the overlap of genes with high DNA methylation variation across the 3 tissues in the A) Control and B) Prenatally Stressed group.

Again, only two genes exhibited high methylation variation in all three PNS group tissues (ENSBTAG00000036102, ENSBTAG00000051147). Both genes are labeled as novel genes in the Ensembl ARS-UCD1.2 reference genome (Cunningham et al., 2021). Pairwise comparisons for each tissue showed minimal overlap in genes with variable methylation (Figure 4.4B). A single promoter region showed variable methylation across all three tissues: the promoter region for Synaptotagmin 4 (*SYN4*). No CpG islands were variable across all three tissues in the PNS group. However, 40 CpG islands were variable in both the anterior pituitary gland and leukocytes. As in the Control cows, no variable CpG shores were shared between the three tissues or between the pairs of tissues.

Pathway analysis revealed that many genes and promoter regions with variable methylation in the pituitary gland and the amygdala are involved in biological pathways such as signaling, response to stimulus, and metabolic processes (AB-1).

4.4 Discussion

The anterior pituitary had the most variable methylated features, followed by the leukocytes. Both had substantially more variation than the amygdala tissue. The same pattern was observed in the number of CpG sites with a beta value SD $\geq$ 0.1. This contrasts with what has been observed in humans, where the amygdala had the most variable methylated regions compared to other tissues harvested from the brain (Rizzardi et al., 2021). Cell type and heterogeneity within a sample can augment the variability of methylation (Jaffe and Irizarry, 2014). Leukocytes consist of numerous cell types, such as monocytes, leukocytes, and neutrophils, which can each have different methylation profiles (Adalsteinsson et al., 2012). The anterior pituitary gland and the amygdala also contain numerous cell types (Tosevski et al., 2002, Le Tissier et al., 2012). The varying cell types in a sample of one tissue can contribute to the high variation relative to another. Isolation of a singular cell type for inter-individual variation analysis could prevent the confounding effect of different methylation profiles in each cell type (Chatterjee et al., 2015).

In all the tissues analyzed, CpG islands and CpG shores were identified with high variation of methylation patterns. Increased inter-individual variation within CpG shores was identified in human blood and cerebellum samples (Zhang et al., 2010; Wang et al., 2012). High levels of inter-individual variation within CpG islands have also been reported in human germ cells (Flanagan et al., 2006). The variability of methylation patterns observed in the three tissues does not follow the general trend of CpG islands being largely and consistently unmethylated (Jeziorska et al., 2017).

However, the number of islands identified to have variable methylation makes up a very small percentage of the CpG islands tested.

Variation in DNA methylation of genes and promoter regions can result in variability in gene expression (Wagner et al., 2014). Variable methylation in the dopamine receptor D4 contributes to variations in gene expression and natural variation in bird behavior and personality (Verhulst et al., 2016). The receptor coded by Melanin-concentrating hormone receptor 2 (*MCHR2*) is influenced by the melanin-concentrating hormone in the amygdala and can control feeling and motivational behavior (Kim and Han, 2016). The Gonadotropin-releasing hormone receptor (*GNRHR*) in the pituitary gland has an essential role in mammalian reproductive function and hormone production (Schang et al., 2012). Both *MCHR2* and *GNRHR* had variable methylation, which could lead to natural variations in behavior and hormone concentration. Variation in methylation patterns of genes involved in these pathways could be responsible for variation in growth, development, and response to environmental stressors. The promoter region for Synaptotagmin 4 (*SYN4*) was variable across all tissues. This gene is expressed in the brain, and the product of *SYN4* plays a vital role in dopamine release (Mendez et al., 2011). Expression levels of *SYN4* have been observed to have an inverse relationship with the methylation levels of the gene (Yang et al., 2021). Variable gene expression of *SYN4* due to DNA methylation could result in differences in behavior.

In leukocytes, numerous variable methylated features were identified to be involved in immune system response. Similar results were found in peripheral blood monocytes in humans (Shen et al., 2013). From studies in human monozygotic twins there were an abundance of variable methylated loci around and within genes that are important for immune response (Córdova-Palomera et al., 2015). In neutrophils, it is hypothesized that the hypervariable sites are essential

in establishing the immune system response (Tejedor and Fraga 2017). Variable methylation in the genes active in the immune system could lead to gene expression differences and contribute to variable immune responses (Lam et al., 2012).

Many of the CpG sites with a beta value SD $\geq$ 0.1 were located within or near short and long interspersed retrotransposable elements and other highly repetitive regions of the genome. This is comparable to what Chatterjee and colleagues (2015) found in methylation profiles of human neutrophils. Differences in DNA methylation patterns of long interspersed retrotransposable elements have been associated with low and high birthweights in humans (Michels et al., 2011). The variable methylation patterns in sites within these elements could lead to phenotypic differences.

There was minimal overlap between the tissues, both genome-wise and feature-wise (Table 4.3). Hannon and colleagues (2015, 2021) observed similar patterns when comparing variation in DNA methylation patterns between whole blood and regions of the brain. In general, between tissue variation has been found to significantly exceed the inter-individual variation within a singular tissue (Davies et al., 2012). These results were consistent with those of Liu et al. (2019) and suggested that hypervariable methylated regions likely harbor tissue-specific expressed genes. The variability in methylation patterns observed between these three tissues potentially contributes to tissue-specific gene expression. The results also show that using one tissue to predict the variability of methylation patterns in another may not be feasible.

While the magnitude and distribution of CpG sites with high standard deviations were similar between the PNS and Control groups, only a slight overlap in the location of the CpG sites was observed in the anterior pituitary and leukocytes. There was also minimal overlap in the features identified to be highly variable in each group. This could mean that prenatal stress in cattle

influences DNA methylation variation. However, the environmental factor rarely acts alone to influence the inter-individual variation of DNA methylation patterns (Chatterjee et al., 2021). Interaction between the environment and the cow genotype could be responsible for the differences in location of the inter-individual variation.

4.5 Conclusion

Analyses of the anterior pituitary gland, leukocytes, and amygdala revealed that a small portion of various genomic features and CpG sites have high variation in DNA methylation patterns in mature cows. The PNS and Control group had high variation in methylation patterns between samples in genes and promoter regions involved in behavior, hormone concentration, and immune response. Inter-individual variation within these genes could potentially contribute to differences in phenotype and performance in cattle. Discordance in DNA methylation patterns between tissues is expected and common. The minimal overlap between tissues observed in this study also suggests that variability in DNA methylation patterns is also tissue specific. The group of cows exposed to prenatal stress exhibited a similar amount of variable methylated sites and features to a control group, but there was minimal overlap of variable features and sites. Each tissue differed in the amount of variable methylated features, which could be due to cell heterogeneity of the sample. Using new and developing technologies that can isolate and determine the methylation profile of a single cell could aid in reducing the noise of different cell types in a sample contributing to the variation.

This study provides the first analysis of inter-individual variation DNA methylation patterns in mature Brahman females across tissues. These regions are important to consider for multiple reasons. Quantifying the inter-individual variation present is also essential to future statistical analyses and understanding how a treatment or stressor affects the variability. These

hypervariable regions in the genome could be linked to important genes or regulatory regions that contribute to complex performance traits in cattle. Additional research is needed to identify the interaction of these variably methylated regions with gene expression and how cow performance is affected.

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5. SUMMARY

Prenatal transportation stress has resulted in altered physiological responses to stress, increased temperament scores, and increased circulating cortisol levels. Stressed-induced changes in DNA methylation could be responsible for those differences. There were vast differences in DNA methylation patterns of cytosines across the genome in 28-day old Brahman heifer calves.. Many differentially methylated cytosines were located within regulatory regions of genes involved in biological pathways such as cell signaling, neurological development, cellular development, immune response, and metabolism (Baker et al., 2020). Changes in gene expression induced by the shifts in DNA methylation patterns of those genes could be responsible for the differences in prenatally stressed offspring.

In amygdala tissues harvested from the same females at 5 years of age, there were minimal differences in DNA methylation patterns between the prenatally stressed cows and the Control. Genome-wide, there were only 29 differentially methylated cytosines. However, comparison of methylation of specific genomic features identified 134 promoter regions, 202 gene bodies, and 210 cytosine-phosphate-guanine islands that were differentially methylated (Baker et al., 2022). The minimal DNA methylation differences were accompanied by an even smaller amount of gene expression differences, with only 2 genes being differentially expressed between the two groups. There was no correspondence between differentially methylated genes and differentially expressed genes. Both DNA methylation and gene expression are dynamic, so any differences that were present at 5 years, like what was observed in the leukocytes at 28 days of age, may no longer be present. This is supported by the comparison of leukocytes harvested at 5 years of age. Relative to the 16,378 differentially methylated cytosines identified at 28 days between the PNS group and the Control, there were 2,637 cytosines that were differentially methylated at 5 years of age (Cilkiz

et al., 2020). Differences in methylation diminished over time in the leukocytes, so it is possible that similar shifts occurred in the amygdala tissue. Comparison of DNA methylation patterns from tissues such as the amygdala at a younger age might reveal more differences.

The data produced by this project provided a unique opportunity to analyze the inter-individual variation of DNA methylation patterns within the Control and the PNS group across numerous tissues. Variations in DNA methylation patterns can contribute to phenotypic variation and potentially influence complex production traits in cattle. For both groups, in the anterior pituitary gland, amygdala and leukocytes, there were CpG sites with high standard deviations throughout the genome, many of them being located within repeat and retrotransposable elements. Numerous promoter regions, gene bodies, CpG islands, and shores showed high variation in DNA methylation. The variable methylation in some genomic features could contribute to natural variation in immune response and behavior.

The pituitary gland consistently had the most amount of highly variable features/sites and the amygdala the least. The cell composition of the sample used to obtain methylation data could be responsible for one tissue having more variation than another because different cell types have different methylomes. For both the sites with high standard deviations and the highly variable genomic features, there was little overlap between tissues and between the PNS group and the Control. These results support the hypothesis that variable DNA methylation of the genome has a role in tissue-specific gene expression. While the environment can explain a portion of the variation in DNA methylation patterns, less is known about the environment's impact on that variation's location. The interaction between the cow genotype and the prenatal environment could be responsible for the lack of overlap between the two groups.

This research provided the first insights into the effect of prenatal transportation stress on tropically adapted female beef cattle. Understanding how the prenatal environment shapes postnatal phenotypes through the epigenome is important to future beef cattle selection and management. However, a comparison of DNA methylation patterns from tissues at a younger age, combined with gene expression data, is needed. Differences at a younger age could result in gene expression and biological alterations during critical developmental periods, potentially impacting future cow performance and productivity. This was the first investigation of the inter-individual variation of DNA methylation patterns across tissues in cattle. More research is needed to identify the contribution of inter-individual variation of DNA methylation to differences in complex production traits and performance, as well as determining if this variation is heritable. Using new technologies that isolate a single cell type for analysis could aid in reducing the variation introduced by having a heterogenous tissue type.

It has long been thought that phenotype is equal to genetic contributions, the environment, and the interaction between the two. However, a window has opened, adding a new component, the epigenome, and its contribution to phenotype. The novel results of these projects are vital to understanding links between environment, epigenome, genome, and phenotypes and provide numerous opportunities for future research avenues.

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APPENDIX A
TABLES

AA-1. Top 30 ($P < 0.0002$; listed from smallest to largest P values) hypermethylated cytosine-guanine sites located within gene body regions of genes in prenatally stressed (PNS) compared with Control heifer calves.

		Mean methylation ratio		
Chr[1]	**Mb**	**PNS**[2]	**Control**	**Gene Name**
23	23.1	0.15	0.00	*Transcription Factor AP-2 Delta; TFAP2D*
27	19.2	0.15	0.00	*Mitochondrial Calcium Uptake Family Member 3; MICU3*
24	32.6	0.17	0.00	*Impact RWD Domain Protein; IMPACT*
23	17.9	0.12	0.00	*Transmembrane Protein 151B; Them151B*
10	81.4	0.20	0.03	*Polypeptide N-Acetylgalactosaminyltransferase 16; GALNT16*
26	22.9	0.12	0.10	*Ribroblast Growth Factor 8; FGF8*
25	37.9	0.14	0.10	*Transmembrane Protein 130; THEM130*
28	25.9	1.00	0.79	*Hexokinase 1; HK1*
5	78.8	0.20	0.05	*DENN Domain Containing 5B; DENND5B*
6	105.3	0.13	0.00	*Evc Ciliary Complex Subunit 1; EVC*
1	83.5	0.14	0.00	*Endothelin Converting Enzyme 2; ECE2*
27	28.5	0.27	0.11	*Ring Finger Protein 122; RNF122*
19	27.7	1.00	0.83	*Transmembrane Protein 256; THEM256*
20	71.8	0.13	0.01	*Aryl-Hydrocarbon Receptor Repressor; AHHR*
X	100.1	0.90	0.80	*Moesin; MSN*
3	27.6	0.22	0.02	*Nescient Helix-Loop-Helix 2; NHLH2*
4	86.6	0.26	0.04	*Wnt Family Member 16; WNT16*
6	92.2	0.22	0.02	*G3BP Stress Granule Assembly Factor 2; G3BP2*
4	87.5	0.13	0.00	*FEZ Family Zinc Finger 1; FEZF1*
3	32.4	0.27	0.03	*DNA Damage Regulated Autophagy Modulator 2; DRAM2*
3	104.1	0.53	0.36	*Y-Box Binding Protein 1; YBX1*
5	32.3	0.18	0.02	*Neural EGFL Like 2; NELL2*
16	80.8	0.21	0.05	*Nuclear Receptor Subfamily 5 Group A Member 2; NR5A2*
3	42.9	0.11	0.01	*G Protein-Coupled Receptor 88; GPR88*
10	86.3	0.76	0.39	*Latent Transforming Growth Factor Beta Binding Protein 2; LTBP2*
28	37.1	0.33	0.05	*Neuregulin 3; NRG3*
2	116.1	0.23	0.04	*Collagen Type IV Alpha 3 Chain; COL4A3*
19	51.6	0.28	0.01	*MAF Bzip Transcription Factor G; MAFG*
11	72.4	0.33	0.11	*Dnaj Heat Shock Protein Family Member C5 Gamma; DNAJCFG*
11	72.4	0.33	0.11	*Solute Carrier Family 30 Member 3; SLC30A3*

[1]Chr = Chromosome
[2]PNS = Prenatally stressed

AA-2. Top 30 ($P < 0.0003$; listed from smallest to largest P values) hypermethylated cytosine-guanine sites located within gene body regions of genes in prenatally stressed (PNS) compared with Control heifer calves.

		Mean methylation ratio		
Chr[1]	**Mb**	**PNS**[2]	**Control**	**Gene Name**
21	20.8	0.90	1.00	*Aggrecan; ACAN*
25	27.5	0.84	1.00	*Branched Chain Keto Acid Dehydrogenase Kinase; BCKDK*
19	21.4	0.84	1.00	*Tumor Protein P53 Inducible Protein 13; TP53I13*
10	20.9	0.02	0.20	*Capping Protein Regulator And Myosin 1 Linker 3; CARMIL3*
18	49.9	0.90	1.00	*AKT Serine/Threonine Kinase 2; AKT2*
17	63.7	0.90	1.00	*2',5'-Oligoadenylate Synthetase 1; OAS1X*
19	56.4	0.86	1.00	*Unk Zinc Finger; UNK*
19	57.3	0.61	0.74	*RAB37, Member RAS Oncogene Family; RAB37*
9	49.2	0.04	0.26	*Potassium Voltage-Gated Channel Subfamily J Member 6; GIRK2*
27	28.9	0.84	0.98	*Ring Finger Protein 122; RNF122*
21	70.9	0.86	1.00	*Adenylosuccinate Synthase Like 1; ADSSL1*
X	40.4	0.18	0.43	*Atpase H+ Transporting Accessory Protein 1; ATP6AP1*
8	70.5	0.11	0.37	*Early Growth Response 3; EGR3*
24	26.4	0.04	0.21	*Desmocollin 3; DSC3*
4	8.0	0.84	1.00	*Cyclin Dependent Kinase 14; CDK14*
15	53.1	0.00	0.19	*Star Related Lipid Transfer Domain Containing 10; STARD10*
2	73.0	0.82	1.00	*GLI Family Zinc Finger 2; GLI2*
4	33.6	0.72	0.83	*KIAA1324 Like; KIAA1224L*
7	44.9	0.03	0.23	*Serine Protease 57; PRSS57*
13	74.4	0.01	0.13	*Recombination Signal Binding Protein Immunoglobulin Kappa J Like; RBPJL*
29	49.5	0.23	0.47	*Potassium Voltage-Gated Channel Subfamily Q Member 1; KCNQ1*
29	41.1	0.03	1.00	*Fatty Acid Desaturase 3; FADS3*
7	63.4	0.82	0.99	*Colony Stimulating Factor 1 Receptor; CSF1R*
25	35.0	0.70	0.94	*Deltex E3 Ubiquitin Ligase 2; DTX2*
5	109.7	0.40	0.20	*BAR/IMD Domain Containing Adaptor Protein 2 Like 2; BAIAP2L2*
25	34.9	0.00	0.16	*Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein;YWANG*
29	49.5	0.40	0.24	*Potassium Voltage-Gated Channel Subfamily Q Member 1; KCNQ1*
18	55.9	0.50	0.79	*Pleckstrin Homology Domain Containing A4; PLEKHA4*
13	16.3	0.01	0.23	*GATA Binding Protein 3; GATA3*
6	7.0	0.76	0.87	*Phosphodiesterase 5A; PDE5A*

[1]Chr = Chromosome
[2]PNS = Prenatally stressed

AA-3. Strongly hypermethylated (methylation difference ≥ 0.33 ratio) CHG sites located within promoter regions of genes in prenatally stressed (PNS) compared with Control heifer calves.[1]

		Mean methylation ratio		
Chr[2]	**Mb**	**PNS**[3]	**Control**	**Gene Name**
17	56.7	0.16	0.00	*PTC7 Protein Phosphatase Homolog; PPTC7*
17	73.5	0.14	0.00	*Beta-Ureidopropionase 1; UPB1*
21	59.8	0.11	0.00	*Serpin Peptidase Inhibitor, Clade A SERPINA3-1*
21	68.6	0.10	0.00	*Heat Shock Protein 90 Alpha Family Class A Member ; HSP90AA1*
28	31.1	0.21	0.00	*Dual Specificity Phosphatase 13; DUSP13*
5	91.7	1.00	0.89	*Capping Actin Protein Of Muscle Z-Line Subunit Alpha 3; CAPZA3*
5	110.1	0.10	0.00	*TRIO And F-Actin Binding Protein; TRIOBP*
X	40.1	0.14	0.00	*Terleukin 1 Receptor Associated Kinase 1; IRAK1*
1	69.1	0.15	0.00	*Kalirin Rhogef Kinase; KALRN*

[1]C= cytosine; G= guanine; H = adenine, cytosine or thymine
[2]Chr = Chromosome
[3]PNS = Prenatally stressed

AA-4. All hypomethylated (at least 10% less hypomethylated) CHG sites located within gene body regions of genes in prenatally stressed (PNS) heifers compared with Control heifer calves.[1]

		Mean methylation ratio		
Chr[2]	**Start**	**PNS**[3]	**Control**	**Gene Name**
9	88.6	0.06	0.16	*Iodotyrosine Deiodinase; IYD*
10	10.5	0.12	0.58	*Spalt Like Transcription Factor 2; SALL2*
21	66.7	0.12	0.41	*ENAH/VASP-Like; EVL*
12	28.8	0.00	0.10	*FRY microtubule binding protein; FRY*
27	28.7	0.09	0.21	*Ring Finger Protein 122; RNF122*
21	67.4	0.59	0.99	*Maternally Expressed 3; MEG3*
27	28.8	0.04	0.16	*Ring Finger Protein 122; RNF122*
21	68.5	0.47	1.00	*Dynein Cytoplasmic 1 Heavy Chain 1; DYNC1H1*
8	65.5	0.00	0.11	*endoplasmic reticulum protein 44; ERP44*
17	55.3	0.45	0.79	*CAP-Gly domain containing linker protein 1; CLIP1*
2	105.0	0.05	0.16	*Membrane Associated Ring-CH-type finger 4; MARCH4*
4	95.1	0.51	0.63	*Coatomer Protein Complex Subunit Gamma 2; COPG2*
X	100.1	0.65	0.87	*Moesin; MSN*
23	20.8	0.59	0.95	*Opsin 5; OPN5*
6	18.5	0.01	0.12	*Hydroxyacyl-CoA Dehydrogenase; HADH*
28	27.0	0.51	0.94	*ADAM Metallopeptidase With Thrombospondin Type 1 Motif 14;ADAMT214*
27	28.5	0.46	0.76	*Ring Finger Protein 122; RNF122*
15	66.3	0.46	0.82	*Pyruvate Dehydrogenase Complex Component X; PHDX*
19	63.4	0.61	0.82	*RecQ like helicase 5; RECQL5*

[1]C= cytosine; G= guanine; H = adenine, cytosine or thymine
[2]Chr = Chromosome
[3]PNS = Prenatally stressed

AA-5. Top 30 ($P < 0.03$; listed from smallest to largest P values) hypermethylated CHG sites located within gene body regions of genes in prenatally stressed (PNS) compared with Control heifer calves.[1]

		Mean methylation		
Chr[2]	**Start**	**PNS**[3]	**Control**	**Gene Name**
4	117.3	0.12	0.00	*Dipeptidyl Peptidase Like 6 DPP6*
7	20.9	0.85	0.38	*SH3 Domain Containing GRB2 Like 1; SH3GL1*
2	15.2	0.11	0.01	*Integrin Subunit Alpha 4; ITGA4*
25	33.5	0.27	0.00	*GTF2I Repeat Domain Containing 1; GTF2*
23	50.6	0.11	0.00	*Serpin Family B Member 1; SERPINB1*
5	110.5	0.11	0.00	*Transmembrane Protein 184B; Them184b*
4	95.1	0.15	0.02	*Coatomer Protein Complex Subunit Gamma 2; COPG2*
5	26.9	0.11	0.00	*Chromosome 5 C12orf10 Homolog; ; C5H12ORF10*
X	100.1	0.37	0.26	*Moesin; MSN*
7	59.8	0.12	0.00	*Protein Phosphatase 2 Regulatory Subunit Bbeta; PPP242B*
2	122.3	0.12	0.00	*IQ Motif Containing C; IQCC*
23	15.7	0.11	0.00	*Cyclin D3; CCND3*
25	38.7	0.14	0.00	*EPH Receptor B4; EPHB4*
16	36.5	0.23	0.08	*Regulator Of G Protein Signaling 7; RGS7*
8	71.0	0.80	0.48	*Tumor Necrosis Factor Receptor Superfamily 10D; TNFRSF10D*
5	91.8	1.00	0.89	*Phospholipase C Zeta 1; PLCZ1*
25	42.0	0.11	0.00	*Integrator Complex Subunit 1; INTS1*
17	71.5	0.11	0.00	*Coiled-Coil Domain Containing 157; CCDC157*
15	33.9	0.39	0.00	*Ubiquitin Associated And SH3 Domain Containing B; UBASH3B*
19	56.6	0.13	0.00	*Small Integral Membrane Protein 5; SMIM5*
21	59.8	0.11	0.00	*Serpin Family A Member 4; SERPINA4-2*
13	59.5	0.18	0.00	*Bone Morphogenetic Protein 7; BMP7*
16	51.9	0.10	0.00	*Protein Kinase C Zeta; PRKCZ*
6	113.6	0.15	0.00	*Member RAS Oncogene Family; RAB28*
23	13.1	0.10	0.00	*Potassium Channel, Subfamily K, Member 5; KCNK5*
8	82.4	0.10	0.00	*Cathepsin L; CTSL*
10	25.7	0.10	0.00	*Homer Scaffold Protein 1; HOMER1*
16	37.8	0.11	0.00	*Basic Leucine Zipper Nuclear Factor 1; BLZF1*
23	51.4	0.15	0.00	*GDP-Mannose 4,6-Dehydratase; GMDS*

[1]C= cytosine; G= guanine; H = adenine, cytosine or thymine
[2]Chr = Chromosome
[3]PNS = Prenatally stressed

AA-6. Hypermethylated CHH sites located within promoter regions of genes in prenatally stressed (PNS) compared with Control heifer calves.[1]

		Mean methylation ratio		
Chr[2]	**Start**	**PNS**[3]	**Control**	**Gene Name**
4	100.6	0.22	0.00	*Myotrophin; MTPN*
7	90.6	0.11	0.00	*Microrna 9-2; MIR9-2*
10	4.4	0.14	0.00	*Transmembrane P24 Trafficking Protein 7; TMED7*
25	1.8	0.11	0.00	*RNA Binding Protein With Serine Rich Domain 1; RNPS1*
21	27.9	0.14	0.00	*M-Phase Phosphoprotein 10; MPHOSPH10*
21	27.9	0.14	0.00	*Methylmalonyl-Coa Epimerase; MCEE*
5	24.1	0.11	0.00	*Plexin C1; PLXNC1*
13	7.7	0.13	0.01	*Mono-ADP Ribosylhydrolase 2; MACROD2*
X	7.9	0.10	0.00	*Stromal Antigen 2; STAG2*
3	60.2	0.10	0.00	*Protein Kinase Camp-Activated Catalytic Subunit Beta; PRKACB*
6	39.0	0.12	0.00	*Ligand Dependent Nuclear Receptor Corepressor Like; LCORL*
19	9.5	0.10	0.00	*Microrna 142; MIR142*
11	102.9	0.11	0.00	*Adenylate Kinase 8; AK8*
11	102.9	0.11	0.00	*Sperm Acrosome Associated 9; SPACA9*
24	37.9	0.11	0.00	*TGFB Induced Factor Homeobox 1; TGIF1*
18	43.9	0.11	0.00	*Solute Carrier Family 7 Member 10; SLC7A10*
4	100.6	0.12	0.00	*Myotrophin; MTPN*
X	40.1	0.14	0.00	*Interleukin 1 Receptor Associated Kinase 1; IRAK1*
29	42.9	0.11	0.00	*REST Corepressor 2; RCOR2*

[1]C= cytosine; G= guanine; H = adenine, cytosine or thymine
[2]Chr = Chromosome
[3]PNS = Prenatally stressed

AA-7. Top 30 ($P < 0.05$; listed from smallest to largest P values) hypomethylated CHH sites located within gene body regions of genes in prenatally stressed (PNS) compared with Control heifer calves.[1]

		Mean methylation ratio		
Chr[2]	**Start**	**PNS**[3]	**Control**	**Gene**
27	28.5	0.16	0.27	*Ring Finger Protein 122; RNF122*
7	64.0	0.09	0.57	*Synaptopodin; SYNPO*
X	100.1	0.30	0.59	*Moesin; MSN*
25	37.8	0.00	0.37	*Ubiquitin Specific Peptidase 42; USP42*
10	75.9	0.15	0.42	*Ras Homolog Family Member J; RHOJ*
27	28.8	0.16	0.27	*Ring Finger Protein 122; RNF122*
21	67.0	0.18	0.65	*WD Repeat Domain 25; WDR25*
27	33.7	0.00	0.13	*Pleckstrin Homology Domain Containing A2; PLEKHA2*
10	28.4	0.00	0.11	*Solute Carrier Family 12 Member 6; SLC12A6*
5	114.9	0.00	0.41	*SAMM Sorting And Assembly Machinery Component; SAMM50*
27	28.8	0.50	0.85	*Ring Finger Protein 122; RNF122*
29	30.4	0.23	0.82	*Kirre Like Nephrin Family Adhesion Molecule 3; KIRREL3*
27	28.6	0.19	0.35	*Ring Finger Protein 122; RNF122*
22	57.3	0.44	0.88	*PPARG-TSEN2*
24	38.1	0.06	0.22	*DLG Associated Protein 1; DLGAP1*
21	56.3	0.23	0.40	*FERM Domain Containing 5; FRMD5*
25	32.4	0.13	0.27	*45S Pre-Ribosomal RNA; RN45S*
29	30.5	0.06	0.55	*Kirre Like Nephrin Family Adhesion Molecule 3; KIRREL3*
25	1.4	0.00	0.11	*Transformation/Transcription Domain Associated Protein; TRRAP*
8	85.2	0.00	0.29	*Osteomodulin; OMD*
22	2.1	0.00	0.11	*Eomesodermin; EOMES*
9	88.3	0.24	0.35	*UL16 Binding Protein 21; ULBP21*
15	78.4	0.21	0.44	*MAP Kinase Activating Death Domain; MADD*
8	71.0	0.49	0.66	*Tumor Necrosis Factor Receptor Superfamily, Member 10d; TNFRSF10D*
27	5.5	0.47	0.78	*Defensin, Beta; DEFB*
7	45.1	0.10	0.33	*AT-Rich Interaction Domain 3A; ARID3A*
5	117.7	0.00	0.12	*Tetratr Icopeptide Repeat Domain 38; TTC38*
3	8.8	0.03	0.13	*CD244 Molecule; CD244*
23	7.3	0.00	0.10	*Collagen Type XI Alpha 2 Chain; COL11A2*
2	62.8	0.00	0.14	*Transmembrane Protein 163; TMEM163*

[1]C= cytosine; G= guanine; H = adenine, cytosine or thymine
[2]Chr = Chromosome
[3]PNS = Prenatally stressed

AA-8. Top 30 ($P < 0.02$; listed from smallest to largest P values) hypermethylated CHH sites located within gene body regions of genes in prenatally stressed (PNS) compared with Control heifer calves.[1]

		Mean methylation ratio		
BTA	**Start**	**PNS**[2]	**Control**	**Gene**
25	2.1	0.11	0.01	*Potassium Channel Tetramerization Domain Containing 5; KCTD5*
29	41.5	0.14	0.00	*Neurexin 2; NRXN2*
26	33.8	0.11	0.00	*Transcription Factor 7 Like 2; TCF7L2*
24	0.6	0.14	0.00	*Par-6 Family Cell Polarity Regulator Gamma; PARD6G*
5	106.6	0.23	0.00	*Poly(ADP-Ribose) Polymerase Family Member 11; PARP11*
6	109.1	0.12	0.00	*Complexin 1;CPLX1*
2	67.3	0.11	0.00	*Dipeptidyl Peptidase Like 10; DPP10*
18	56.7	0.15	0.00	*PTOV1 Extended AT-Hook Containing Adaptor Protein; PTOV1*
13	68.3	0.92	0.55	*Protein Phosphatase 1 Regulatory Subunit 16B; PPP1R16B*
14	78.9	0.11	0.00	*Atpase H+ Transporting V0 Subunit D2; ATP6V0D2*
10	1.6	0.11	0.00	*Erythrocyte Membrane Protein Band 4.1 Like 4A; EPB41L4A*
18	43.9	0.19	0.01	*Solute Carrier Family 7 Member 10; SLC7A10*
1	45.5	0.62	0.14	*Adhesion G Protein-Coupled Receptor G7; ADGRG7*
12	52.7	0.34	0.18	*MYC Binding Protein 2; MYCBP2*
19	57.4	0.29	0.13	*RAB37, Member RAS Oncogene Family; RAB37*
23	49.2	0.53	0.08	*Phenylalanyl-Trna Synthetase 2, Mitochondrial; FARS2*
5	114.9	0.15	0.03	*SAMM50 Sorting And Assembly Machinery Component; SAMM50*
25	41.1	0.12	0.00	*18S Ribosomal RNA; RN18S1*
11	15.0	0.10	0.00	*Baculoviral IAP Repeat Containing 6; BIRC6*
7	90.6	0.11	0.00	*Uncharacterized LOC100616526**;** LOC100616526*
17	47.0	0.35	0.00	*Adhesion G Protein-Coupled Receptor D1; ADGRD1*
6	107.3	0.13	0.00	*Neuronal Vesicle Trafficking Associated 1; NSG1*
4	114.8	0.16	0.00	*Ras Homolog, Mtorc1 Binding; RHEB*
29	43.5	0.47	0.13	*Asparaginase And Isoaspartyl Peptidase 1; ASRGL1*
16	80.9	0.85	0.27	*Nuclear Receptor Subfamily 5 Group A Member 2; NR5A2*
26	34.3	0.12	0.00	*Hyaluronan Binding Protein 2; HABP2*
6	107.3	0.44	0.02	*Neuronal Vesicle Trafficking Associated 1; NSG1*
4	113.5	0.81	0.53	*SCO-Spondin; SSPO*
15	77.2	0.11	0.00	*Diacylglycerol Kinase Zeta; DGKZ*
19	37.0	0.00	0.11	*Xylosyltransferase 2; XYLT2*

[1]C= cytosine; G= guanine; H = adenine, cytosine or thymine
[2]PNS = Prenatally stressed

AA-9a. Genes with Hypomethylated CpG sites located in promoter regions that contribute to "Psychological Disorders "

Categories	Diseases or Functions Annotation	p-value	Molecules	# Molecules
Hereditary Disorder, Neurological Disease, Organismal Injury and Abnormalities,Psychological Disorders, Skeletal and Muscular Disorders	Huntington's Disease	0.0000692	*CKB,DRD1,DRD5,FDFT1,FGG,GABRD,GAD2,GDNF,GRIK2,LY6E,MLF2,NGRN,NPM1,NTF3,NTRK2,PARP1,PDK3,PLK2,PSMB8,PTPN5,RASD2,RHOG,SLC1A4,STOM,UCK2*	25
Psychological Disorders	Mood Disorders	0.00043	*ACTL6B,AR,CACNA1A,DEGS2,DRD1,DRD5,FDPS,GABRD,GABRQ,GAD2,GDNF,GRIK2,HTR5A,MAOA,NGFR,NTRK2,OGG1,PTPRU,SLC1A4*	19
Psychological Disorders	Anxiety Disorders	0.000458	*CACNA1A,DRD1,DRD5,GABRD,GABRQ,GAD2,HTR5A,MAOA,SLC1A4*	9
Neurological Disease, Psychological Disorders	Post-traumatic stress disorder	0.000517	*CACNA1A,DRD1,DRD5,GABRD,GABRQ,GAD2,HTR5A*	7
Hereditary Disorder, Neurological Disease, Organismal Injury and Abnormalities,Psychological Disorders, Skeletal and Muscular Disorders	Early-onset Huntington disease	0.000546	*GRIK2,NTRK2*	2
Developmental Disorder, Neurological Disease, Psychological Disorders	Speech disorder	0.000629	*CHAMP1,DRD1,GABRD,HTR5A,SLC6A8*	5
Neurological Disease, Psychological Disorders	Bipolar I disorder	0.000819	*DRD1,DRD5,GABRD,GAD2,HTR5A*	5
Developmental Disorder, Neurological Disease, Psychological Disorders	Speech and language disorders	0.00135	*CHAMP1,DRD1,DRD5,GABRD,HTR5A,SLC6A8*	6
Neurological Disease, Organismal Injury and Abnormalities,Psychological Disorders	Dementia	0.00158	*AR,CACNA1A,CNKSR2,DRD1,DRD5,EEF1G,FDFT1,FDPS,GABRD,GABRQ,GAD2,GDNF,LY6E,MAOA,MEOX2,MYC,NGFR,NR1H2,NTRK2,NTRK3,OGG1,PLK2,PTPN5,TUBA4A*	24
Neurological Disease, Psychological Disorders	Bipolar disorder	0.00172	*DEGS2,DRD1,DRD5,GABRD,GABRQ,GAD2,GRIK2,HTR5A,MAOA,NTRK2,OGG1,PTPRU*	12
Neurological Disease, Organismal Injury and Abnormalities,Psychological Disorders	Tauopathy	0.00173	*AR,CNKSR2,DRD1,DRD5,EEF1G,FDFT1,FDPS,GABRD,GAD2,GDNF,LY6E,MAOA,MEOX2,MYC,NGFR,NR1H2,NTRK2,NTRK3,OGG1,PLK2,PTPN5,SLC1A4,TUBA4A*	23
Psychological Disorders	Agitation	0.00177	*DRD1,DRD5,GABRD,GAD2,HTR5A*	5
Neurological Disease, Psychological Disorders	Primary restless legs syndrome	0.00179	*DRD1,DRD5*	2
Psychological Disorders	Depressive disorder	0.00241	*ACTL6B,AR,CACNA1A,DRD1,DRD5,FDPS,GABRD,GABRQ,GAD2,GDNF,MAOA,NGFR,SLC1A4*	13
Neurological Disease, Organismal Injury and	Amyotrophic lateral sclerosis	0.00246	*COL1A2,DRD1,DRD5,FHL3,GABRD,GAD2,MAGED2,NEFM,NGFR,SLC1A4,TUBA4A*	11

Abnormalities,Psychological Disorders				
Neurological Disease, Organismal Injury and Abnormalities,Psychological Disorders	Disorder of basal ganglia	0.00262	*ABCD1,CKB,DRD1,DRD5,FDFT1,FGG,GABRD,GAD2,GDNF,GRIK2,LY6E,MLF2,NGRN,NPM1,NTF3,NTRK2,PARP1,PDK3,PLK2,PSMB8,PTPN5,RASD2,RHOG,SLC1A4,STOM,TUBA4A,UCK2*	27
Neurological Disease, Organismal Injury and Abnormalities,Psychological Disorders, Skeletal and Muscular Disorders	Advanced idiopathic Parkinson disease	0.00266	*DRD1,DRD5*	2

AA-9b. Genes with Hypermethylated CpG sites located in promoter regions that contribute to "Psychological Disorders"

Categories	Diseases or Functions Annotation	p-value	Molecules	# Molecules
Psychological Disorders	Anxiety Disorders	0.0000244	*ADRA2B,CA3,CHRM1,CNR1,DRD1,HTR5A,HTR6,MAOA,OXT,PGR,SCN1B,SLC1A1,SLC6A11,SLC6A13,SLC6A2*	15
Neurological Disease,Psychological Disorders	Hebephrenic schizophrenia	0.000154	*ADRA2B,CNR1,DRD1,HTR5A,HTR6*	5
Neurological Disease,Organismal Injury and Abnormalities,Psychological Disorders	Tauopathy	0.000445	*ADRA2B,AMPH,BRCA1,CALB1,CCL4,CEMIP2,CHRM1,CNKSR2,CNR1,DRD1,DYSF,EPHA1,FGF2,HNRNPU,HTR6,HTRA2,ICAM1,IGSF8,IL1A,MAOA,NPTX1,PDIA3,PGR,PIK3IP1,PPTC7,PRKACB,PRKN,PTGER4,SCN1B,SERPINA3,SET,SLC1A1,SLC52A2,SLC6A2,SNAP91,STIP1,TUBA4A,TUBB4A,VIM*	39
Psychological Disorders	Social anxiety disorder	0.000447	*ADRA2B,CA3,DRD1,OXT,SCN1B,SLC1A1,SLC6A11,SLC6A13,SLC6A2*	9
Cellular Compromise, Neurological Disease, Organismal Injury and Abnormalities,Psychological Disorders	Injury of cortical neurons	0.000536	*CNR1,FAIM2,FGF2*	3
Neurological Disease, Psychological Disorders	Schizophrenia	0.0006	*ADRA2B,AMPH,ATF2,CA3,CALB1,CCK,CHRM1,CNNM2,CNR1,DRD1,GAD1,GOT1,GRIK3,GRM1,GSN,HTR5A,HTR6,LHB,MAOA,MEST,NPTX1,NTNG2,OXT,RARG,RPS19BP1,SCN1B,SETD1A,SLC12A5,SLC1A1,SLC35F3,SLC6A2*	31
Psychological Disorders	Schizophrenia spectrum disorder	0.000814	*ADRA2B,AMPH,ATF2,CA3,CALB1,CCK,CHRM1,CNNM2,CNR1,DRD1,GAD1,GOT1,GRIK3,GRM1,GSN,HTR5A,HTR6,LHB,MAOA,MEST,NPTX1,NTNG2,OXT,RARG,RPL10,RPS19BP1,SCN1B,SETD1A,SLC12A5,SLC1A1,SLC35F3,SLC6A2*	32
Neurological Disease, Organismal Injury and Abnormalities,Psychological Disorders	Cerebral degeneration	0.000895	*ADAMTS1,ADRA2B,CAPN2,CHCHD10,CHRM1,COL1A2,DRD1,ENO3,FUCA1,GSN,HTR6,PFKP,PGR,SCN1B,SERPINA3,SLC1A1,TUBA4A,TUBB4A,VIM*	19
Psychological Disorders	Drug dependence	0.000904	*ADRA2B,CA3,CHRM1,CNR1,DRD1,HTR6,MAOA,OXT,PGR,SCN1B,SLC52A2,SLC6A2*	12
Neurological Disease, Organismal Injury and Abnormalities,Psychological Disorders	Frontotemporal lobar degeneration or amyotrophic lateral sclerosis	0.00102	*ADAMTS1,ADRA2B,CAPN2,CHCHD10,CHRM1,COL1A2,DRD1,ENO3,FUCA1,GSN,HTR6,PFKP,PGR,SCN1B,SERPINA3,SLC1A1,TUBA4A,VIM*	18
Nutritional Disease, Psychological Disorders	Eating Disorders	0.0011	*CA3,CCK,CCKBR,CHRM1,CNR1,DRD1,HTR6,MAOA,NPY2R,PGR,SLC52A2,SLC6A2,TUBA4A,TUBB4A*	14
Psychological Disorders	Alcoholism	0.00113	*ADRA2B,CA3,CHRM1,DRD1,HTR6,MAOA,OXT,PGR,SCN1B,SLC52A2,SLC6A2*	11
Neurological Disease,O rganismal Injury and Abnormalities,Psychological Disorders	Dementia	0.0012	*ADRA2B,AMPH,BRCA1,CA3,CALB1,CCL4,CEMIP2,CHCHD10,CHRM1,CNKSR2,CNR1,DRD1,DYSF,EPHA1,FGF2,HNRNPU,HTR6,HTRA2,ICAM1,IGSF8,IL1A,MAOA,NPTX1,PDIA3,PGR,PIK3IP1,PPTC7,PRKACB,PRKN,PTGER4,SCN1B,SERPINA3,SET,SLC52A2,SLC6A2,SNAP91,STIP1,TUBA4A,VIM*	39
Neurological Disease, Psychological Disorders	Post-traumatic stress disorder	0.00129	*ADRA2B,CA3,CNR1,DRD1,HTR5A,HTR6,OXT,PGR,SLC6A2*	9
Neurological Disease, Psychological Disorders	Undifferentiated schizophrenia	0.00147	*ADRA2B,DRD1,HTR5A,HTR6*	4

Metabolic Disease, Neurological Disease, Organismal Injury and Abnormalities,Psychological Disorders	Alzheimer disease	0.00147	*ADRA2B,AMPH,BRCA1,CALB1,CCL4,CEMIP2,CHRM1,CNKSR2,CNR1,DRD1,DYSF,EPHA1,FGF2,HNRNPU,HTR6,HTRA2,ICAM1,IGSF8,IL1A,MAOA,NPTX1,PDIA3,PGR,PIK3IP1,PPTC7,PRKACB,PRKN,PTGER4,SCN1B,SERPINA3,SET,SLC52A2,SLC6A2,SNAP91,STIP1,VIM*	36
Neurological Disease, Organismal Injury and Abnormalities,Psychological Disorders	Amyotrophic lateral sclerosis	0.00162	*ADAMTS1,ADRA2B,CAPN2,CHCHD10,CHRM1,COL1A2,DRD1,ENO3,FUCA1,GSN,HTR6,PFKP,PGR,SCN1B,SLC1A1,TUBA4A,VIM*	17
Hereditary Disorder, Neurological Disease, Organismal Injury and Abnormalities,Psychological Disorders	Familial frontotemporal dementia	0.00178	*CHCHD10,CHRM1,SERPINA3,TUBA4A*	4
Cardiovascular Disease, Neurological Disease, Organismal Injury and Abnormalities,Psychological Disorders	Ischemic injury of brain	0.00183	*CNR1,GSN,RASGRF1,STIP1,UCP2*	5
Neurological Disease, Psychological Disorders	Paranoid schizophrenia	0.00213	*ADRA2B,DRD1,HTR5A,HTR6*	4
Neurological Disease, Organismal Injury and Abnormalities,Psychological Disorders	Disorder of basal ganglia	0.00275	*AADAT,ADRA2B,ALDH6A1,ARIH2,ATP2B1,CALB1,CCKBR,CCL4,CHRM1,CKB,CNR1,CTNNB1,DONSON,DRD1,ENO3,ETV5,FGF2,GPR107,GPS2,HNRNPU,HSP90AA1,HTR6,HTRA2,IL1A,JUNB,NDRG1,OCLN,PRKN,RNASEH2B,RPL13,RYR1,SCN1B,SERGEF,SERPINA3,SLC1A1,SLC6A2,SLIRP,SRM,SRPX,TOP1,TSN,TUBA4A,TUBB4A,VIM*	44
Psychological Disorders	Restlessness	0.00306	*ADRA2B,CHRM1,DRD1*	3
Neurological Disease, Organismal Injury and Abnormalities,Psychological Disorders	Cocaine-related disorder	0.00325	*ADRA2B,CA3,CHRM1,DRD1,OXT,PGR,SLC6A2*	7
Neurological Disease, Psychological Disorders	Major depression	0.00326	*ADRA2B,CCKBR,CHRM1,CRHR2,DRD1,HTR6,IL1A,MAOA,PGR,PSMD9,RNASEH2B,SCN1B,SLC1A1,SLC6A2,UBE2S,VIM*	16
Psychological Disorders	Personality disorder	0.00354	*ADRA2B,CA3,CHRM1,DRD1,HTR6,MAOA*	6

AA-10a. Canonical pathways involving genes with hypomethylated sites in the prenatally stressed heifers compared to control heifers

Ingenuity Canonical Pathways	-log(p-value)	Molecules
Thyroid Cancer Signaling	4.19	*MYC,NTF3,RASD2C3:C41,NTRK2,GDNF,NTRK3*
Neurotrophin/TRK Signaling	3.71	*NTF3,RASD2,NTRK2,GRB2,NTRK3,NGFR,SORCS1*
Amyotrophic Lateral Sclerosis Signaling*	3.05	*GDNF,**GRB2**,NEFM,**RAB5B**,**GRIK2**,CACNA1A,**SSR4***
CDK5 Signaling*	2.45	***RASD2**,**NTRK2**,DRD1,**NGFFR**,DRD5,**CACNA1A***
Ceramide Biosynthesis	2.43	*SPTSSB,DEGS2*
Human Embryonic Stem Cell Pluripotency*	2.4	***NTF3**,**NTRK2**,**GRB2**,**NTRK3**,**BMP8B**,**WNT6,**BMP7*
Hereditary Breast Cancer Signaling	2.27	*NPM1,RASD2,GRB2,H2AFX,POLR2H,ACTL6B,CHEK2*
Glioma Invasiveness Signaling	2.12	*RASD2,RHOG,GRB2,RHOT1,ITGAV*
GABA Receptor Signaling*	2.02	*GABRQ,GAD2,**GPR37**,**GABRD,CACNA1A***
Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency*	2.01	***RASD2,LIF,GRB2,BMP8B,WNT6,**BMP7*
BER pathway	1.95	*OGG1,PARP1*
ERK5 Signaling	1.78	*MYC,RASD2,LIF,MEF2A*
Actin Nucleation by ARP-WASP Complex	1.78	*RASD2,RHOG,GRB2,RHOT1*
Intrinsic Prothrombin Activation Pathway	1.71	*COL1A2,KLK9,FGG*
Molecular Mechanisms of Cancer*	1.66	***MYC,RASD2,RHOG**,**RABIF**,**GRB2,RHOT1,BMP8B,CDKN2C,WNT6**,BMP7,**CHEK2***
Cholecystokinin/Gastrin-mediated Signaling	1.63	*RASD2,RHOG,GRB2,RHOT1,MEF2A*
ErbB2-ErbB3 Signaling	1.63	*MYC,RASD2,GRB2,NRG3*
Telomerase Signaling	1.59	*MYC,RASD2,GRB2,TERF2IP,ELK3*
Epoxysqualene Biosynthesis	1.57	*FDFT1*
Glutamate Dependent Acid Resistance	1.57	*GAD2*
IL-8 Signaling*	1.55	***RASD2,RHOG,GRB2,RHOT1,ITGAV,CSTB,IRAK1***
Glioma Signaling	1.55	*RASD2,CAMK1,GRB2,IDH3G,CDKN2C*
BMP signaling pathway*	1.54	***RASD2,GRB2,**BMP8B,BMP7*
PTEN Signaling	1.54	*RASD2,NTRK2,NTRK3,GRB2,NGFR*
mTOR Signaling	1.5	*RASD2,RPS16,RHOG,RHOT1,GRB2,RPS21,MLST8*
Maturity Onset Diabetes of Young (MODY) Signaling	1.49	*HNF1B,CACNA1A*

Thrombin Signaling*	1.48	***RASD2****,CAMK1,****RHOG,GRB2,RHOT1****,****GATA3****,GATA2*
cAMP-mediated signaling*	1.44	*PDE8A,DUSP9,CAMK1,VIPR1,DRD1,HTR5A,DRD5*
Glioblastoma Multiforme Signaling*	1.44	***MYC****,****RASD2****,****RHOG****,****GRB2****,****RHOT1****,****WNT6***
STAT3 Pathway	1.43	*MYC,RASD2,NTRK2,NTRK3,NGFR*
GP6 Signaling Pathway	1.43	*COL1A2,RHOG,COL4A1,GRB2,FGG*
HMGB1 Signaling	1.43	*RASD2,RHOG,LIF,GRB2,RHOT1,NGFR*
Neuregulin Signaling*	1.38	***MYC,RASD2,GRB2,NRG3***
G-Protein Coupled Receptor Signaling*	1.37	***PDE8A,RASD2,DUSP9,****DRD1,VIPR1,HTR5A,****GRB2****,DRD5*
PEDF Signaling	1.35	*HNF1B,RASD2,GDNF,GRB2*
NF-κB Signaling	1.34	*RASD2,NTRK2,NTRK3,GRB2,NGFR,IRAK1*
Sirtuin Signaling Pathway	1.33	*MYC,POLR1D,NR1H2,TIMM10,PPIF,TUBA4A,OGG1,PARP1*
Apelin Liver Signaling Pathway	1.32	*COL1A2,APLN*
Glucocorticoid Receptor Signaling	1.3	*RASD2,AR,VIPR1,GRB2,POLR2H,GTF2H5,KRT80,ACTL6B,FGG*

* Canonical pathways that were identified in both heifer and bull analysis

Bolded genes are genes within shared pathways that are unquie to the heifer analysis

AA-10b. Canonical pathways involving genes with hypermethylated sites in the prenatally stressed heifers compared to control heifers

Ingenuity Canonical Pathways	-log(p-value)	Molecules
Gαs Signaling*	5.46	***GNG4,PRKACB***,*HTR6,DRD1,VIPR1,HTR5A,****CNR1***,*CREBBP,****MRAS****,* ***RYR1,****CHRM1,****PTGER4,ATF2***
PCP pathway*	4.64	*FZD10,****ROR2***,*WNT7A,****VANGL1,****SMO,****JUND***,*JUNB,****LGR4,ATF2***
Axonal Guidance Signaling*	3.62	***EFNA2,GNG4,PRKACB,****FZD10,****PLXNC1,ARHGEF12,ADAMTS1,*** ***KALRN***,*ADAMTS14,ARPC1B,NTN4,****PDIA3***,*EPHA1,****PTCH1,MMP15*** *,TUBA4A,****EFNA1,SEMA6D,WNT7A,GAB1,SRGAP1,NTNG2***,*SMO,* ***MRAS,TUBB4A,SRGAP2,MYL12B***
Gap Junction Signaling*	2.96	***PRKACB,NOV,GAB1***,*DRD1,****PDIA3,GAD1***,*GRIK3,****CSNK1A1,MRAS*** *,****GJD2,****TUBA4A,****TUBB4A,CTNNB1,GJB2***
Wnt/β-catenin Signaling*	2.77	***SOX17,APPL1,****FZD10,****WNT7A***,*CDH3,CREBBP,****CSNK1A1***,*SMO,SO X14,****CSNK2B,CTNNB1,RARG***
G-Protein Coupled Receptor Signaling*	2.53	***ADRA2B,PRKACB***,*HTR6,HTR5A,GRK2,****GRM1,CNR1***,*CREBBP,CH RM1,****ATF2,DUSP9,****DRD1,****GAB1***,*VIPR1,****MRAS,PTGER4***
L-cysteine Degradation I	2.42	*GOT1,CDO1*
Synaptogenesis Signaling Pathway	2.33	*EFNA2,PRKACB,KALRN,ARPC1B,GRM1,EPHA1,CREBBP,CDH15, EFNA1,ATF2,RASGRF1,DNAJC5,GAB1,CDH3,THBS2,MRAS,CTNN B1*
cAMP-mediated signaling*	2.24	***PRKACB,ADRA2B***,*HTR6,****DUSP9,****DRD1,VIPR1,****GRK2***,*HTR5A,****CNR 1***,*CREBBP,CHRM1,****PTGER4,ATF2***
Lysine Degradation II	2.21	*AADAT,AASDHPPT*
Lysine Degradation V	2.21	*AADAT,AASDHPPT*
Ovarian Cancer Signaling	2.18	*PRKACB,FZD10,PMS2,WNT7A,GAB1,MRAS,SMO,BRCA1,CTNNB1, LHB*
Sonic Hedgehog Signaling	2.17	*PRKACB,GRK2,PTCH1,SMO*
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis*	1.99	*FZD10,****IL1A***,*ICAM1,****PDIA3,FGF2***,*CREBBP,CSNK1A1,CEBPG,****IRA K1,ATF2,ROR2,WNT7A,GAB1***,*SMO,****MRAS,CTNNB1***
Corticotropin Releasing Hormone Signaling*	1.95	*PRKACB,CRHR2,GAD1,CNR1,PTCH1,CREBBP,SMO,JUND,ATF2*
Transcriptional Regulatory Network in Embryonic Stem Cells*	1.92	***SET,****LHX5,****ZIC3,GJD2,O****TX1*
TR/RXR Activation	1.85	*KLF9,UCP2,GAB1,GPS2,SLC16A2,PFKP,DIO3*
Superpathway of Methionine Degradation	1.85	*PRMT5,MCEE,GOT1,CDO1*
Hereditary Breast Cancer Signaling	1.74	*PMS2,POLR2F,HDAC8,GAB1,CREBBP,MRAS,BRCA1,CHEK2,FAN CA*
Endocannabinoid Developing Neuron Pathway	1.71	*PRKACB,GAB1,CNR1,CREBBP,MRAS,CTNNB1,BRCA1,ATF2*
Wnt/Ca+ pathway*	1.68	*FZD10,PDIA3,CREBBP,SMO,****ATF2***
Sertoli Cell-Sertoli Cell Junction Signaling*	1.67	***PRKACB,EPN2,CLDN4***,*KEAP1,****MRAS,****TUBA4A,****TUBB4A,CTNNB1 ,ATF2,OCLN***
Acyl Carrier Protein Metabolism	1.59	*AASDHPPT*
UDP-N-acetyl-D-galactosamine Biosynthesis I	1.59	*GALE*

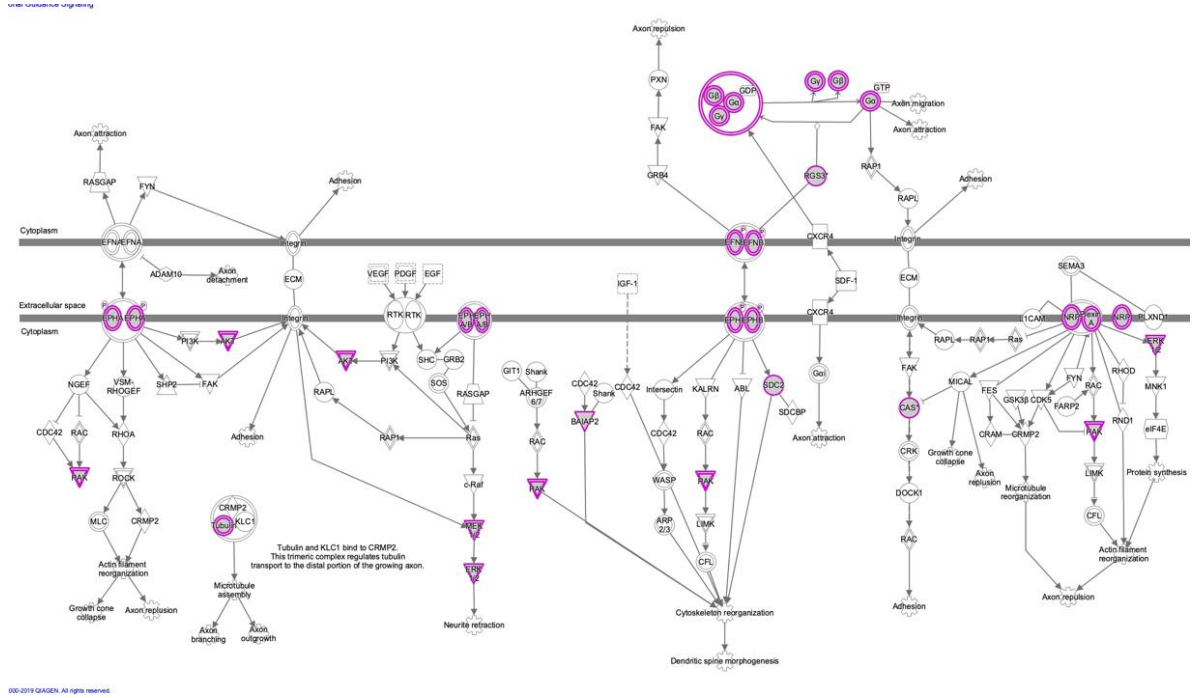
Role of IL-17F in Allergic Inflammatory Airway Diseases	1.56	*CCL4,CREBBP,RPS6KA4,ATF2*
Huntington's Disease Signaling*	1.54	***GNG4,POLR2F,****DNAJC5,****HDAC8,GAB1,GRM1,****CREBBP,****CAPN2,RCOR2,****HIP1,****UBE2S,ATF2***
PFKFB4 Signaling Pathway	1.53	*PRKACB,FGF2,CREBBP,ATF2*
UDP-N-acetyl-D-galactosamine Biosynthesis II	1.51	*PGM3,GALE*
AMPK Signaling	1.51	*ADRA2B,PRKACB,GAB1,AK8,CREBBP,MRAS,AK4,PFKP,CHRM1,PPM1G,ATF2*
Remodeling of Epithelial Adherens Junctions	1.5	*ARPC1B,TUBA4A,ZYX,TUBB4A,CTNNB1*
Factors Promoting Cardiogenesis in Vertebrates*	1.5	***TBX5,FZD10,SMO,NPPA,CTNNB1,ATF2***
Human Embryonic Stem Cell Pluripotency*	1.45	*FZD10****,WNT7A,ZIC3****,GAB1,****FGF2****,SMO,****MRAS,CTNNB1***
Cleavage and Polyadenylation of Pre-mRNA	1.44	*CPSF2,CSTF2*
Assembly of RNA Polymerase I Complex	1.44	*POLR2F,POLR1A*
ERK5 Signaling	1.43	*GAB1,CREBBP,MRAS,RPS6KA4,ATF2*
Basal Cell Carcinoma Signaling	1.41	*FZD10,WNT7A,PTCH1,SMO,CTNNB1*
GPCR-Mediated Integration of Enteroendocrine Signaling Exemplified by an L Cell*	1.41	*PRKACB,VIPR1,PDIA3,NPY2R,CCK*
Amyloid Processing	1.39	*PRKACB,CSNK1A1,CAPN2,CSNK2B*
RhoGDI Signaling*	1.38	***GNG4****,****ARHGEF12****,ARPC1B,PIP5K1C,CDH3,CREBBP,MRAS,****CDH15,MYL12B***
Ephrin Receptor Signaling*	1.38	***GNG4,EFNA2,ARPC1B,****KALRN,EPHA1,CREBBP,****MRAS,ATF2,EFNA1***
Oleate Biosynthesis II (Animals)	1.38	*FADS6,ALDH6A1*
Glycogen Degradation II	1.38	*GDPGP1,PGM3*
Colorectal Cancer Metastasis Signaling	1.37	*GNG4,PRKACB,APPL1,FZD10,WNT7A,GRK2,GAB1,MMP15,MRAS,SMO,CTNNB1,PTGER4*
Epithelial Adherens Junction Signaling	1.37	*EPN2,ARPC1B,KEAP1,TUBA4A,MRAS,ZYX,TUBB4A,CTNNB1*
Osteoarthritis Pathway*	1.34	*FZD10,****FGF2,ANKH****,PTCH1,CREBBP,****PTHLH****,SMO,IHH,****CTNNB1****,****ATF2***
Synaptic Long Term Potentiation	1.32	*PRKACB,GRM1,PDIA3,PPP1R7,CREBBP,MRAS,ATF2*
Phenylalanine Degradation IV (Mammalian, via Side Chain)	1.32	*GOT1,MAOA*
NGF Signaling*	1.31	***FZD10,WNT7A,CSNK1A1,SMO,CTNNB1***
β-alanine Degradation I	1.3	*ALDH6A1*
Taurine Biosynthesis	1.3	*CDO1*

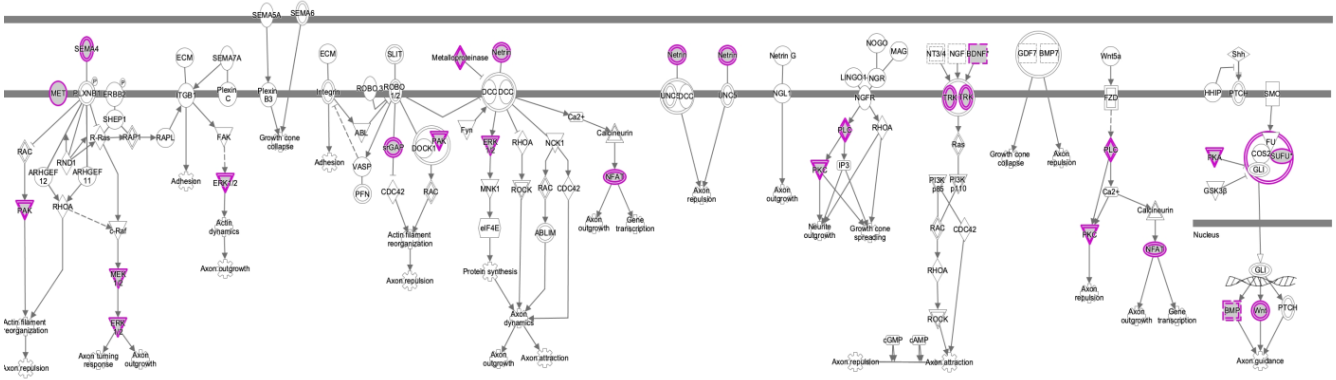
Spermidine Biosynthesis I	1.3	*SRM*
Adenine and Adenosine Salvage I	1.3	*APRT*
Glutamate Dependent Acid Resistance	1.3	*GAD1*

* Canonical pathways that were identified in both heifer and bull analysis
Bolded genes are genes within shared pathways that are unquie to the heifer analysis

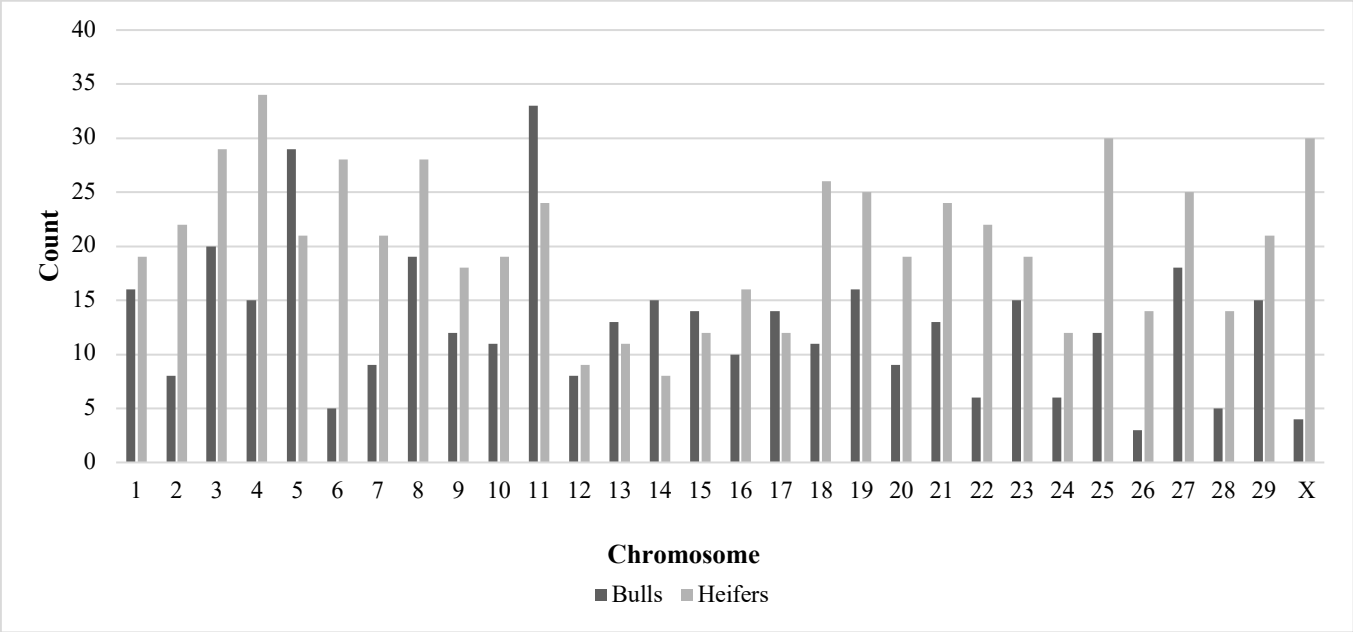
APPENDIX A

FIGURES

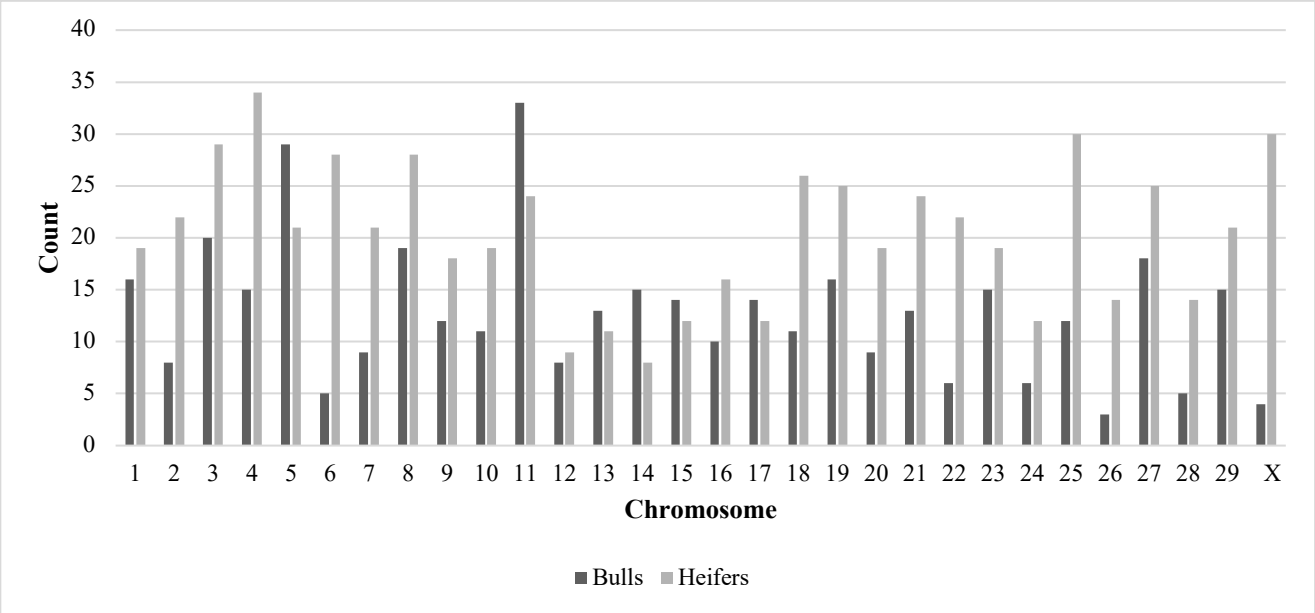




A-11. Axon Signaling Pathway: Genes highlighted pink had differentially methylated cytosine-guanine sites located within the promoter regions.



AA- 12. Distribution of significant CHG (H = adenine, cytosine or thymine) by chromosome for prenatally stressed bulls and heifers.



AA-13. Distribution of significant CHH (H = adenine, cytosine or thymine) by chromosome for prenatally stressed bulls and heifers.

APPENDIX B
TABLES

AB-1. Differentially methylated genes in amygdala tissue of prenatally stressed mature Brahman cows relative to Control cows.

Gene	Chr	Start	End	FDR	Difference[1]
SENP7	1	45816823	45988234	0.10239995	-10.455321
BOC	1	57945013	58023212	0.08055885	-7.7289743
IGSF11	1	63749635	63892877	0.04973763	-8.752317
HSPBAP1	1	67055375	67120252	0.08055885	13.418031
NCEH1	1	94818555	94882838	0.08706868	-10.272999
ENSBTAG00000049676	1	97057201	97058241	0.10934962	15.159061
RYK	1	135082834	135191082	0.08055885	8.928837
ENSBTAG00000046447	1	146865776	146877553	0.11906554	22.764719
ENSBTAG00000055206	1	157501070	157505106	0.1015848	12.949747
COL3A1	2	7375289	7414912	0.12965506	10.470315
IFIH1	2	34111730	34166890	0.11515915	-17.165384
PSMD14	2	34972722	35083065	0.08751004	12.692742
NMI	2	44826000	44846258	0.04046289	17.573175
GPR39	2	64935007	65196499	0.04973763	-7.60767
IKZF2	2	101012199	101190609	0.08067776	11.076058
BARD1	2	102760020	102854286	0.10357999	-10.248572
ABCA12	2	103002440	103202440	0.04193123	-11.698329
CNOT9	2	106532969	106566527	0.09922317	-11.317347
PSMD1	2	118865174	118946608	0.04183229	11.1653385
FAM43B	2	132076687	132077658	0.02485114	-14.899159
TMCO4	2	132915932	133020007	0.04548043	4.2697687
RF00416	2	133418331	133418462	0.09431379	-29.589947
HAX1	3	16250174	16252803	0.08055885	14.571835
S100A2	3	16805481	16811823	0.04046289	13.934963
SLC44A5	3	69467291	69583186	0.08055885	14.647983
ITGB3BP	3	82052300	82157897	0.10358545	-12.447068
MROH7	3	91567418	91621073	0.07619905	9.911443
ENSBTAG00000053378	3	106957972	106993645	0.05885069	12.056766
NDUFS5	3	106970587	106976098	0.08055885	21.374765
ENSBTAG00000050311	3	119733372	119736703	0.10239995	-14.60452
ZNF277	4	55985141	56120652	0.08055885	-6.9447637
WNT16	4	85839505	85850836	0.11707792	-8.879396
CEP41	4	94144405	94192546	0.14612688	12.581438
ENSBTAG00000051724	4	104316587	104452633	0.13822861	5.482247
ENSBTAG00000053682	4	105597508	105599525	0.08055885	-28.33998
TRPV6	4	106181051	106197865	0.0426268	12.620437

OSBPL8	5	5839779	6001503	0.00538802	9.881604
ACSS3	5	10671183	10846953	0.0426268	7.9681783
DUSP6	5	19213683	19217415	0.02639504	-9.941793
ENSBTAG00000016166	5	27660828	27673135	0.12443317	-11.240663
HELB	5	47481804	47519482	0.08927897	-13.566505
GNS	5	49053812	49103597	0.14179182	-10.152886
TIMELESS	5	56937139	56964713	0.08055885	-8.865396
STAT2	5	56997911	57009477	0.09905289	17.458893
NABP2	5	57097196	57103340	0.10363378	-10.103758
KCTD17	5	75479863	75490596	0.08751004	-7.8307943
TAPBPL	5	103922269	103929825	0.13822861	12.756297
IL17REL	5	119366047	119390141	0.08310065	-6.133422
BBS7	6	3385320	3423390	0.01556856	23.072868
MCUB	6	15717981	15813728	0.14331916	-7.9600143
NPNT	6	19173926	19254856	0.13770875	-6.1439633
MANBA	6	22062326	22189956	0.02485114	-10.626316
HPGDS	6	30269614	30303214	0.08055885	23.523632
ENSBTAG00000048013	6	84189236	84210644	0.09004046	45.07505
SEC31A	6	97653816	97713798	0.04973763	-16.679014
OTOP1	6	104881314	104932641	0.08055885	-12.444698
DGKQ	6	117399827	117410601	0.0426268	-7.3917727
ENSBTAG00000053194	7	4759093	4762902	0.08310065	13.559833
ENSBTAG00000035777	7	14350562	14381871	0.14090647	-13.706535
EPOR	7	15775449	15781166	0.08310065	10.811068
SNAPC2	7	16674793	16679420	0.10357999	-7.285522
SH2D3A	7	17732309	17747008	0.01067229	11.893044
NRTN	7	18518580	18532314	0.11560852	12.772074
ENSBTAG00000053812	7	20343967	20346465	0.13233256	-34.809727
ARL10	7	37840043	37846096	0.12408821	-9.734785
EDIL3	7	83846108	84324020	0.03312637	-7.6059146
ANXA10	8	495871	571472	0.11560852	-10.570888
PALLD	8	702606	1069003	0.04046289	3.1453402
PLGRKT	8	39279965	39316564	0.08310065	-14.640904
RCL1	8	39548658	39743532	5.52E-04	-13.902611
DMRT3	8	43580997	43593395	0.07396033	8.026344
STMN4	8	74339655	74364212	0.08310065	17.590965
CENPP	8	83951574	84187164	0.04183229	-12.664855
ACTL7A	8	98466288	98467807	0.08055885	29.759043
PALM2	8	99312764	99721951	0.05125592	-3.86279
C5	8	110338434	110435907	0.02485114	9.696053

ENSBTAG00000003921	9	23783735	23829745	0.07211056	8.028332
GPR6	9	40221514	40223897	0.05887487	-15.369354
FAXC	9	50419564	50488286	0.08055885	-8.706862
SPG21	10	11906117	11926485	2.52E-04	23.283916
KHNYN	10	20736077	20744735	0.0562095	-8.424653
ENSBTAG00000049196	10	21507163	21509872	0.05885069	-31.706348
MMP14	10	21967557	21978162	0.08091702	-9.081854
ENSBTAG00000048109	10	27030777	27031739	0.11560852	-43.89333
TMCO5A	10	33565746	33580261	0.09905289	55.165344
SAV1	10	43523556	43555390	0.0299807	-13.363056
C2CD4B	10	47902447	47904131	0.05401727	9.839191
DAAM1	10	71479280	71670966	0.05523539	7.396218
ARG2	10	79498980	79534600	0.06564941	-9.48084
ACTN1	10	80672883	80775228	0.08055885	-4.7000756
BOLA3	11	10504933	10514090	0.11560852	-10.543231
PAX8	11	46917080	46980824	0.12402434	5.056192
IMMT	11	48673645	48716318	0.07684372	-12.396902
WDR34	11	99159372	99179482	0.00507213	10.078437
CYSRT1	11	106240675	106241679	0.05885069	22.077307
NRARP	11	106326244	106326588	0.05523539	13.503956
TDRD3	12	1586960	1813642	0.0426268	-10.798498
PCCA	12	76812919	77174969	0.08055885	4.393822
ERCC5	12	79079749	79107518	0.08055885	8.593557
HSPA12B	13	51444141	51461167	0.05885069	-12.006369
ZGPAT	13	54034397	54047311	0.14785379	-7.160184
CHMP4B	13	63226304	63271073	0.13170381	8.964003
ENSBTAG00000046614	13	73786382	73797126	0.05401727	16.864708
TKDP4	13	74106673	74114980	0.0562095	-31.964869
SNX21	13	74603457	74608558	0.09004046	9.602252
CSE1L	13	77193956	77229841	0.06564941	12.707356
CYC1	14	738124	740518	0.09044099	12.541129
RHPN1	14	1262194	1269340	0.08312428	-7.1904235
ENSBTAG00000049478	14	16327058	16335278	0.08055885	8.500687
ANGPTL5	15	6889168	6906278	0.08055885	20.34807
ENSBTAG00000045881	15	13888639	13888722	0.14090647	47.718254
DSCAML1	15	28095216	28194601	0.13661687	-4.327785
FXYD2	15	28480087	28485276	0.09460237	13.052121
TRIM29	15	30684847	30712927	0.13822861	-7.562095
OR51I2	15	48161137	48216925	0.0562095	-26.14822
ARHGEF17	15	52798117	52856744	0.08310065	-5.356974

C2CD3	15	53454345	53526389	0.04262528	-10.612026
THAP12	15	55762482	55791234	0.11795015	-16.342508
ENSBTAG00000006060	15	61692631	62145149	0.08312428	-6.977913
LPXN	15	81845565	81887132	0.02639504	-10.08148
KCNT2	16	6388192	6826502	0.05816152	8.129614
RGS13	16	12596362	12623734	0.08751004	-25.60083
LIN9	16	29239152	29323608	0.10064777	-9.356739
STUM	16	29549972	29611628	0.08894859	7.418273
ENSBTAG00000024791	16	54214285	54220938	0.08751004	21.849363
ENSBTAG00000046630	16	54285818	54291206	0.14911702	30.013227
CRB1	16	76188124	76401805	0.00507213	-6.874449
ENSBTAG00000054727	16	80633603	80634826	0.09922317	19.272028
TMED2	17	52081940	52091382	0.11946971	-9.930153
CCDC60	17	55876002	56046137	0.07211056	-5.6608367
ENSBTAG00000055141	17	64687886	64720472	0.02485114	-8.527846
MN1	17	67173546	67221633	0.02639504	4.7563634
MED15	17	72431576	72476026	0.0850291	-4.0577784
DNAJA2	18	15375166	15386724	0.11761849	15.013463
MT1E	18	24029352	24032186	0.12004414	19.243813
OGFOD1	18	24154462	24184693	0.08055885	12.555247
AGRP	18	35086439	35087189	0.08055885	-25.63677
SLC7A9	18	43263397	43296804	0.0269549	12.144528
RHPN2	18	43404074	43474596	0.09114608	-8.253365
ENSBTAG00000050377	18	48525183	48527414	0.04973763	-29.960827
CRX	18	54704534	54716131	0.03312637	-13.696613
NOSIP	18	55990005	56002003	0.1214736	-7.1251154
ENSBTAG00000049494	18	57101773	57106993	0.02485114	-36.856384
ENSBTAG00000049736	18	58390922	58413756	0.05747092	-21.498652
ENSBTAG00000011052	18	58474268	58533857	0.08055885	-8.489392
RAB34	19	20139709	20143944	0.1171535	9.942293
ANKFY1	19	24725920	24776748	0.10498131	8.42412
G6PC	19	42949691	42960484	0.12004414	14.587959
AARSD1	19	42993675	43002885	0.01154433	-15.920333
RF00001	20	112377	112495	0.0426268	-14.934749
CREBRF	20	4776513	4831767	0.13233256	-8.723614
NSG2	20	5750258	5822147	0.06564941	-8.7111635
MRPS27	20	9285735	9390606	0.05448543	9.370926
IRX4	20	70777557	70786491	0.10075219	-4.1542287
ADAMTS7	21	30312315	30373658	0.04973763	6.9160256
RF00001	21	33002799	33002917	0.00311139	-2.2406046

ARID3B	21	34083678	34131883	0.04046289	7.2232895
ITPK1	21	57589349	57752955	0.10295309	-2.6991556
STAC	22	10127665	10240558	0.06407131	5.756243
ITGA9	22	10908546	11272063	0.10502975	-3.6321723
CTNNB1	22	13798884	13845848	0.08055885	-10.107223
LHFPL4	22	17087253	17118687	0.04183229	14.214472
SCAP	22	52163455	52264116	0.08055885	5.1119075
MGLL	22	59639050	59826846	0.04046289	-2.7216818
MLIP	23	6197413	6480455	0.08202975	6.586527
PSMB8	23	7173038	7176410	0.13822861	-10.619718
FOXP4	23	15366276	15417429	0.05401727	5.1421494
PPP1R11	23	28909903	28913055	0.06419515	14.219636
ENSBTAG00000025398	23	29027762	29074861	0.10502975	13.884531
CDKAL1	23	36745642	37444814	0.0850291	4.2813225
TMEM170B	23	44802116	44835671	0.0426268	13.835053
RREB1	23	48048576	48110128	0.03312637	-4.93398
CEP76	24	43057463	43072701	0.12453467	-14.56066
ENSBTAG00000053940	25	930440	932741	0.02639504	10.705613
DEXI	25	9604176	9618759	0.08055885	9.148698
GPR139	25	17645402	17687968	0.08055885	9.959709
COG7	25	21049528	21139768	0.02639504	-7.6824007
GSG1L	25	25339151	25591807	0.08055885	-3.7281926
bta-mir-2387	25	32930343	32930420	0.08751004	49.747475
TMEM120A	25	34213516	34218982	0.08310065	-8.790573
FAM20C	25	42135192	42169725	0.13233256	4.1166377
ENSBTAG00000006239	25	42246841	42262415	0.12004414	-13.934631
MINPP1	26	9137086	9201162	0.10502975	10.969415
NEURL1	26	24085526	24160165	0.00807545	-3.9000108
TACC2	26	41877231	42112620	0.1214736	2.676822
BUB3	26	42947094	42959878	0.01556856	14.276612
DOCK1	26	46392481	46948728	0.11761849	-2.2653499
CNOT7	27	19986516	20001556	0.08055885	-31.057346
DUSP26	27	29281841	29288445	0.00571753	13.108827
THAP1	27	37547355	37553519	0.08055885	-9.764168
ENSBTAG00000012132	28	4236177	4236836	0.07619905	-32.804234
ADAMTS14	28	26827396	26922026	0.08055885	5.245592
NDST2	28	29696608	29705236	0.00167585	-14.976065
ANXA8L1	28	41953894	41970260	0.00311139	23.369226
HEPACAM	29	28381619	28398677	0.00167585	14.751014
CABP4	29	45343481	45346973	0.05885069	12.876217

MRPL21	29	46258414	46269659	0.04183229	11.293135
ENSBTAG00000055043	X	28693036	28697532	0.1015848	16.971563
ENSBTAG00000049983	X	77056647	77123946	0.04973763	-17.344778
WDR45	X	87156551	87161774	0.11486064	-25.739037
GK	X	112861218	112935556	0.08462626	11.838968
FANCB	X	128633411	128661957	0.08055885	13.179837

[1]A positive (negative) difference indicates the prenatally stressed cows had decreased (increased) methylation of the gene relative to the Control cows.

AB-2 Panther Classification of genes with differentially methylation gene bodies in amygdala tissue of prenatally stressed mature Brahman cows relative to Control cows.

Gene	Biological Processes	Panther Protein Class
[Heparan sulfate]-glucosamine N-sulfotransferase	Metabolic Process	transferase
AA_TRNA_LIGASE_II_ALA domain-containing protein	Biological Regulation	RNA metabolism protein
ADAM metallopeptidase with thrombospondin type 1 motif 14	Cellular Process	metalloprotease
Agouti domain-containing protein	Behavior Biological Regulation Cellular Process Multicellular Organismal Process Response To Stimulus Signaling	intercellular signal molecule
ANK_REP_REGION domain-containing protein	Localization	ion channel
Annexin A8	Localization Metabolic Process	calcium-binding protein
AT-rich interaction domain 3B	Biological Regulation Cellular Process Metabolic Process	DNA-binding transcription factor
ATP binding cassette subfamily A member 12	Localization	ATP-binding cassette
Bardet-Biedl syndrome 7 protein homolog	Localization	
Beta-mannosidase	Cellular Process Metabolic Process	protein modifying enzyme
BTB domain-containing protein	Biological Regulation Cellular Process Localization Metabolic Process	
BZIP domain-containing protein	Biological Regulation Metabolic Process	
Calcium uniporter regulatory subunit MCUb	Biological Regulation Localization	
Catenin beta-1	Biological Regulation Metabolic Process	
CCR4-NOT transcription complex subunit 7	Biological Regulation Metabolic Process	mRNA polyadenylation factor
CHMP4B protein	Localization	membrane traffic protein
Collagen alpha-1(III) chain	Cellular Process	extracellular matrix structural protein
Complex I-15 kDa	Cellular Process	dehydrogenase
Component of oligomeric Golgi complex 7	Localization	
CS domain-containing protein	Cellular Process	chaperone
DIRP domain-containing protein	Biological Regulation Cellular Process Metabolic Process	DNA metabolism protein

DM domain-containing protein	Reproduction Reproduction Process Developmental Process	DNA-binding transcription factor
Dual specificity protein phosphatase 6	Developmental Process Multicellular Organismal Process Response To Stimulus Signaling	protein phosphatase
E3 ubiquitin-protein ligase PPP1R11	Biological Regulation Metabolic Process	ubiquitin-protein ligase
Excision repair cross-complementing rodent repair deficiency, complementation group 5	Response To Stimulus	DNA metabolism protein
FA complementation group B	Biological Regulation Cellular Process Metabolic Process Response To Stimulus	
FAM20C golgi associated secretory pathway kinase	Cellular Process Metabolic Process	
Fork-head domain-containing protein	Biological Regulation Metabolic Process	
G_PROTEIN_RECEP_F1_2 domain-containing protein	Biological Regulation Cellular Process Metabolic Process Response To Stimulus Signaling	G-protein coupled receptor
Glycerol kinase	Cellular Process Metabolic Process	carbohydrate kinase
GOLD domain-containing protein	Cellular Process Localization	vesicle coat protein
HCLS1-associated protein X-1	Biological Regulation Cellular Process	
Homeobox domain-containing protein	Biological Regulation Developmental Process Metabolic Process Metabolic Process Multicellular Organismal Process	homeodomain transcription factor
Ig-like domain-containing protein	Biological Adhesion Biological Regulation Cellular Process Response To Stimulus Signaling	immunoglobulin superfamily cell adhesion molecule
IKAROS family zinc finger 2	Biological Regulation Cellular Process Metabolic Process	C2H2 zinc finger transcription factor
Inositol-tetrakisphosphate 1-kinase	Metabolic Process	kinase
Ion channel TACAN	Cellular Process Developmental Process	

Leupaxin	Biological Adhesion Biological Regulation Locomotion Cellular Process Developmental Process Immune System Process Localization Multicellular Organismal Process Response To Stimulus Signaling	actin or actin-binding cytoskeletal protein
LHFPL tetraspan subfamily member 4 protein	Cellular Process Localization Multicellular Organismal Process	
Metallothionein	Biological Regulation Response To Stimulus	
MICOS complex subunit MIC60	Cellular Process Developmental Process	
NADH dehydrogenase [ubiquinone] iron-sulfur protein 5	Cellular Process	
Neuralized E3 ubiquitin protein ligase 1	Biological Regulation Developmental Process Multicellular Organismal Process Response To Stimulus Signaling	ubiquitin-protein ligase
Neuronal vesicle trafficking-associated protein 2	Localization	
Paired domain-containing protein	Developmental Process Metabolic Process	
Palladin, cytoskeletal associated protein	Biological Adhesion Locomotion Developmental Process Multicellular Organismal Process Response To Stimulus	scaffold/adaptor protein
Protein Wnt	Biological Regulation Cellular Process Developmental Process Multicellular Organismal Process Response To Stimulus Signaling	intercellular signal molecule
Ras responsive element binding protein 1	Biological Regulation Cellular Process Metabolic Process	
RNA helicase	Immune System Process Interspecies Interaction Between Organisms Multicellular Organismal Process Response To Stimulus	
RNase_Zc3h12a domain-containing protein	Metabolic Process	endoribonuclease
Salvador family WW domain containing protein 1	Response To Stimulus Signaling	
SH2 domain containing 3A	Biological Regulation Metabolic Process	

SH3 and cysteine-rich domain-containing protein	Biological Regulation Cellular Process Localization Multicellular Organismal Process	
Signal transducer and activator of transcription	Immune System Process Interspecies Interaction Between Organisms Metabolic Process Response To Stimulus Signaling	DNA-binding transcription factor
Solute carrier family 44 member 5	Localization	secondary carrier transporter
Solute carrier family 7 (Cationic amino acid transporter, y+ system), member 9	Localization	transporter
SOSS complex subunit B1	Response To Stimulus	nucleic acid metabolism protein
specificity protein phosphatase 6	Growth	protein phosphatase
Stathmin-4	Developmental Process Multicellular Organismal Process	
TACC_C domain-containing protein	Cellular Process Developmental Process Multicellular Organismal Process	
Timeless circadian regulator	Biological Regulation Metabolic Process Response To Stimulus	
UvrD_C_2 domain-containing protein	Biological Regulation Metabolic Process Response To Stimulus	RNA helicase
WD repeat domain phosphoinositide-interacting protein 4	Cellular Process Localization Metabolic Process	
WD_REPEATS_REGION domain-containing protein	Localization	vesicle coat protein
ENSBTAG00000011052	Biological Regulation Metabolic Process	
ENSBTAG00000038706	Biological Regulation Cellular Process Immune System Process Localization Response To Stimulus	
ENSBTAG00000025398	Biological Regulation Immune System Process Interspecies Interaction Between Organisms Metabolic Process Response To Stimulus Signallng	
ENSBTAG00000012132	Biological Regulation Cellular Process Immune System Process Localization Response to Stimulus	
ENSBTAG00000016999	Developmental Process Metabolic Process Multicellular Organism Process	
ENSBTAG00000046129	Localization	
ENSBTAG00000053940	Localization	

AB-3. Differentially methylated promoter[1] regions of genes in amygdala of prenatally stressed mature Brahman cows relative to the Control.

Promoter Region Of Gene	Chr	Start	End	FDR	Difference[2]
EIF5A2	1	96540492	96541992	0.0440214	15.864462
GK5	1	126735659	126737159	0.11195214	-12.384089
ENSBTAG00000049676	1	97056701	97058201	0.13191943	15.159061
HOXD1	2	20725509	20727009	0.06068113	11.453134
NMI	2	44825500	44827000	0.0784824	17.706318
STK36	2	106632721	106634221	0.0784824	22.094421
FAM43B	2	132076658	132078158	0.09070953	-10.584254
bta-mir-2358	2	135235643	135237143	0.1103739	-15.181887
PSMD1	2	118864674	118866174	0.1103739	11.012016
ENSBTAG00000040210	2	125044661	125046161	0.11664288	20.34308
FASTKD2	2	95064220	95065720	0.11720979	-20.698772
MDH1B	2	95063408	95064908	0.11720979	-20.698772
SSB	2	26553248	26554748	0.11720979	-19.552204
FGR	2	125688047	125689547	0.13401553	12.1650505
NDUFS5	3	106975098	106976598	0.0784824	22.37125
SNIP1	3	108337606	108339106	0.0784824	24.136435
RAB3B	3	94333517	94335017	0.08714517	-20.493038
CHRNB2	3	16009188	16010688	0.0920618	-14.643203
ENSBTAG00000048336	3	16009850	16011350	0.0920618	-14.643203
HSPB11	3	92262579	92264079	0.1103739	-21.00205
ENSBTAG00000054946	3	19173000	19174500	0.12828086	28.732193
ACP6	3	21960026	21961526	0.1340965	-12.010084
HAX1	3	16251803	16253303	0.14552826	12.262102
CEP41	4	94191546	94193046	0.0440214	25.610773
ENSBTAG00000053682	4	105597008	105598508	0.09994129	-28.33998
ADCK2	4	103993901	103995401	0.1458904	-10.160612
RASSF3	5	49189776	49191276	0.06752499	10.0584345
ENSBTAG00000016166	5	27660328	27661828	0.0818289	-16.624817
RF00026	5	44504613	44506113	0.11884585	-37.907047
SERHL2	5	113416641	113418141	0.14552826	-12.069026
SLC34A2	6	45177985	45179485	0.08714517	-27.994108
ATP8A1	6	61477178	61478678	0.11720979	-13.03858
BBS7	6	3384820	3386320	0.13104619	20.681067
CDKL3	7	45940170	45941670	0.0784824	19.936378
HARS	7	51816052	51817552	0.0818289	12.780387
HARS2	7	51816594	51818094	0.0818289	12.780387
HIST3H2BB	7	2572494	2573994	0.08218186	11.979676

MAT2B	7	75186953	75188453	0.08526111	13.497207
PLVAP	7	5762252	5763752	0.08703735	-15.885114
GIN1	7	102141034	102142534	0.09012403	16.833632
TBC1D9B	7	1374003	1375503	0.12828086	-11.136779
REXO1	7	44168237	44169737	0.13079715	-11.484821
STK32A	7	58457053	58458553	0.13079715	13.15607
KHSRP	7	18034888	18036388	0.13561586	-20.864365
BTBD2	7	44301294	44302794	0.13809216	12.754726
DNAJA1	8	74790116	74791616	0.0784824	15.529491
ACTL7A	8	98465788	98467288	0.0920618	30.216917
TMEM215	8	11334552	11336052	0.13463095	12.754243
GPR6	9	40222897	40224397	0.0818289	-15.369354
SLC35A1	9	62444501	62446001	0.12262049	-10.76525
SAV1	10	43554390	43555890	0.00283451	-17.046978
PSEN1	10	84619190	84620690	0.0784824	-14.272879
ENSBTAG00000049196	10	21508872	21510372	0.0818289	-31.706348
ENSBTAG00000051260	10	58436594	58438094	0.0920618	-23.416115
CCNB1IP1	10	26794463	26795963	0.1103739	-34.32697
F2R	10	7945404	7946904	0.11884585	-10.153249
TRIM36	10	4014816	4016316	0.12828086	11.230626
ZFYVE26	10	79666575	79668075	0.12954988	21.350533
ENSBTAG00000048109	10	27030739	27032239	0.13561586	-43.89333
CYSRT1	11	106240175	106241675	0.0784824	20.890074
FCN1	11	105449692	105451192	0.0784824	30.08309
ENSBTAG00000053674	11	104100532	104102032	0.0818289	-18.515192
IMMT	11	48673145	48674645	0.1103739	-13.37757
NDUFA8	11	93045862	93047362	0.1103739	16.17201
SLC3A1	11	26700906	26702406	0.11664288	33.581097
DAB2IP	11	92473856	92475356	0.12372064	21.397684
NRARP	11	106325588	106327088	0.12874347	8.726269
ASB6	11	100035799	100037299	0.13079715	-13.630757
MTIF3	12	32482258	32483758	0.0784824	24.633791
FAM155A	12	83412896	83414396	0.13079715	10.360734
PHF20L1	14	8542137	8543637	0.11664288	-14.648264
ENY2	14	55086610	55088110	0.11720979	14.231379
CYC1	14	739518	741018	0.13079715	12.774602
RHPN1	14	1268340	1269840	0.1458904	-12.441449
BDNF	15	58421131	58422631	0.0784824	-10.808294
THAP12	15	55790234	55791734	0.0784824	-17.56266
CELF1	15	77367002	77368502	0.11884585	13.892899

TRIM68	15	50412431	50413931	0.12798077	-12.211134
PTPMT1	15	77367719	77369219	0.1373871	12.143205
ENSBTAG00000052297	15	55791110	55792610	0.14959848	-15.205834
ENSBTAG00000050748	16	1580530	1582030	0.0784824	-23.721552
TIMM17A	16	80542813	80544313	0.0784824	-19.5594
LIN9	16	29322608	29324108	0.08526111	-10.646973
ENSBTAG00000055141	17	64719472	64720972	0.0784824	-13.280887
TCHP	17	63364001	63365501	0.0784824	13.153582
RSPH14	17	71815710	71817210	0.1103739	-13.175939
BEAN1	18	34194053	34195553	0.00283451	15.8675165
TOMM40	18	52618277	52619777	0.04530949	17.81364
NUDT21	18	24184406	24185906	0.0784824	14.91077
OGFOD1	18	24183693	24185193	0.0784824	14.91077
RAB4B	18	50035878	50037378	0.0784824	21.286337
NLRP8	18	63454958	63456458	0.09070953	-26.328257
PPP1R14A	18	48068952	48070452	0.0920618	-13.992401
FBXO17	18	48652122	48653622	0.11368779	-21.925058
GALNS	18	14031348	14032848	0.11664288	-10.716908
TRAPPC2L	18	14032088	14033588	0.11664288	-12.576086
KANSL1	19	46178281	46179781	0.0784824	26.386414
TIMP2	19	53460171	53461671	0.0784824	13.228864
NSF	19	45590669	45592169	0.1103739	-12.817368
DLX3	19	36661921	36663421	0.11664288	14.752321
CREBRF	20	4776013	4777513	0.0784824	-16.098417
RF00001	20	111877	113377	0.08218186	-12.620383
RF00001	21	33002299	33003799	5.68E-18	-3.338676
STARD5	21	27109334	27110834	0.0784824	21.163773
ZNF710	21	21402917	21404417	0.0784824	-14.137433
RF00003	21	44980500	44982000	0.13561586	-22.120235
CYP8B1	22	14948705	14950205	0.0920618	-36.492306
NCKIPSD	22	51200755	51202255	0.1103739	11.718233
EPM2AIP1	22	10550878	10552378	0.11664288	12.014217
ENSBTAG00000048652	23	43076484	43077984	0.1103739	-15.793822
MRTFB	25	12981880	12983380	0.0784824	15.783358
CACNA1H	25	930115	931615	0.0920618	9.494366
ENSBTAG00000006239	25	42261415	42262915	0.12828086	-16.041954
OAT	26	44063391	44064891	0.04650527	12.567262
ADGRA1	26	50722720	50724220	0.11122266	-10.797248
SH3PXD2A	26	24184907	24186407	0.11884585	27.283272
RF00002	27	6221800	6223300	0.00283451	-0.9971664

CNOT7	27	19986016	19987516	0.11720979	-15.481467
TRMT9B	27	24057110	24058610	0.11930687	12.115524
MTMR7	27	19847525	19849025	0.13561586	-12.403482
NDST2	28	29704236	29705736	0.14025979	-13.648562
MRPL21	29	46268659	46270159	0.0440214	29.031803
bta-mir-708	29	16595562	16597062	0.0784824	32.07527
KCNJ1	29	32243810	32245310	0.0784824	-27.71117
RF01684	29	43678068	43679568	0.0784824	33.49958
FLI1	29	32051049	32052549	0.0872757	12.629973
GAB2	29	17610031	17611531	0.08948711	-9.776492
IGHMBP2	29	46267978	46269478	0.1103739	16.660202
HNRNPUL2	29	41041489	41042989	0.14090458	-8.597105
KIF4A	X	80152340	80153840	0.0784824	-27.504435
PDZD11	X	80152961	80154461	0.0784824	-27.504435
SYN1	X	85991826	85993326	0.0920618	20.70853
GK	X	112934556	112936056	0.14016895	12.317889
SLC6A8	X	36913789	36915289	0.1458904	-11.856002

[1]Promoter regions were defined as 1000 base pairs upstream and 500 base pairs downstream from the transcription start site of the gene.

[2] A positive (negative) difference indicates the prenatally stressed cows had decreased (increased) methylation of the promoter region relative to control cows.

AB- 4. Differentially methylated CpG Islands1 in amygdala tissue of prenatally stressed mature Brahman cows relative to the Control.

Chr	Start	End	FDR	Difference
1	21327855	21328815	0.14489412	12.145946
1	88366237	88369031	0.14489412	19.594866
1	126735924	126736630	0.12981069	-12.384089
1	135431218	135431861	0.12693226	-25.97825
2	20725317	20726712	0.07114318	10.512508
2	21711342	21712479	0.13607466	14.495579
2	26553754	26554354	0.13854703	-19.552204
2	95064331	95064733	0.068468705	-29.626097
2	107451561	107452278	0.14489412	-13.560942
2	120803540	120804190	0.09857339	21.93038
2	121257369	121257812	0.1212729	18.356642
2	121571589	121576022	0.06906675	-0.972367
2	130682794	130684271	0.069648564	-12.100336
2	134188358	134189208	0.14489412	-16.733187
3	21227680	21228657	0.12160571	-19.703747
3	94333847	94334422	0.10163742	-20.493038
3	98535567	98535968	0.1214736	18.982307
3	108062645	108063158	0.14489412	-15.35155
3	115484235	115484705	0.1212729	50.45316
3	120132993	120133451	0.13607466	-22.16022
3	120287472	120288055	0.14489412	-35.393818
4	209862	210887	0.070508845	2.0327425
4	94192085	94192593	0.039706513	26.187975
4	104013158	104013884	0.07903983	21.839346
4	112507117	112508987	0.14489412	-10.769279
4	119127134	119128129	0.12628135	-23.942549
5	27660509	27661056	0.13607466	-15.5934925
5	32462290	32463599	0.12693226	11.963529
5	49190079	49191444	0.068468705	10.8877535
5	116581096	116582033	0.098364934	14.800256
5	116910979	116914323	0.12981069	-12.3218155
5	118986118	118986790	0.077380985	24.142744
6	45178532	45178952	0.10163742	-27.994108
6	116762144	116762699	0.119157724	23.578781
6	117313599	117314218	0.14489412	16.366083
7	1663058	1664049	0.07515866	-16.082977
7	2572475	2574236	0.068468705	12.628663

7	8157583	8158206	0.068468705	-20.438505
7	17744539	17745609	0.14489412	10.46968
7	106955324	106955725	0.057861846	-35.107075
8	1951766	1952273	0.13854703	-16.184608
8	11335580	11336259	0.12663691	12.841094
8	74790043	74791115	0.068468705	15.529491
8	112182251	112183435	0.14489412	16.861137
9	23784270	23786401	0.14489412	8.73909
9	62445007	62445799	0.058504775	-14.087485
9	87095881	87097345	0.058538288	-19.013952
10	7944928	7946673	0.14489412	-9.357961
10	36918247	36921059	0.14489412	-8.483359
10	43554822	43555817	0.018044801	-18.353825
10	47902330	47904071	0.09857339	9.46308
10	80769502	80771739	0.07903983	-9.18809
10	84619419	84619987	0.119157724	-15.536146
10	85383845	85384713	0.058504775	-20.242792
11	68469081	68469784	0.13559434	-13.178784
11	100036078	100037084	0.14489412	-13.630757
11	103412945	103414870	0.14489412	14.958873
11	104578337	104578780	0.14489412	23.702679
11	105739281	105740354	0.11481134	21.575684
11	106168512	106169605	0.091110736	-17.129986
13	2020030	2021874	0.10470228	-12.238297
13	27800477	27801059	0.14489412	-19.809866
14	739697	740838	0.12693226	13.847962
14	1268837	1269659	0.14489412	-12.65311
14	8542396	8543158	0.13607466	-14.648264
14	44856435	44857319	0.14489412	-13.998491
14	62132377	62132931	0.14489412	16.606443
14	81276069	81277345	0.06560621	-12.712286
15	26344772	26345623	0.13607466	21.173195
15	55790819	55791912	0.068468705	-17.56266
15	58421514	58422936	0.14489412	-9.354227
16	47324909	47326146	0.057861846	-16.632252
16	50616824	50617526	0.061019354	-22.911331
16	60632097	60633641	0.13607466	-9.86738
16	80543303	80544270	0.08611717	-19.5594
17	51223292	51224917	0.058504775	11.412326
17	53052763	53053209	0.14489412	28.952677

17	64719671	64720319	0.068468705	-14.345258
17	71653253	71653869	0.11481134	-29.387466
17	71664693	71665300	0.098364934	-35.77381
18	1178756	1179351	0.14489412	23.25662
18	5566886	5567503	0.068468705	-19.697992
18	14031996	14033015	0.058504775	-13.989094
18	34194169	34195605	0.003489547	15.8675165
18	52618574	52619637	0.12781523	15.972969
18	59225165	59228125	0.10358603	-1.1116353
18	64038403	64039035	0.14669716	-14.4734535
19	14504006	14504410	0.1212729	-29.008615
19	17228097	17228795	0.14489412	-21.569008
19	39187146	39188303	0.13607466	-11.614799
19	42189834	42190481	0.14669716	-26.69414
19	49916310	49917178	0.057861846	20.846664
19	62947747	62948313	0.058504775	-24.976372
20	112298	112821	0.098364934	-12.620383
20	4776189	4777637	0.068468705	-16.081154
20	71009252	71009654	0.057861846	30.508352
21	5284513	5285968	0.12156962	10.265126
21	27110177	27110620	0.058504775	23.573555
21	30374233	30374848	0.1384632	22.579184
21	31336702	31337220	0.119157724	26.495945
21	33001944	33003266	6.99E-18	-3.338676
21	33023989	33026059	0.001672166	2.3634098
21	58537284	58538064	0.061019354	-18.44024
22	10127376	10128210	0.068468705	12.086294
22	45565536	45566201	0.14489412	17.832607
22	46845829	46846276	0.098364934	-18.238861
22	51200977	51201673	0.12663691	11.718233
22	59748520	59749600	0.068468705	-28.574736
22	59805538	59807288	0.07114318	-18.507301
23	16551912	16552325	0.13607466	-16.414312
23	48178994	48179574	0.12693226	-21.415386
24	382623	383382	0.12663691	-21.870962
24	33563941	33565049	0.14489412	-12.335613
25	576519	576940	0.077380985	21.463896
25	2451862	2452362	0.14489412	-18.310242
25	12982104	12982986	0.057861846	17.016039
25	39162700	39163161	0.116253704	28.863672

25	42261615	42262457	0.14489412	-16.041954
26	42947711	42948827	0.068468705	14.494594
26	44063740	44064800	0.057861846	13.139896
27	14953824	14956280	0.098364934	-9.881515
27	16448217	16448934	0.14489412	-20.254025
29	12504979	12505967	0.117201	-16.05458
29	17610083	17611142	0.12628135	-10.437281
29	42549665	42551050	0.002384404	14.031609
29	46268140	46269101	0.12693226	16.660202
29	48492363	48493007	0.14489412	-19.816578
29	48645570	48646135	0.14489412	17.592733
29	50667134	50667577	0.14489412	-31.44485
X	64634437	64634871	0.14489412	28.797499
X	85992295	85993119	0.117201	20.70853
X	103344150	103346915	0.09857339	9.5680895
X	133145428	133145880	0.08509484	-36.059883

[1]Cytosine-Phosphate-Guanine rich locations within the genome

[2] A positive (negative) difference indicates the prenatally stressed cows had decreased (increased) methylation of the promoter region relative to control cows.

AB-5. Differentially methylated CpG[1] sites within amygdala tissue of prenatal stressed mature Brahman cows relative to Control cows.

Chr	Locus	LogFC[2]	FDR	Annotation	Gene Name
1	145199443	-2.52	0.09	Intergenic	
1	148969359	4.22	0.149	Intergenic	
2	135488751	-2.48	0.09	Intron	*Protein-arginine deiminase type-2*
2	136199564	3.59	0.114	Intergenic	
4	74891603	-4.65	0.09	Intron	*Tensin 3*
9	97903579	4.74	0.07	Intron	*Parkin*
11	103612150	-3.76	0.077	Intergenic	]
11	103612147	-3.26	0.09	Intergenic	
11	105605519	-2.34	0.112	Intergenic	
14	22230539	-5.03	0.01	Intron	*SRY-Box Transcription Factor 17*
14	7280340	-2.43	0.09	Intergenic	
17	72559623	2.2	0.09	Intergenic	
17	71213263	2.91	0.149	Exon	*Zinc Finger Protein 70*
17	13849058	-3.44	0.149	Intergenic	
18	62261931	-3.93	0.141	Intron	*Troponin I*
18	65212650	-3.88	0.149	Intergenic	
20	40994789	-4.3	0.09	Exon	*Natriuretic Peptide Receptor 3*
20	62608028	-4.02	0.147	Exon	*Ankyrin repeat domain 33B*
21	20861947	-2.87	0.09	Intergenic	
21	60328786	-2.79	0.125	Intron	*Spectrin Repeat Containing Nuclear Envelope Family Member 3*
21	20884739	2.44	0.147	Intergenic	
24	57999173	4.51	0.09	Intron	*Zinc Finger Protein 532*
25	2273343	-3.65	0.141	Exon	*Serine protease 33*
25	240979	2.79	0.149	Intron	*Luc7-like 1*
25	21705350	-3.5	0.149	Intron	*Protein Kinase C Beta*
28	41900953	-2.7	0.09	Intergenic	
29	11042233	-5.49	0.007	Intergenic	
29	46942706	-4.52	0.09	Promoter	*Fibroblast growth factor 19*
29	28949723	-3	0.125	Promoter	*Fasciculation & Elongation Protein Zeta 1*

[1] Cytosine-Phosphate-Guanine

[2] Positive (negative) fold change indicates the prenatally stressed cows had decreased (increased) methylation of the site compared to the Control