EPIGENETIC PROGRAMMING OF PHYSIOLOGICAL FUNCTIONS BY A PRENATAL STRESSOR AND GENETIC PARAMETERS OF TEMPERAMENT IN CATTLE

A Dissertation

by

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ABSTRACT

This project consisted of two main objectives. Objective 1 assessed the influences of prenatal stress on 1) postnatal physiological functions and 2) the postnatal presence and prevalence of epigenetic differences, specifically degree of DNA methylation, in immune cells of calves. Objective 2 assessed the genetic parameters of temperament across an age continuum in cattle. Calves studied in Objective 1 were progeny from Brahman cows that were either transported at 60, 80, 100, 120, and 140 ± 5 d of gestation (the prenatally stressed group, PNS) or were designated as the non-transported Control group. After weaning, response to an endotoxin challenge was assessed in 16 PNS and 16 Control bull calves. In response to LPS, PNS bull calves exhibited increased rectal temperatures, IFN-γ, and TNF-α, as well as decreased serum IL-6. Additionally, a subset of bull calves (n=7 PNS; n= 7 Control) was selected from the total population for evaluation of genome-wide DNA methylation in white blood cells. There were 16,128 CpG sites, 226 CHG sites, and 391 CHH sites differentially methylated in PNS compared to Control calves. An enrichment analysis was used to associate differentially methylated sites in PNS calves with predicted alterations to biological pathways. Enrichment analysis revealed alterations to biological pathways related to functions such as immune function, HPA axis activity, and neurotransmitter signaling. Objective 2 sought to further understand the genetic components of temperament. Random regression procedures estimated genetic parameters of temperament across an age continuum in a population of commercial beef cattle. As the

cattle matured over time there was an increased influence of permanent environmental effects and a decreased influence of additive genetic effects based on random regression analyses.

DEDICATION

To my family and loved ones that have supported me along the way.

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NOMENCLATURE

11β-HSD2	11β-hydroxy steroid dehydrogenase type II
5-HT	Hydroxytryptamine
AcH	Acetylcholine
ACTH	Adrenocorticotropic hormone
ADHD	Attention deficit hyperactivity disorder
APP	Acute phase proteins
APR	Acute phase response
BCP	1-bromo-3-chloropropane
c^2	Permanent environmental variance as a proportion of the phenotypic variance
cAMP	Cyclic adenosine monophosphate
CBC	Complete blood count
COMT	Catechol o-methyl transferase
CRH	Corticotrophin-releasing hormone
E	Epinephrine
EV	Exit velocity
GABA	Gamma-aminobutyric acid
GR	Glucocorticoid receptor
HPA	Hypothalamic-pituitary-adrenal
IFN-γ	Interferon-gamma

IL-6	Interleukin-6
IPA	Ingenuity Pathway Analysis software
LPS	Lipopolysaccharide
LSM	Least squares mean
MR	Mineralocorticoid receptor
MTC	Mature temperament score
NE	Norepinephrine
NR3C1	Glucocorticoid receptor gene
PEPCK	Phosphoenolpyruvate carboxykinas
PNS	Prenatal stress
POMC	Proopiomelanocortin
PS	Pen score
PVN	Paraventricular nucleus
RT	Rectal temperature
s^2	Variance
SAM	Sympathetic-adrenal-medullary
SBS	Sickness behavior score
SEM	Standard error of the mean
TNF-α	Tumor necrosis factor-alpha
TpH	Tryptophan 5-hydroxylase
TS	Temperament score
VP	Arginine vasopressin

WBC	White blood cells

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

There is a growing demand for and world trade in animal derived protein (USDA, 2013). This demand for beef products starts with the cow-calf industry and necessitates increased production of wholesome and efficiently produced meat products. Animal health in beef cattle production systems is a key welfare and economic concern. Increased morbidity rates result in additional medical costs, reduced growth rates, and losses in productivity, especially at the feedlot stage of production (Cernicchiaro et al., 2013). Furthermore, feedlot cattle with more excitable and nervous temperaments have been reported to have increased morbidity rates relative to calm cattle (Fell et al., 1999). Both temperament and immune function have been reported to be altered in prenatally stressed animals, including calves (Littlejohn et al., 2016; Carroll et al.; 2017). Therefore, it is important to understand how early life, especially prenatal development, can shape physiological functions. Prenatal stress is known to affect development of the fetus and lead to adverse health issues later in life (Barker et al., 2002). A primary mechanism believed to regulate the effects of prenatal stress is epigenetics, specifically DNA methylation (Szyf, 2012).

Transportation is a common occurrence in many production operations for pregnant cows, and is known to be a stressor in pregnant Brahman cows (Lay et al., 1996, Price et al., 2015) . Specifically, transportation caused increased rectal temperature

as well as circulating concentrations of cortisol and glucose (Price et al., 2015). Pregnant Brahman cows were transported at 60, 80, 100, 120, and 140 ± 5 d of gestation. Our lab reported these progeny to have more excitable temperaments and increased circulating concentrations of cortisol through weaning (Littlejohn et al., 2016). We then evaluated innate immune response to an endotoxin challenge in a subset of prenatally stressed bull calves. Additionally, a small subset of bulls from this population was evaluated for genome-wide differential methylation. An enrichment analysis was used to associate differentially methylated sites with predicted alterations to biological pathways. Enrichment analysis revealed alterations to biological pathways related to immune function, HPA axis activity, and neurotransmitter signaling. Because temperament and stress responsiveness were the predominant phenotypic and methylomic related alterations observed in prenatally stressed calves, our lab sought to further understand not only the epigenetic, but also the genetic components of temperament. Random regression procedures were used to estimate genetic parameters of temperament as a calf ages in an independent population of commercial cattle. The following review of the literature discusses how prenatal stressors could affect physiological functions by transcriptomic and epigenomic regulation, and genetic parameters of temperament in cattle.

LITERATURE REVIEW

Physiology of Stress

General Concepts of Stress. Stress can be defined as the biologic responses by which animals cope with real or perceived threats to homeostasis (Moberg, 1999; Chrousos, 2009). Homeostasis is defined as the maintenance of physiological variables within acceptable ranges (Cannon, 1929). Claude Bernard first reported ideas that would lead to the understanding of homeostasis in 1878 with the statement, "It is the fixity of the 'milieu interieur' which is the condition of free and independent life all the vital mechanisms, however varied they may be, have only one object, that of preserving constant the conditions of life in the internal environment." Hans Selye later (1936) introduced the early concepts of stress to the literature as, ''the nonspecific response of the body to any demand upon it.'' Selye further described a process of events occurring in response to a stressor, which he labeled as the "General Adaptation Syndrome". The "General Adaptation Syndrome" consists of an initial "alarm reaction," followed by a period of adaptation to the stressor, and ending with a final state of exhaustion (Selye, 1974). Selye reported that when injected with various tissue extracts or formalin, rats had bleeding gastrointestinal ulcers, larger adrenal glands, and degenerated lymphoid tissue in the thymus, spleen, and lymph nodes (Selye, 1952). It has long been established that individuals react differently to stress, thus biological responses to stress are different in every individual (Selye, 1973). Stress may elicit positive, negative, or no effect on biological systems. Different types and severities of stress have diverse effects on biological systems. Stress as a whole is neither negative nor positive, but situational.

Selye put it best when he wrote that stress is unavoidable and necessary. He made the point that during normal daily activities, even while sleeping, your heart, lungs, and digestive system are continuously working. The body is constantly under some degree of stress to maintain homeostasis and adapt to a constantly changing environment (Selye, 1973). Two key components of a physiological response to a stressor include the hypothalamic-pituitary-adrenal (**HPA**) axis and the sympathetic-adrenal-medullary (**SAM**) axis.

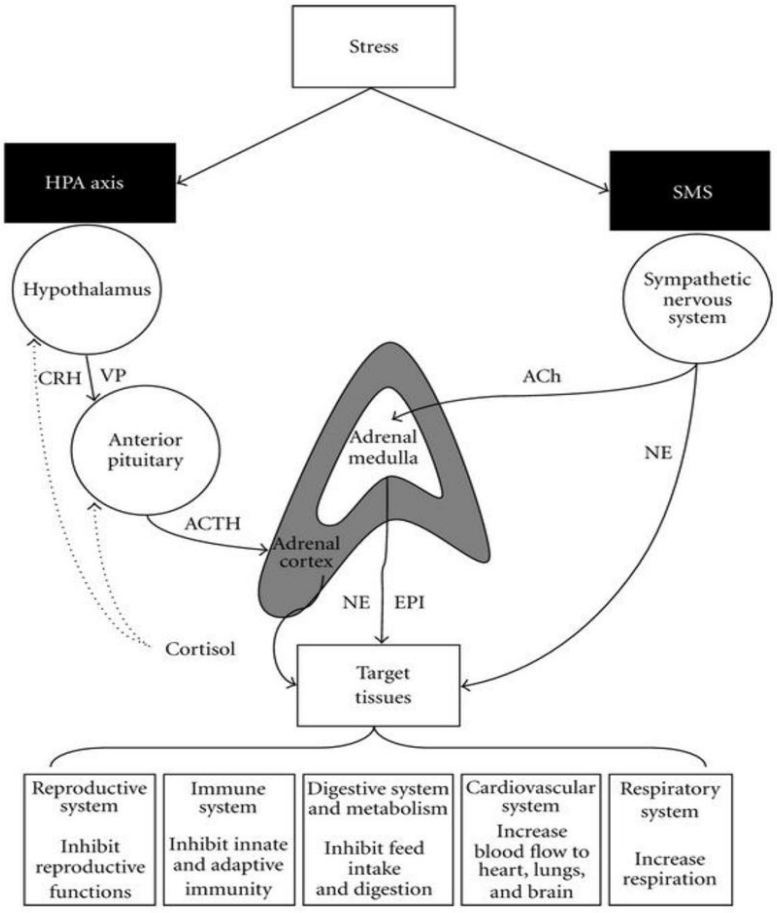

Figure 1-1. Impact of stress on the hypothalamic-pituitary-adrenal axis and sympathetic-adrenal-medullary axis (Reprinted from Burdick Sanchez et al., 2011).

Hypothalamic-Pituitary-Adrenal Axis. It has been established that stressors cause activation of the HPA axis to mediate a stress response, resulting in elevated circulating concentrations of cortisol in the blood (Butcher and Lord, 2004; Figure 1-1). In response to stress, neurosecretory neurons in the periventricular nucleus (**PVN**) of the hypothalamus synthesize corticotrophin-releasing hormone (**CRH**) and arginine vasopressin (**VP**; Brown, 1994). Corticotrophin-releasing hormone is a 41 amino acid peptide that regulates secretions of the anterior pituitary gland (Vale et al., 1981), which is increased in response to stress (Rivier and Vale, 1983; Gibbs, 1985; Plotsky et al., 1985). The hypothalamus is the primary, but not sole origin of CRH. It is also secreted by other tissues such as the placenta; thereby, affecting maternal and fetal circulation (Challis et al., 1995). Corticotrophin-releasing hormone travels through the hypophyseal portal vessels to act on corticotrophes in the anterior pituitary gland. Corticotrophes in the anterior pituitary gland produce a pre-cursor protein known as proopiomelanocortin (**POMC**), which can be cleaved into the peptides adrenocorticotrophic hormone (**ACTH**) and β-endorphin, an endogenous opioid (Nakanishi et al., 1979; Herbert, 1981; Blalock, 1994; Brown, 1994). Adrenocorticotrophic hormone is released from the anterior pituitary gland and travels through the circulation to the zona fasciculata of the adrenal cortex to stimulate synthesis of glucocorticoids, primarily cortisol in mammals (Vale et al., 1981; Axelrod and Reisine, 1984; Antoni, 1986; Plotsky, 1987; Charmandari et al., 2005; Brown, 1994). Glucocorticoids act on both glucocorticoid receptors (**GR**) and mineralocorticoid receptors (**MR**) in target tissues, causing modifications to the production of glucocorticoids from the adrenal cortex by altering

the regulation of gene expression in the hypothalamus and anterior pituitary gland (Evans, 1989; Burdick et al., 2011; Herman et al., 2012). Once glucocorticoids bind to the GR, the ligand-receptor complex will migrate into the nucleus from the cytoplasm, where it will bind to glucocorticoid response elements, causing changes in gene expression (Jones, 2012). Both ACTH and cortisol are generally secreted in a pulsatile manner and can be affected by stimuli such as feeding patterns, length of day, and encounters with stressors (Charmandari et al., 2005). In most species, the magnitude of CRH pulses is greatest in the early morning, resulting in increased ACTH and cortisol secretion at that time (Charmandari et al., 2005). High concentrations of glucocorticoids in circulating blood act on the hypothalamus to terminate the secretion of CRH and act on the anterior pituitary gland to terminate the secretion of ACTH. This negative feedback mechanism is involved in reducing HPA activity and terminating the stress response (Charmandari et al., 2005). The return of glucocorticoids to basal concentrations is essential, as glucocorticoids are known to have many effects, such as to decrease muscle protein synthesis and increase muscle protein degradation. They also act to allocate resources to produce increased blood glucose for energy expenditure and have catabolic, lipogenic, anti-reproductive and immunosuppressive effects, in which prolonged exposure can be detrimental to the body (Sapolsky et al., 2000; Gerrard and Grant, 2003; Charmandari et al., 2005; Chrousos, 2009).

Sympathetic-Adrenal-Medullary Axis. Upon encountering a stressor, the body will activate the sympathetic-adrenal-medullary axis (**SAM**) to initiate an immediate response. This immediate response to a stressor was termed by Walter Cannon as the

"fight or flight response" and is associated with acute changes in adrenal gland secretion (Cannon and Lissak, 1939). Selye (1973) described this response as an "alarm reaction". When an individual encounters a stressor, acetylcholine (**AcH**) is secreted from the pre-ganglionic nerve fibers in the adrenal medulla. This initiates the secretion of catecholamines, epinephrine (**E**), norepinephrine (**NE**), and dopamine from the medulla of the adrenal gland (Verbrugghe et al., 2012; Figure 1-1). Catecholamines function to act on cardiac muscle and blood vessels to maintain blood circulation and pressure, mobilize glycogen for energy, initiate a proinflammatory response, and increase body temperature and respiration rate to prepare for "fight or flight" and mediate the acute stress response (Gerrard and Grant, 2003; Verbrugghe et al., 2012).

Prenatal Programming by a Maternally Encountered Stressor

Maternally encountered stressors alter physiological parameters in the dam such as glucocorticoids and catecholamines, which can result in an altered fetal environment. Potential stressors incurred by a gestating bovine could include predation, handling, restraint, or transportation (Cooke et al., 2013; Grandin and Shivley, 2015). Prenatal programming is defined as the fetal response to a specific challenge during a critical developmental period that alters the trajectory of development (Nathanielsz et al., 2007). Such alterations in fetal environment due to prenatal stress are involved in programming fetal systems to select for enhanced vigilance to better prepare the neonate to survive in a stressful postnatal environment (Matthews, 2002).

Potential mechanisms by which alterations to fetal systems, especially the HPA axis, occur due to prenatal stress are complex. Glucocorticoids are known to pass across the placenta from maternal to fetal circulation in rats and humans (Zarrow et al., 1970; Merlot et al., 2008). Under normal conditions, the glucocorticoid cortisol is converted to an inactive form called cortisone by the placental barrier enzyme 11β-hydroxy steroid dehydrogenase type II (11β-HSD2; Benediktsson et al., 1997). This mechanism is believed to play a crucial role in maintaining a 13-fold lower concentration of cortisol in fetal blood circulation compared to maternal blood circulation (Gitau et al., 2001). This regulated amount of cortisol allowed to enter the fetal circulation is necessary for proper fetal development. For example, glucocorticoids are known to promote fetal lung maturation. Often premature human infants are even supplemented with synthetic glucocorticoids to promote lung development (Garbrecht et al., 2006). However, under conditions of maternal stress, elevated cortisol concentrations in maternal blood circulation may cause the suppression of 11β-HSD2. Holmes et al. (2006) found that blocking 11β-HSD2 with carbenoxolone, an 11β-HSD2 inhibitor, in pregnant mice caused an upregulation of glucocorticoid receptors in basolateral, central and medial nuclei in the amygdala portion of the brain as well as greater basal concentrations of corticosterone in the blood of the offspring as adults. Mechanisms by which altered fetal environment give rise to alterations in trajectories of developing biological processes, is regulated in part by epigenetic modifications.

Epigenetic Regulation Programs Developmental Trajectories of Prenatally Stressed Individuals

Epigenetic modifications act to regulate genome function, without altering the DNA sequence itself (Waddington, 1957). The epigenome is most sensitive to change during embryogenesis and perinatal development (Reik, 2007). Epigenetic signaling involves chemical modifications to the DNA or the regions surrounding the DNA, known as histones (Zhang and Meaney, 2010; Roth and Sweatt, 2011). Histones are proteins that package and condense eukaryotic DNA within the nucleus, including H3, H4, H2A, H2B, and H1 (Strahl and Allis, 2000; Roth and Sweatt, 2011). Chromatin, the unified combination of DNA and histones, is organized within the nucleosome. It has been established that DNA emits a negative electrostatic charge while associated histones emit a positive electrostatic charge. This creates an affinity between DNA and associated histones that result in a physically close relationship between DNA and histones. Under normal conditions, this close relationship prevents the existence of space or gaps between DNA and histones. As a result, transcription factors are unable to bind to regulatory sites on DNA or histones, which is necessary to alter gene transcription and modification. However, when this affinity between DNA and histone charges is decreased, the close proximity of DNA and histones will consequently decrease, creating a space between DNA and histones and resulting in transcription factors gaining access to regulatory sites on DNA or histones (Zhang and Meaney, 2010). The status of this structural conformation of chromatin is regulated by epigenetic modifications. The two primary types of epigenetic modifications include histone modifications and DNA

methylation. DNA methylation is a covalent modification in which a methyl group is added to the 5' position of a cytosine nucleotide (Wyatt, 1950; Razin and Riggs et al., 1980; Figure 1-2). DNA methylation is the most studied epigenetic modification involved in prenatal programming. This chemical modification to DNA plays a crucial role in regulating the unique function between different cell types, in spite of the fact that each of cell type has an identical genome (Razin and Riggs, 1980). Additionally, DNA methylation can be altered by external stimuli, which can result in alterations in gene expression and phenotype (Feinberg, 2010; Szyf, 2012). These alterations are not limited to the X chromosome, and most of DNA methylation occurs on autosomal chromosomes (Massart et al., 2016). Increased DNA methylation in promoter regions of a gene has been shown to result in repressed transcription and gene expression (Levine et al., 1991; Tate and Bird, 1993), while increased DNA methylation in gene bodies has been shown to result in increased gene expression (Hellman and Chess, 2007). However, the consistency of these relationships is disputable. Nonetheless, DNA methylation can provide insight into how prenatal environment alters postnatal phenotype. Phenotype in beef cattle is of great economic importance with regard to health, behavior, and production traits.

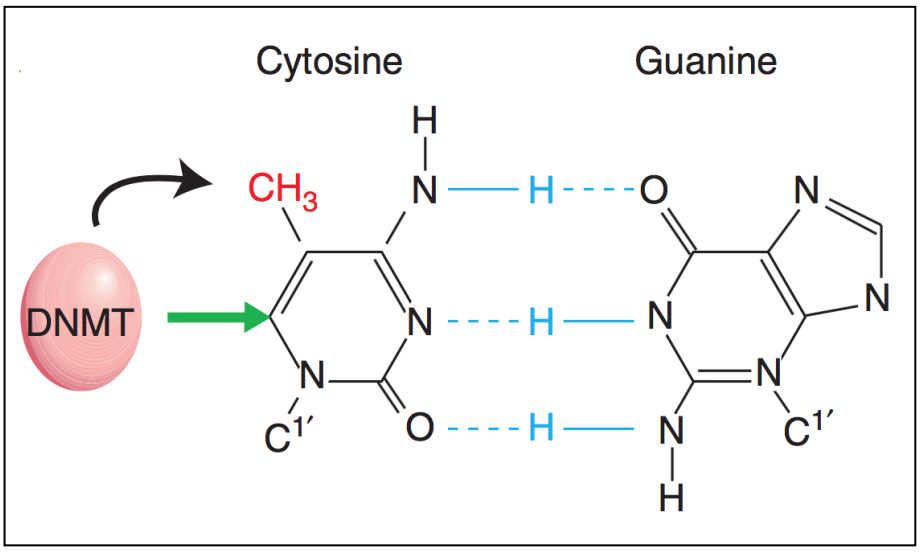

Figure 1-2. Methylation of a cytosine nucleotide (Adapted from Li and Zhang, 2014).

Influence of Prenatal Stress and Epigenetic Regulation on Immune Response and Inflammation

The developmental trajectory of the immune system is of critical importance to an animal's life-long health status. The prenatal environment mediates the first processes to shape postnatal immune response in mammals (McDade and Kuzawa, 2004). Prenatal programming due to prenatal stress has been shown to result in alterations to immune function as evidenced by differences in hematology and cytokine concentrations in swine, primates, and rodents (Couret et al., 2009; Reyes and Coe, 1997; Vanbesien-Mailliot et al., 2007). The majority of literature regarding the effects of prenatal physiological stressors on immune function is in rodent models (Kay et al., 1998; Götz et al., 2007). However, limited studies have evaluated the influence of other prenatal challenges in cattle. For example, calves whose dams were administered LPS during gestation exhibited altered vaginal temperatures, sickness behaviors, and IL-6 in response to an LPS challenge (Carroll et al., 2017). Between 4 weeks of age and shortly

after weaning, a calf becomes less supported by passive immunity and more dependent on its own developing innate and adaptive immune systems (Chase et al., 2008), which may not reach maturity until near puberty (Chase et al., 2008; Reber et al., 2006). Likewise, weaning and shipping stressors typically occur at sensitive times relative to immune system development, resulting in compromised immune response (Blecha et al., 1984) and increased incidence of morbidity and mortality (Knowles, 1995; Sanderson et al., 2008). Increased morbidity rates in beef cattle production systems result in additional medical costs, reduced growth rates, and losses in productivity, especially at the feedlot stage of production (Cernicchiaro et al., 2013). Therefore, it is important to understand how early life, especially prenatal development, can shape postnatal immune response.

Variations in immune response and inflammation have been associated with diverse phenotypic, methylomic, and transcriptomic alterations due to perinatal stress. The majority of the literature suggests suppressed immune function in prenatally stressed individuals. For example, rats whose dams were exposed to weekly noise and light stress throughout gestation had decreased circulating and splenic lymphocytes in response to pokeweed mitogen at 2 months of age (Kay et al., 1998). Furthermore, prenatally stressed adult male rats whose dams underwent 2 h of confrontation with another female rat daily for a 2-month period differed in neutrophil, monocyte, T cell, and NK cell numbers as well as lymphocyte proliferation compared to their non-prenatally stressed counterparts (Götz et al., 2007). In response to an LPS challenge, pigs whose dams were exposed to 5 minutes of restraint stress every day from 84 to 112 days of gestation had increased circulating concentrations of TNF-a, IL-6, epinephrine, and norepinephrine

(Collier et al., 2011). Prenatal stress results in transcriptomic alterations to regulators of immune response such as, cytokines (Szczesny et al., 2014; Vanbesien-Mailliot et al., 2007) and immune cell populations. Additionally epigenomic alterations have been associated with differences in immune function of prenatally stressed individuals (Richetto et al., 2016; Cao-Lei et al., 2014). The following sections discuss the influence of prenatal stress on various biological responses to immune challenges, including rectal temperatures, sickness scores, cytokines, immune cell parameters, and acute phase proteins.

Rectal Temperatures. Rectal temperature is an indicator of health status and inflammation in cattle (Burdick et al., 2010; Carroll et al., 2009; Jacobsen et al., 2005). An increase in body temperature is necessary for pathogen clearance (Hasday et al., 2000). The secretion of the cytokines TNF-α, IL-1b, and IL-6 are known to be involved in the fever response to increase body temperature (Dinarello, 1996). Limited research is available describing the effects of prenatal stress on rectal or core body temperature in response to an immune challenge. Fever response to LPS was greater in 8-week-old female pigs whose dams were administered cortisol during early and late gestation compared to Controls (de Groot et al., 2007). Fever response in response to IL-1B was lower in monkeys whose dams were administered ACTH during pregnancy (Reyes and Coe; 1997). The ability of prenatal stress to regulate the physiological control of rectal temperature through mechanisms associated with cytokine signaling at the level of the transcriptome and epigenome are outlined in the following sections.

Sickness Behaviors. Sickness behavior (**SBS**) can be defined as coordinated

behavioral and physiological changes that occur in response to an infection due to the secretion of pro-inflammatory cytokines (Dantzer et al., 1996). These behavioral changes might include weakness, malaise, listlessness, inability to concentrate, depression, lethargy, lack of attention to surroundings, and decreased intake of food and water (Dantzer et al., 1996). Sickness behavior is induced by pro-inflammatory cytokines such as IL-1, IL-6, TNF-α, and interferons (Dantzer, 2001). Limited literature is available describing SBS in prenatally stressed animals. Increased sickness behavior has been reported in response to LPS in rodents, pigs, and cattle (Avitsur and Sheridan, 2009; Burdick et al., 2010; de Groot et al., 2007; Lay et al., 2011). Pigs whose dams were handled roughly during gestation had lower SBS than pigs whose dams were either administered ACTH during gestation or were maintained as Controls (Lay et al., 2011). Mice that underwent neonatal maternal separation had altered sickness behavior in the form of decreased food consumption compared with Controls in response to LPS administration (Avitsur and Sheridan, 2009). Furthermore, pigs whose dams were administered cortisol during pregnancy recovered from an endotoxin challenge more quickly (had shortened periods of sickness behavior) compared with Controls (de Groot et al., 2007). The ability of prenatal stress to regulate the physiological control of sickness behavior through mechanisms associated with cytokine signaling at the level of the transcriptome and epigenome are outlined in the following section.

Cytokines. Cytokines have been defined as intercellular signaling polypeptides produced by activated cells (Gabay and Kushner, 1999). Proinflammatory cytokines are involved in processes causing fever response (Dinarello, 1996) and sickness behavior

(Dantzer, 2001). Stress has been associated with increased proinflammatory cytokines, TNF-α, IL-6, and IFN-γ, in humans (Maes et al., 1998). There is contrasting literature regarding cytokine activity in response to an immune challenge in prenatally stressed animals. Increased circulating IFN-γ in response to phytohemagglutinin-A was reported in prenatally stressed rats whose dams underwent restraint stress during the final 11 d of gestation (Vanbesien-Mailliot et al., 2007). In response to LPS administration, prenatally stressed pigs across sex classes whose dams underwent restraint stress during gestation had increased circulating TNF-α and IL-6 compared to Controls (Collier et al., 2011). Monkeys whose dams were administered ACTH on d 120 through 133 of gestation had decreased circulating concentrations of IL-6 (Reyes and Coe, 1997). Furthermore, rats whose dams underwent restraint and light stress from d 14 of gestation to partition exhibited depressive-like behaviour, increased basal mRNA expression of hippocampal IL-1β, and increased LPS-stimulated hippocampal IL-1β, TNF-α, and IL-6 (Szczesny et al., 2014). Additionally, adult rats whose dams were exposed to restraint and light stress during the final 11 d of gestation had increased mRNA expression of IFN-γ in peripheral blood mononuclear cells (Vanbesien-Mailliot et al., 2007). With regard to epigenomic regulation, enrichment analysis of genome-wide methylation in T-cells from children whose mothers were in the ice storm of 1998 in Québec revealed IL-4, IL-6 and IL-8 signaling pathways to be significantly altered (Cao-Lei et al., 2015).

Immune Cell Populations. Immune cells are key mediators of the innate and adaptive immune response. Immune cells both regulate and are regulated by other immune cells by cytokine production (Arai et al., 1990). Altered immune function has

been reported in prenatally stressed animals as shown by differential hematology and cytokine concentrations in swine, primates, and rodents (Couret et al., 2009; Reyes and Coe, 1997; Vanbesien-Mailliot et al., 2007). There is contrasting literature regarding effects of prenatal stress on basal and exogenously induced populations of immune cells. Piglets whose dams were exposed to social stress (housing with unknown gilts) during late gestation had decreased basal white blood cells and lymphocytes but increased lymphocyte proliferation in response to Concanavalin A (Couret et al., 2009). Male rats whose dams were exposed to hanging stress (daily 2 h periods from 14 d of gestation to term) had decreased basal leukocytes and lymphocytes but increased basal neutrophils and eosinophils (Llorente et al., 2002). However, leukocyte counts in response to LPS were not different in weaned pigs whose dams were exposed to restraint stress daily between d 84 and 112 of gestation compared to Controls (Collier et al., 2011). With regard to epigenomic regulation, Richetto et al. (2016) reported an enrichment analysis of genome-wide methylation data in which "Leukocyte Differentiation" was significantly altered in mice that were prenatally exposed to a viral challenge on gestational d 9 or 17. Additionally, Cao-Lei et al. (2014) reported that children whose mothers were in the 1998 ice storm in Quebec during gestation had altered genome-wide DNA methylation in T cells at 13 years of age. Six of the top 10 pathways in an enrichment analysis were related to T lymphocyte function (Cao-Lei et al., 2014). The ability of prenatal stress to regulate the physiological control of immune cell populations through mechanisms associated with cytokine signaling at the level of the transcriptome and epigenome are outlined in the previous section.

Acute Phase Response. Acute phase proteins (**APP**) have been defined as plasma proteins that increase or decrease by 25% during an inflammatory state (Gabay and Kushner, 1999; Samols et al., 2002). The acute phase response (**APR**) has been defined as a systemic reaction to inflammation (Gabay and Kushner, 1999). The APR includes changes in both APP as well as neuroendocrine, hemapoietic, metabolic, hepatic, and plasma concentrations of molecules other than proteins (Gabay and Kushner, 1999). Acute phase proteins are primarily stimulated by IL-6 (Gauldie et al., 1987). Elevated concentrations of IL-6 and C-reactive protein (a positive acute phase protein) have been associated with stress during pregnancy (Coussons-Read et al., 2007). Rats whose dams consumed low protein diets throughout gestation exhibited a blunted acute phase response with alterations in serum albumin concentrations (a negative acute phase protein) after an endotoxin challenge (Langley et al., 1994). A greater C-reactive protein response to LPS was observed in rats whose dams were administered LPS on d 18 of gestation (Ginsberg et al., 2012). Components of the acute phase response have been associated with stress during pregnancy as well as prenatal stress suggesting an impact on inflammation and innate immune response.

Influence of Prenatal Stress on the Physiological Control of Temperament, Stress Response, and Neural Function

Perhaps the most studied impact of prenatal stress is its capacity to alter behavioral temperament, stress response, and neural function, which are all interrelated. Differences in the transcriptome, epigenome, phenotype and interactions thereof have

been reported in prenatally stressed individuals. The following sections explore the relationships of prenatal stress and the physiological control of temperament, stress response, and neural function.

Temperament. Temperament can be defined as behavioral reactivity to humans and novel environments, willingness to take risks, exploration, aggression, sociality, and excitability (Fordyce et al., 1988; Reale et al., 2007). Elevated temperament or increased excitability in cattle is not desirable from a managerial standpoint, because temperamental cattle have been reported to have compromised immune function (Fell et al., 1999), reduced growth (Tulloh, 1961), inferior carcass characteristics (King et al., 2006; Voisinet et al., 1997), and to be more difficult to manage (Hassall et al., 1974). Behavior, stress response, and neural function are largely regulated by the limbic system. Though the official components of the limbic system are not universally agreed upon, the limbic system is generally regarded to include the hypothalamus, limbic cortex (cingulate gyrus and parahippocampal gyrus), hippocampal formation (dentate gyrus, hippocampus, and subicular complex), amygdala, and the septal area of the brain (Rajmohan and Mohandas, 2007). The hypothalamus is involved in endocrine regulation of various hormones, including glucocorticoids by the HPA axis as mentioned in the above paragraphs. Temperament is interrelated with HPA axis function in the bovine, with temperamental Brahman calves having greater circulating concentrations of cortisol relative to calmer herdmates (Curley et al., 2006, 2008). The cingulate gyrus is involved in social cognition and memory, while the parahippocampal gyrus is involved in spatial memory (Rajmohan and Mohandas, 2007). The hippocampus is involved in spontaneous

activity and long-term memory storage, which is important in recovering fear memories (Green, 1964; Rajmohan and Mohandas, 2007). The amygdala is involved in social cognition, anxiety, emotional memory, fear conditioning, and aggression (Rajmohan and Mohandas, 2007). The septal area may also be involved in aggression (Rajmohan and Mohandas, 2007). Dysfunctions of the limbic system, especially at the level of the amygdala, are thought to be associated with various neurobehavioral disorders such as schitzophrenia, demensia, anxiety, attention deficit hyperactivity disorder (**ADHD**), and autism (Rajmohan and Mohandas, 2007; Aggleton et al., 1993). The components of the limbic system are key regulators of behavioral temperament.

Prenatal stress has been reported to alter behavioral temperament across species such as nonhuman primates, rodents, sheep, and cattle (Clarke et al., 1996; Fride and Weinstock, 1988; Coulon et al., 2011; Littlejohn et al., 2016). The vast majority of the literature suggests prenatal stress results in selection for a more depressive, anxious, or excitable temperament phenotype. For example, rats whose dams were exposed to perigestational stress spent less time in open areas when placed in a maze that was partially enclosed by walls and partially without walls at 6 mo of age, suggesting an anxious phenotype compared with Control rats (Fride and Weinstock, 1988). In concurrence, calves whose dams were transported during pregnancy exhibited more excitable temperaments along with increased circulating concentrations of cortisol through weaning (Littlejohn et al., 2016). In many cases, these alterations in behavioral temperament appear to be stable as animals mature. Four year old monkeys whose dams were placed in a small dark cage and exposed to loud noise 5 times each week from 90

to 145 d of gestation were less exploratory, more vocal (indicating distress), and exhibited greater locomotion and disturbance when separated from cagemates (Clarke et al., 1996). The ability of prenatal stress to regulate the physiological control of temperament through mechanisms associated with HPA axis and neurotransmitter signaling at the level of the transcriptome and epigenome are outlined in the following sections.

The HPA Axis and Stress Response. Function of the HPA axis and stress response is interrelated with behavior and neural function, and has been well documented to be altered by prenatal stressors. Cortisol is involved in mediating the stress response, and is an indicator of stress in cattle (Lefcourt and Elsasser, 1995). The HPA axis is susceptible to programming during the fetal and perinatal periods of life (Matthews, 2000; Meaney, 2001; Seaman-Bridges et al., 2003). Prenatal stress results in fetal alterations that favor enhanced vigilance in attempts to better prepare the neonate to survive in a stressful postnatal environment (Matthews, 2000). Perhaps increased HPA axis activity is a mechanism to increase survivability. Altered HPA axis activity due to prenatal or early life stress has been consistently reported across many species such as mice, pigs, sheep, and calves (Meaney et al., 2000; Haussmann et al., 2000; Roussel et al., 2004; Lay et al., 1997a; Lay et al., 1997b).

Prenatally stressed pigs whose dams were restrained by snout snare for 5 min each day between 84 and 112 d of gestation had greater serum concentrations of cortisol relative to Controls (Collier et al., 2011). Additionally, blue foxes whose dams were exposed to 1 min of daily handling stress during the last trimester of pregnancy showed

increased signs of distress when held by a human evaluator (crawling, fighting, yelping, aggression, etc.) than Controls when held by a human (Braastad et al., 1998). With regard to cattle, prenatal transportation stress (dams transported for 2 h at 60, 80, 100, 120, and 140 ± 5 d of gestation) has been reported to be associated with greater circulating concentrations of cortisol for a longer duration of time in response to restraint stress compared with Control calves. Additionally, prenatally stressed calves cleared a bolus dose of cortisol at a slower rate than Control calves (Lay et al., 1997b). Using a similar transportation stress model, a more recent study reported prenatally stressed calves to have greater basal concentrations of serum cortisol through weaning (Littlejohn et al., 2016). Diverse alterations at various levels involving the HPA axis have been reported due to prenatal stressors.

Prenatal stress is known to cause alterations and damage to the limbic system as well as other regions of the brain (Anderson et al., 1985; Coe et al., 2002; Coe et al., 2003). Prenatal stress was reported to cause reduced GR expression in the hippocampus and cortex of the limbic system in rats (Reul et al., 1994; Maccari et al., 1995). Additional studies have shown that reduced GR in the limbic system of mice results in reduced sensitivity to negative feedback, which has been reported to cause a prolonged and amplified stress response in PNS individuals (Kapoor et al., 2006). Other prenatal challenges, such as prenatal protein restriction, have been associated with decreased methylation of a glucocorticoid receptor promoter region resulting in increased expression of glucocorticoid receptor and decreased 11β -HSD2 expression in the liver, lung, kidney, and brain of rats (Lillycrop et al., 2005).

Increasing reports of the association of prenatal or early life stress with differences in methylation of genes involved in HPA axis regulation have been made, especially at the level of the glucocorticoid receptor gene (**NR3C1**; Oberlander et al., 2008; Mulligan et al., 2012; Perroud et al., 2014; Kertes et al., 2016). Differential methylation was reported within the promoter region of NR3C1in cord blood of newborns whose mothers were exposed to the stress of war in the Democratic Republic of Congo during gestation (Mulligan et al., 2012). Increased anxiety-like behavior and decreased DNA methylation were observed within the promoter region of the CRH gene in the hypothalamus of rats whose dams underwent restraint and light stress for 30-min intervals twice each day between d 8 and 21 of gestation (Xu et al., 2014). Overall, the HPA axis and stress response signaling has been associated with various phenotypic, methylomic, and transcriptomic alterations associated with prenatal and early life stress.

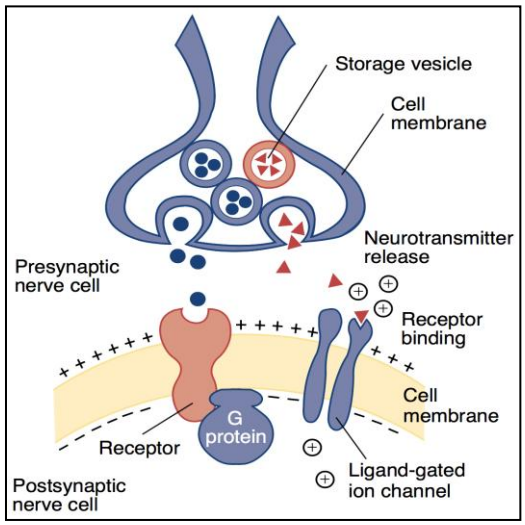

Figure 1-3. General representation of signaling mechanisms between neurons (Adapted from the article, "The principles of nerve cell communication").

Neural Function and Neurotransmitter Signaling. Many biological processes, including behavior and stress response are regulated, in part, by neural function, specifically neurotransmitter signaling. Generally, different neurons communicate with each other via transmitters. Typically, neurotransmitters are synthesized and stored in vesicles within the presynaptic nerve cell and then released by exocytosis into the synaptic cleft. Those neurotransmitters then interact with various receptors on the postsynaptic nerve cell (Figure 1-3; The principles of nerve cell communication). Alterations to neurotransmitter pathways, such as dopamine, GABA, and serotonin have been associated with psychiatric disorders such as depression, anxiety, psychosis, and schizophrenia (Markham and Koenig, 2011). Alterations in neural function and neurotransmitter signaling have been reported as a consequence of prenatal stress across various species. In many cases, such alterations were related to differences in behavior and/or stress response.

Salm et al. (2004) reported that prenatal stress (i.e., randomly handling in a new environment and intramuscular saline injection once a day) was associated with increased volume, neuronal density, number of neurons, and number of glial cells in the lateral nucleus of the amygdala of rats. Contrasting results were reported in 25 d old rats whose dams were randomly handled in a new environment and received an i.m. injection of saline once a day, with prenatally stressed rats exhibiting decreased volume, number of neurons, and number of glial cells in the basolateral, central, and lateral nuclei of the amygdala (Kraszpulski et al., 2006). However, at 45 days of age, no differences in volume, number of neurons, or number of glial cells were observed (Kraszpulski et al.,

2006). This suggests that prenatal stress had short-term effects on the physiology of the amygdala in rats. Lasting neural alterations have also been observed into adulthood in prenatally stressed animals. Prenatal stress in the form of fetal exposure to dexamethasone, a synthetic glucocorticoid, has been shown to decrease calretinin in the lateral amygdala of female rats as adults with no significant effect of dexamethasone treatment on males within the same study (Zuloaga et al., 2012). Calretinin is a calcium binding protein found in the cytosol of some nerve cells (Baimbridge and Miller, 1984). Calretinin proteins are present in early fetal development and are believed to be involved in cell differentiation (Ellis et al., 1991). Prenatal stress has also been shown to affect the hippocampal portion of the brain, which contains glucocorticoid receptors that cause inhibition of hypothalamic release of CRH in times of elevated concentrations of glucocorticoids (Sapolsky et al., 1986). Prenatal stress has been associated with decreased neurogenesis in the dentate gyrus portion of the hippocampus (Coe et al., 2003; Lemaire et al., 2000), decreased hippocampal volume (Coe et al., 2003; Schmitz et al., 2002), decreased hippocampal weight (Szuran et al., 1994), and decreased number of granule cells (Lemaire et al., 2000) in the hippocampus.

Prenatal stress results in transcriptomic alterations to regulators of neural function, such as DPYSL2 and DIO3 gene expression. The DPYSL2 (dihydropyrimidinase-like 2) gene is a collapsin response mediator protein that is involved in neurotransmission and synapse function. Rats whose dams underwent gestational stressors (restraint stress, food deprivation, forced swimming, reversed light-dark cycles, and overcrowding stress during dark cycles) between 14 d of gestation and

birth exhibited decreased DPYSL2 expression and potentially increased susceptibility to schizophrenic characteristics (Lee et al., 2015). Furthermore, rats whose dams were stressed by placing them on an elevated platform made of Plexiglass® twice each day for a 10-min period between 12 and 16 d of gestation resulted in genome-wide alterations in gene expression, including expression of DPYSL2 (Mychasiuk et al., 2011). The DIO3 gene is an imprinted gene in cattle, mice, and other species that encodes iodothyronine deiodinase 3, which inactivates thyroid hormones (Yang et al., 2017; Tsai et al., 2002). Thyroid hormones are involved in central nervous system development (Bernal, 2005) and behavior (Stohn et al., 2018). Male and female mice that were deficient in DIO3 exhibited increased aggression in response to an intruder (Stohn et al., 2018). Furthermore, rats whose dams consumed ethanol from day 8 to 21 of gestation had increased placental DIO3 mRNA compared to Controls (Shukla et al., 2011), suggesting the ability of prenatal environment to shape nervous system development.

There has been increased literature on differential DNA methylation as a regulator of neural differences in prenatally stressed individuals. For example, rats whose dams underwent daily restraint stress for 2-h periods between embryonic days 11 and 20 exhibited lower numbers of reelin-positive neurons, lower reelin gene expression, and altered DNA methylation within the promoter region of the reelin gene (Palacios-García et al., 2015). The reelin gene is expressed during cortical development in Cajal-Retzius cells and is involved in cortical lamination and synaptic maturation (Palacios-García et al., 2015). Furthermore, genome-wide DNA methylation was assessed in mice

whose dams were exposed to a gestational viral challenge on d 9 or 17 of gestation and enrichment analysis showed "Neuronal Differentiation" to be the most enriched gene ontology term. Significant subterms of "Neuronal Differentiation" included gamma-aminobutyric acidergic differentiation, central nervous system differentiation, noradrenergic system differentiation, and dopamine differentiation (Richetto et al., 2016).

The preceding studies suggest that prenatal stress is associated with various physiological alterations in the brain of the affected offspring. Prenatal stress has been associated with alterations in many specific neurotransmitter systems such as dopamine, GABA, and serotonin signaling.

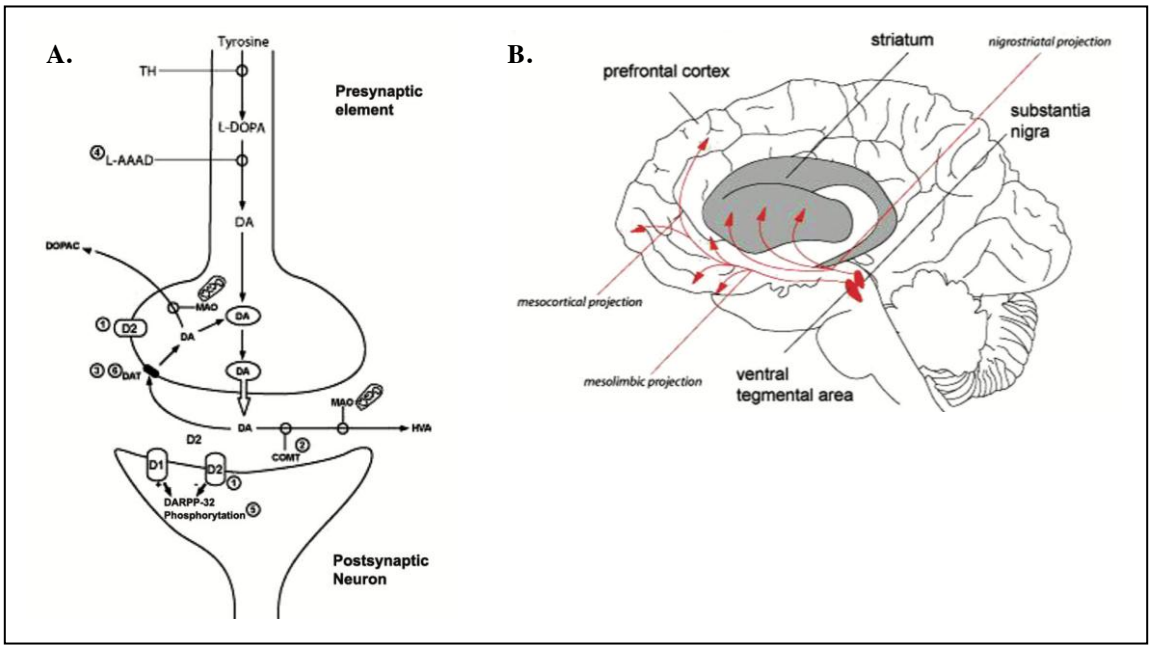

Figure 1-4. A. Dopamine synthesis by a dopaminergic neuron (Adapted from Cools, 2008). B. Innervation of dopaminergic neurons to various brain regions (Adapted from Cools, 2008).

Dopamine. Dopamine is a monoamine, specifically a catecholamine, neurotransmitter produced from a tyrosine amino acid precursor (Figure 1-4A; Wilkinson and Brown, 2015; Cools, 2008). Dopamine acts on G-protein-coupled receptors (Wilkinson and Brown, 2015). Dopamine signaling is involved in the physiological control of cognition, motivation, reward/punishment, and physiological control of behavior (Cools, 2008; Beaulieu and Gainetdinov, 2011). Dopaminergic control of behavior is complex due to the diversity of brain regions innervated by dopaminergic neurons (Figure 1-4B; Cools, 2008). Components of the dopamine signaling pathway have been associated with temperament in humans, rodents, and cattle (Keltikangas-Järvinen and Salo, 2009; Ray et al., 2006; Garza-Brenner et al., 2017). Dopamine signaling has also been associated with many neurobehavioral and neurodegenerative disorders, such as bipolar disorder (Cousins et al., 2009), Huntington's disease (Bäckman et al., 1997), attention deficit hyperactivity disorder (Li et al., 2006), and especially schizophrenia (Howes et al., 2015).

Previous reports suggest an influence of prenatal stress on dopamine signaling, especially at the level of COMT (Thompson et al., 2012) and dopamine receptors (Berger et al., 2002). Catechol o-methyl transferase (**COMT**) is an enzyme that is responsible for degrading catecholamines (Wilkinson and Brown, 2015). Maternal stress (ranked using the Perceived Stress Scale) during gestation was associated with behavioral issues in human offspring with a polymorphism of an allele in the COMT gene (Thompson et al., 2012). Adult rats whose dams were exposed to dexamethasone on d 18 and 19 of gestation had decreased dopamine and increased DRD2 gene

expression in the nucleus accumbens (Rodrigues et al., 2012). As mentioned in the temperament section above, rats whose dams were exposed to perigestational stress spent less time in open areas when placed in a maze that was partially enclosed by walls and partially without walls at 6 mo of age, suggesting an anxious phenotype compared with Control rats (Fride and Weinstock, 1988). These rats also had altered dopamine activity in left compared to right regions of the prefrontal cortex, caudate nucleus, and nucleus accumbens (Fride and Weinstock, 1988). The DRD1 gene encodes for dopamine receptor D1, and has been associated with in behavioral disorders such as psychosis and schizophrenia (Andreou et al., 2016). Rat pups that were separated from their mothers for 6-h periods each day during the first 2 weeks of life had downregulated DRD1 gene expression in the nucleus accumbens (Zhu et al., 2010). Rat pups that were separated from their mothers for 3-h periods had decreased mid-brain tyrosine hydroxylase-immunoreactive dopaminergic neurons as juveniles (15 d of age) but increased numbers as adolescents (35 d of age) and adults (70 d of age; Chocyk et al., 2011). These varying results suggest that alterations to the DRD1 gene due to prenatal or early life stress are specific to life stage. Humans diagnosed with schizophrenia had decreased methylation rates in the promoter region of the DRD2 gene (Yoshino et al., 2016). Maternal consumption of alcohol during gestation has been associated with increased methylation of DRD4 in human offspring (Fransquet et al., 2016). Overall, dopamine signaling has been associated with various phenotypic, methylomic, and transcriptomic alterations associated with both psychiatric disorders and perinatal stress.

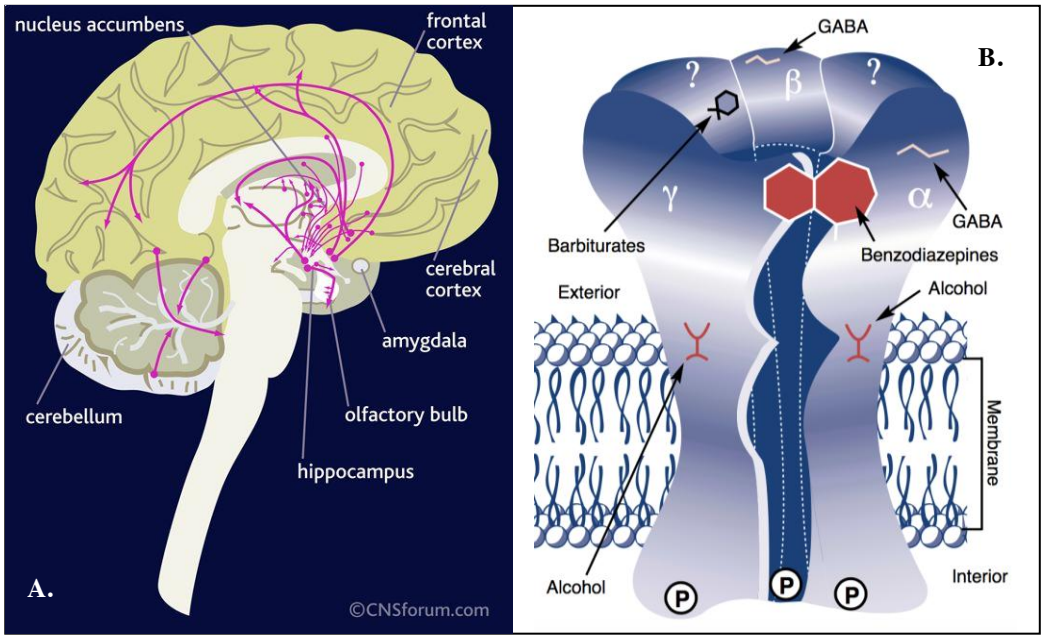

Figure 1-5. A. Innervation of GABA neurons to various brain regions (Adapted from CNSforum.com). B. $GABA_A$ receptor (Adapted from Mihic and Harris, 1997).

GABA. Gamma-aminobutyric acid (**GABA**), inhibitory amino acid neurotransmitter. GABA is the most prevalent neurotransmitter in the brain, with up to 50% of the total synapses estimated to be GABAergic in nature (Young and Chu, 1990), and spans diverse regions of the brain (Figure 1-5A). The glutamic acid decarboxylase enzyme converts the precursor glutamic acid to GABA (Wilkinson and Brown, 2015). GABA acts on $GABA_A$ receptors, which consist of 5 protein molecules to form an ion channel (Figure 1-5B). Interaction of GABA and $GABA_A$ open those channels to the postsynaptic neuron, inhibiting electrophysiological activity (Wilkinson and Brown, 2015). Tranquilizers, anesthetics, barbiturates, and alcohol elicit their effects by acting on $GABA_A$, thereby suppressing anxious behaviors (Wilkinson and Brown, 2015). GABA signaling is involved in the physiological control of cognition, motor learning,

fear, and behavior (Stagg et al., 2011; Earnheart et al., 2007). Components of the GABA signaling pathways have been associated with behavioral temperament (Paredes and Agmo, 1992; Stratton et al., 2014). Dysfunctions in GABA signaling have also been associated with many neural and neurobehavioral disorders, such as depression (Stratton et al., 2014), anxiety (Berger et al., 2002; Stratton et al., 2014), and ADHD (Bollman et al., 2015).

Previous reports suggest an influence of prenatal stress on components of the GABA signaling pathway (Lussier and Stevens, 2016; Ehrlich et al., 2015; Vangeel et al., 2017). Mice whose dams underwent restraint and light stress from embryonic d 12 until parturition exhibited increased anxiety-like characteristics and developmental delays in GABAergic cell counts (Lussier and Stevens, 2016). Rats whose dams were exposed to various gestational stressors between d 9 and 20 of gestation exhibited anxious behaviors and altered gene expression of the GABAergic regulators, KCC2 and NKCC1 (chloride transporters; Ehrlich et al., 2015). Children born to mothers with high anxiety levels during gestation exhibited genome-wide differences in DNA methylation, including GABBR1 (GABA-B receptor subunit 1 gene; Vangeel et al., 2017). Overall, GABA signaling has been associated with various phenotypic, methylomic, and transcriptomic alterations associated with both psychiatric disorders and perinatal stress.

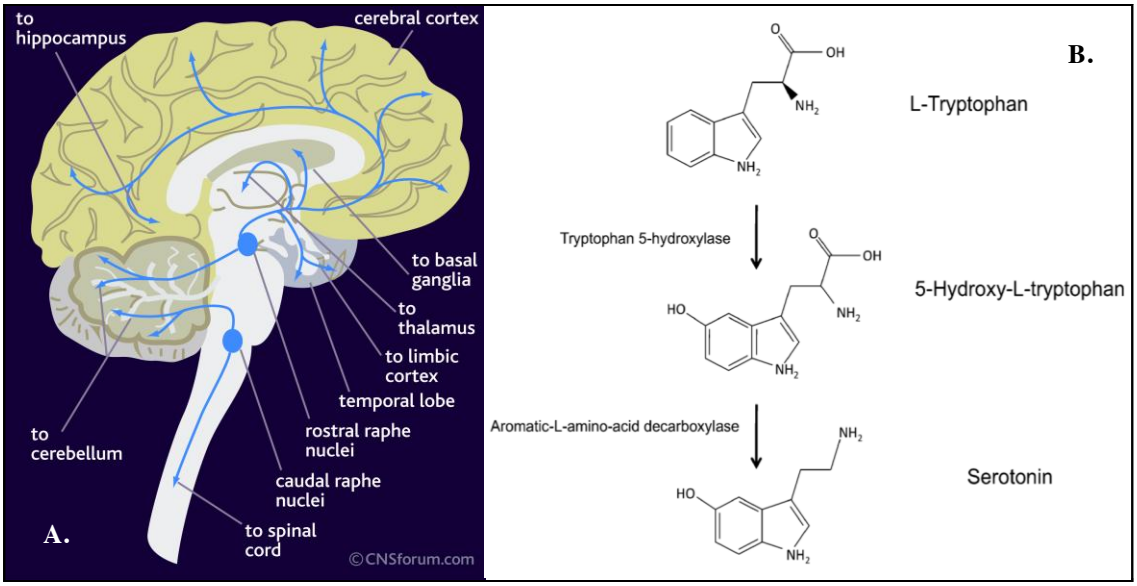

Figure 1-6. A. Innervation of serotonin neurons to various brain regions (Adapted from CNSforum.com). B. Synthesis of serotonin (Adapted from Fidalgo et al., 2013).

Serotonin. Serotonin, also known as hydroxytryptamine (**5-HT**), is a monoamine, specifically an indoleamine, neurotransmitter (Wilkinson and Brown, 2015). The majority of serotonin neuron cell bodies are derived from the Raphé region of the brain, but reach to regions throughout the brain (Figure 1-6A; Wilkinson and Brown, 2015). Serotonin is derived from tryptophan attained from the diet (Figure 1-6B; Fidalgo et al., 2013). Components of the serotonin signaling pathways have been associated with behavioral temperament in humans, nonhuman primates, and cattle (Raleigh et al., 1980; Knutson et al., 1998; Garza-Brenner et al., 2017). Dysfunctions in serotonin signaling have been associated with disorders such as anxiety (Marcinkiewcz et al., 2016), aggression (Olivier, 2004), autism spectrum disorder (Chugani, 2002) and schizophrenia (Hashimoto et al., 1991).

Prenatal stress has been associated with differences in serotonin receptor binding, serotonin synthesis, and associated behavioral alterations (Van den Hove et al., 2006; Peters, 1986). Mice whose dams were exposed to daily 6-h periods of restraint stress between 5.5 and 17.5 of gestation exhibited increased anxiety-like behavior and an increase in cells within the raphe nuclei that expressed 5-HT (Miyagawa et al., 2011). The conversion of L-Tryptophan to 5-Hydroxy-L-Tryptophan by Tryptophan 5-hydroxylase (**TpH**) is the rate-limiting step in serotonin production. There are two isoforms of Tph, TpH1 and TpH2, which are primarily used in this conversion in the brain (Fidalgo et al., 2013). Female rats whose dams were injected with dexamethasone daily between 18 and 22 d of gestation exhibited increased TpH2 mRNA at 7 d of age but decreased TpH2 mRNA in adulthood in the caudal dorsal raphe nucleus (Hiroi et al., 2016). In conjunction with alterations to TpH2, the same female rats also exhibited increased anxiety-like and depression-like behaviors (Hiroi et al., 2016). Newborns whose mothers experienced anxiety or depression during pregnancy and took serotonin reuptake inhibitor medication exhibited differential methylation of a CpG site within the SLC6A4 gene (encodes for a serotonin transporter) in cord blood (Non et al., 2014). Overall, serotonin signaling has been associated with various phenotypic, methylomic, and transcriptomic alterations associated with both psychiatric disorders and perinatal stress.

Genetic Parameters of Temperament

Phenotypic variation attributed to epigenetic alterations. The above paragraphs highlight an example of a non-genetic, epigenetically controlled, parameter of temperament. Generally, phenotypic variation is known to be attributed to a combination of genotypic and environmental variance (Bourdon, 2000). Genotypic variance is composed of additive and non-additive (dominance and epistasis) components. The word 'environment' was first introduced to population genetics literature in 1918 by R. A. Fisher as random external effects (Fisher, 1918). Classically, environmental variance was considered to include "tangible" environmental variation. Analysis of the proportion of phenotypic variation attributed to genotypic and environmental variance has been studied in highly outbred guinea pig lines with regard to coat color pattern, finding only 42% attributed to genotypic variation and an extremely small portion attributed to environmental variation. The remaining almost 58% of phenotypic variation was neither attributable to genotype or environment (Wright, 1920). Furthermore, Wong et al. (2005) reported little difference in phenotypic variation between monozygotic twins raised together compared with those raised separately, suggesting a small proportion of the phenotypic variation due to "tangible" environmental effects. This remaining variance is thought to be a component of "intangible" environmental variance otherwise known as "stochastic variance" (Peaston and Whitelaw, 2006; Burga and Lehner, 2012). Phenotypic variation attributed to epigenetic regulation is thought to fall within this category (Peaston and Whitelaw, 2006; Burga and Lehner, 2012). Therefore, alterations

in temperament due to prenatal stress outlined in the above sections could be considered to be an intangible environmental component of phenotype.

Phenotypic variation attributed to additive genetic variation. Heritability can be defined as the additive genetic variance as a proportion of the phenotypic variance (Bourdon, 2000). Heritability estimates consist of a scale of 0 to 1 (0 = lowest heritability estimate and 1 = greatest heritability estimate). Characteristics with low heritability estimates will likely have a low selection response, which is important in terms of trait selection in breeding programs. Temperament has been shown to be moderately to highly heritable across various breeds of cattle. Breed is responsible for a significant *(P* < 0.001) amount of variation in measures of temperament in cattle (Hoppe et al., 2010). Specifically, Brahman crossed cattle are more temperamental relative to British breeds (Fordyce et al., 1982). Additionally, heifers are known to be more temperamental than bulls (Shrode and Hammack, 1971).

Bos indicus and *Bos indicus*-cross cattle have been reported to have heritabilities between 0.19 and 0.51 for various temperament traits. Brahman and Brahman-Hereford (F1) crossed cattle (n=1,209; Schmidt et al., 2014) in Texas had a heritability estimate of 0.49 for pen score (subjective scale of 1-5), 0.27 for exit velocity (m/s upon exiting a squeeze chute), and 0.43 for temperament score. Brahman, Belmont Red, and Santa Gertrudis heifers and steers (n=3,594; Kadel et al., 2006) in Australia had a heritability estimate of 0.30 for flight time, 0.21 for flight speed (m/s), and 0.19 for chute score (subjective scale of 1-15) after weaning. Africander-cross cattle (n=1,871; Burrow, 2001) in Australia had a heritability estimate of 0.40 for flight speed. Nellore-Angus

cross cattle (n=1,816; United States; Riley et al., 2014) had a heritability estimate of 0.47 for temperament score (subjective scale of 1-9), 0.51 for aggressiveness (subjective scale of 1-9), 0.40 for nervousness (subjective scale of 1-9), 0.45 for flightiness (subjective scale of 1-9), and 0.49 for gregariousness (subjective scale of 1-9).

Bos taurus cattle have been reported to have heritability estimates between 0.19 and 0.49 for various temperament traits. Charolais cattle (n=6,649; Vallée et al., 2015) in France had a heritability estimate of 0.19 for aggression at parturition (subjective scale of 1-7). *Bos taurus* crossbred cattle (n=302; Nkrumah et al., 2007) in a Canadian population had a heritability estimate of 0.49 for flight speed (m/s). Hereford cattle (n=697; Hoppe et al., 2010) in Germany had a heritability estimate of 0.33 for chute score (subjective scale of 1-5) and 0.36 for flight speed (4 subjective categories).

Overall, reports of heritability of temperament characteristics in cattle have been consistently moderately to highly heritable across breedtypes.

Overall Concepts

Prenatal environment is known to shape postnatal outcomes of physiological functions across species. The experiments described in the following chapters sought to understand how a prenatal stressor, transportation in this case (a common practice in the process of beef cattle production systems), could alter the developmental trajectory of economically relevant traits in beef cattle. Because temperament and the physiological control of temperament were found to be altered by the epigenetic control of a prenatal

stressor, our lab sought to examine the genetic parameters of temperament in an independent population.

Specifically, the goals of these studies were to:

1. Characterize innate immune response to an endotoxin challenge in prenatally stressed compared with Control bull calves;
2. Evaluate genome-wide differential methylation between prenatally stressed and Control bull calves; and,
3. Estimate genetic parameters of temperament across an age continuum in an independent population of commercial beef cattle.

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CHAPTER II

PRENATAL TRANSPORTATION STRESS ALTERS INNATE IMMUNE RESPONSE TO AN ENDOTOXIN CHALLENGE IN WEANED BRAHMAN BULLS

INTRODUCTION

Prenatal environment shapes postnatal immune response in mammals (McDade and Kuzawa, 2004). Stress encountered by a dam during gestation can result in decreased activity of placental 11β-HSD2, which is responsible for converting cortisol to an inactive form (Benediktsson et al., 1997). This can result in increased exposure of the fetus to cortisol. Such experiences alter fetal environment and can affect the developmental trajectory of biological systems in utero, a process known as prenatal programming (Nathanielsz et al., 2007). Prenatal programming due to prenatal stress has been shown to result in alterations to immune function as evidenced by differences in hematology and cytokine concentrations in swine, primates, and rodents, (Couret et al., 2009; Reyes and Coe, 1997; Vanbesien-Mailliot et al., 2007). Potential stressors incurred by a gestating bovine may include predation, handling, restraint, or transportation (Cooke et al., 2013; Grandin and Shivley, 2015). Transportation results in elevated body temperature as well as increased systemic concentrations of cortisol and glucose, as shown in pregnant Brahman cows (Price et al., 2015) that gave birth to the population of prenatally stressed calves from which a subset will be discussed in this study. Furthermore, calves in that population had altered temperament and elevated serum cortisol through weaning (Littlejohn et al., 2016). Cattle with more excitable

temperaments have been reported to have altered immune responses (Burdick et al., 2010a; Hulbert et al., 2011). The bovine immune system may not be complete in its maturation until near puberty (Chase et al., 2008; Reber et al., 2006). The average age at puberty has been estimated to be over 460 days in Bos indicus breeds (Fields et al., 1982; Neuendorff et al., 1985) and over 280 days in Bos taurus breeds (Lunstra et al., 1978). Between 4 weeks of age and shortly after weaning (the standard weaning age in cattle is between 160 to 250 days; Cundiff et al., 2016) a calf becomes less supported by passive immunity and more dependent on its own developing innate and adaptive immune systems (Chase et al., 2008). Likewise, weaning and shipping stressors typically occur at sensitive times relative to immune system development, resulting in compromised immune response (Blecha et al., 1984) and increased incidence of morbidity and mortality (Knowles, 1995; Sanderson et al., 2008). Furthermore, feedlot cattle with more excitable and nervous temperaments have been reported to have increased morbidity rates relative to Calm cattle (Fell et al., 1999). Increased morbidity rates in beef cattle production systems result in additional medical costs, reduced growth rates, and losses in productivity, especially at the feedlot stage of production (Cernicchiaro et al., 2013). Consequently, it is important to understand how early life, especially prenatal development, can shape postnatal immune response. Such information has implications for both animal and human health, implications to improve the production of wholesome meat products and provide models to improve human health. Such information is pertinent to 1) increased understanding of animal health, 2) enhanced production of wholesome meat products, and 3) development of a dual

purpose and benefit animal model to improve human health. Therefore, the primary objective of this study was to assess the influence of prenatal stress on the post-weaning innate immune response to an endotoxin challenge in Brahman bulls.

MATERIALS AND METHODS

Animals

All experimental procedures were in compliance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010) and were approved by the Texas A & M AgriLife Research and USDA-ARS-LIRU Animal Care and Use Committees.

To test the hypothesis that prenatal stress alters the post-weaning immune response to an endotoxin challenge in Brahman bull calves, the following procedures were employed. Ninety-six pregnant Brahman cows (Bos indicus) were determined pregnant in 2011 by palpation per rectum 45 days after the last breeding date. At that time, each cow was assigned to one of two treatment groups [Transportation stress (Transport) = 48; Control = 48] based on age, parity, and temperament. Dam temperament was balanced (Table 2-1) between treatment groups using each cow's pen score, exit velocity, and temperament score at weaning, as well as each cow's mature temperament classification. The stressor consisted of 5 transportation events at 60, 80, 100, 120, and 140 ± 5 days of gestation (Price et al., 2015). Immediately before and after each transportation event, blood samples were collected from the tail. Cows were transported in the same 3-section trailer (2.4 x 7.3 m) that was towed by a ¾ ton truck on

Table 2-1. Temperament classification of pregnant dams within Control and Transported treatment groups[1,2]

Item	Control (n = 44)	Transport (n = 41)	P-Value
[2]Weaning PS	2.74 ± 0.17	2.66 ± 0.17	0.74
[2]Weaning EV	2.54 ± 0.17	2.51 ± 0.18	0.90
[2]Weaning TS	2.64 ± 0.14	2.59 ± 0.15	0.79
[2]MTC	2.02 ± 0.10	2.0 ± 0.10	0.87

[1]Data compare Control and Transported dam treatment groups and are presented as least squares means (± SE).
[2]Data are represented for dams' weaning pen score (PS, subjective scale of 1 to 5), weaning exit velocity (EV, m/s), weaning temperament score [TS, TS = (PS + EV)/2], and mature temperament score (MTC, subjective scale of 1-3).
[3]This table was adapted from Littlejohn et al., 2016.

smooth highways for a total of 2 h. This treatment was modeled after a previous study that resulted in differences in the function of the HPA axis in calves born to cows transported at these stages of gestation (Lay et al., 1997). The transportation process resulted in elevated body temperatures, cortisol concentrations, and glucose concentrations in this population of pregnant Brahman cows (Price et al., 2015). Control cows were maintained in the same manner as Transport cows with the exception of being transported. The two groups were housed in the same pasture (at the Texas A & M AgriLife Research Center in Overton, TX) and fed the same diet. The diet consisted of ad libitum coastal Bermuda grass in pastures during the summer and fall. The same pastures were overseeded with rye (Secale cereal) and ryegrass (Lolium multiflorum). Cows were supplemented with coastal Bermuda grass (Cynodon dactylon) hay and a 3:1 corn:soybean meal mix as required depending on forage quality and availability. From these cows, Controls gave birth (Spring 2012) to 26 males and 18 females (Control), while Transport cows gave birth to 20 males and 21 females (Prenatally stressed; PNS). Male calves were maintained as bulls (uncastrated) throughout the study. Temperament was evaluated when the bulls were weaned, at an average age of 175 days of age. It is important to note that preweaning analyses of the entire population from which these bulls were derived showed that PNS calves were more temperamental relative to Control calves (Littlejohn et al., 2016). Furthermore, temperament has been shown to have an influence on immune function in cattle (Burdick et al., 2010a; Hulbert et al., 2011), illuminating the importance of assessing the influence of temperament on immune response to an endotoxin challenge in these PNS calves.

Evaluation of temperament

Three measures of calf temperament, pen score (**PS**; a subjective measurement; Hammond et al., 1996), exit velocity (**EV**; an objective measurement; Burrow et al., 1988; Curley et al., 2006), and an overall temperament score (**TS**; Curley et al., 2006) were assessed at weaning. The PS was recorded prior to restraining animals for other measurements. To determine PS, an experienced observer evaluated individual calves (scored on a scale of 1-5; Table 2-2) in groups of 3-5 within an uncovered pen. Subsequently, each group was herded into an adjacent uncovered pen, where they remained until PS was recorded for all calves. Following pen scoring, calves were walked into an enclosed handling facility. Then, they individually entered a squeeze chute and were restrained for normal weaning management procedures. Calves were restrained for less than 5 min. An exit velocity measurement was then collected. Exit velocity was defined as the rate, measured in m/s, at which an animal traversed 1.83 m upon exiting the squeeze chute using an infrared beam sensor system (FarmTek Inc., North Wylie, TX; Burdick et al., 2009; Burrow et al., 1988; Curley et al., 2006). Temperament score [TS = (PS + EV)/2] was determined as previously reported (Curley et al., 2006; King et al., 2006). Bull calves (n=46) produced from the Transport and Control dams with a temperament score less than 1.78 were classified as Calm. Calves with a temperament score between 1.78 and 2.9 were classified as Intermediate, and calves with a temperament score greater than 2.9 were classified as Temperamental.

Table 2-2. Descriptions of subjective pen score classifications (Hammond et al., 1996).

Score	Description
1	Calves walked slowly and were not excited by evaluator
2	Calves ran along fences and kept distance from evaluator
3	Calves heads were high, avoided the evaluator, and ran when approached by the evaluator but stopped before hitting fences
4	Calves stayed at the back of the group with their heads high, were very aware of humans, and often ran into fences
5	Calves were very excited or aggressive, ran into fences, and ran over anything in their path

Endotoxin challenge

Of the 46 bulls produced from the Transport and Control dams, 32 were selected based on prenatal treatment (16 Controls and 16 PNS bulls) for an endotoxin challenge. Groups were further balanced by sire to control for genetic variation. The Control group consisted of 11 Calm, 2 Intermediate, and 3 Temperamental bull calves, and the PNS group consisted of 7 Calm, 5 Intermediate, and 4 Temperamental bull calves. This imbalance in temperament was expected, because the population of PNS bulls was more temperamental than the population of Control bulls (Littlejohn et al., 2016). Because the bulls were selected based on prenatal treatment within sire groups, it was not feasible to balance treatment groups by temperament. The average age of the selected bull calves at the time of the endotoxin challenge was 237 days of age.

On day -3 (3 days prior to the endotoxin challenge) each of the 32 bulls was fitted with an indwelling rectal temperature-recording device as previously described (Reuter et al., 2010, Burdick et al., 2010b). These rectal temperature-recording devices measured rectal temperature (**RT**) in the absence of a human operator. Rectal temperatures were measured continuously every 5 min and then averaged into hourly intervals for statistical analysis. On day -2 (2 days prior to the endotoxin challenge) bulls were transported by trailer for 9 h (for 750 km) from Overton to Lubbock, TX. On day -1 (1 day prior to the endotoxin challenge) bulls were fitted with indwelling jugular vein cannulas. For the jugular cannulation procedure, a small (2–3 cm) incision was made in the skin to more easily access the jugular vein. Temporary indwelling jugular catheters, consisting of 30.48 cm of sterile Tygon® tubing (AAQ04133; US Plastics, Lima, OH,

USA; 1.27 mm i.d. and 2.286 mm o.d.), were inserted into the jugular vein using a 14-gauge by 5.08-cm thin-walled stainless steel biomedical needle (o.d. 3 mm). The catheter was held in place using tag cement and a 2.08-cm wide porous surgical tape around the incision site, and then the entire neck region of each bull was wrapped with vet wrap (VetrapTM; 3M Animal Care Products, St. Paul, MN, USA) to ensure stability of the catheterization site. The remaining tubing not inserted into the bull served as the extension portion of the cannula for collection of blood samples. During these procedures, each bull was restrained in a working chute for approximately 10–15 min.

Following these procedures, each bull was stalled in an adjacent novel environment in a light and temperature-controlled building. This building contained individual stalls (2.13 m long x 0.76 m wide), which housed all 32 bulls through the duration of the LPS challenge. During the challenge the bulls had ad libitum access to feed and water. The extension tubing of the cannula was extended above the stall to allow researchers to collect blood throughout the study without disturbing the bulls, whether the bulls were standing or lying down.

On day 0 (the day of the endotoxin challenge), whole blood samples (9 ml) were collected into Monovette tubes containing no additive (Sarstedt, Inc., Newton, NC, USA). Samples were collected every 0.5 h, beginning 2 h before and continuing 8 h after the administration of LPS (0.5 μg/kg body weight; LPS from Escherichia coli O111:B4; Sigma-Aldrich, St. Louis MO, USA) and again at 24 h. Whole blood was allowed to clot for 30 min at room temperature, after which blood was centrifuged at 1250 x g for 20 min at 4°C and serum collected. Serum was stored at -80°C until analyzed for cortisol

and cytokine concentrations. A second 4-mL sample was collected at -2, 0, 2, 4, 6, 8, and 24 h in a vacutainer containing EDTA for determination of complete blood count (CBC) variables using a ProCyte Dx Hematology Analyzer (IDEXX, Westbrook, ME, USA).

Sickness behavior scoring

On day 0, sickness behavior scores (**SBS**) were recorded at 0.5-h intervals from -2 to 8 h and again at 24 h relative to LPS challenge (Time 0 h = LPS administration). After each blood sample was collected during the LPS challenge, a trained observer, who was blind to the treatments, assessed and recorded each bull's SBS by visual observation (Burdick et al., 2010a). Bulls were scored on a scale of 1 (active or agitated; showing the least amount of sickness behavior) to 5 (lying on side with labored breathing; showing the greatest amount of sickness behavior; Table 2-3). The same observer assigned all SBS throughout the experiment.

Table 2-3. Sickness Behavior Score descriptions of visual signs of sickness (Burdick et al., 2010a).

Score	Description
1	Normal, alert, ears erect; head level or high, eyes open, standing, locomotor activity, responsive, performing maintenance behaviors
2	Calm but less alert, less activity, less responsive, standing or lying ventral, semi-lateral
3	Lying, Calm, head distended or tucked, less alert, signs of some mild respiratory problems (coughing, wheezing)
4	Clinical signs of sickness, respiratory problems, not responsive, head distended, lethargic
5	All/most respiratory problems, mucus/foam; head distended, not responsive—medical intervention required

Assays for cortisol and cytokines

All serum samples were analyzed in duplicate. Serum cortisol concentrations were determined using a commercially available enzyme immunoassay kit (Arbor Assays, Ann Arbor, MI, USA) according to the manufacturer's directions by comparison of unknowns to standard curves generated with known concentrations of cortisol (Burdick Sanchez et al., 2015). The minimum detectable cortisol concentration was 45.4 pg/ml, and the intra- and inter-assay coefficients of variation were 7.9% and 17.7%, respectively. Data are presented as ng/ml.

Serum cytokine concentrations for interferon-gamma (**IFN-γ**), tumor necrosis factor-alpha (**TNF-α**), and interleukin-6 (**IL-6**) were determined by a custom bovine three-plex sandwich-based chemiluminescence ELISA kit (Searchlight-Aushon BioSystems Inc., Billerica, MA, USA; Burdick Sanchez et al., 2015). The minimum detectable concentrations were 0.5, 0.1, and 3.3 pg/ml for TNF-α, IFN- γ, and IL-6, respectively. Intra-assay coefficients of variation were < 6.44% and all inter-assay coefficients of variation were < 21% for all assays. Data are presented as pg/ml.

Statistical analyses

The 2-h period prior to LPS administration (Pre-LPS) was analyzed separately from the 24-h period following LPS administration (Post-LPS). Pre-LPS and Post-LPS data for RT, SBS, cortisol, and cytokines were analyzed using the MIXED procedure of SAS (SAS, Inc., Cary, NC, USA) specific for repeated measures with treatment, temperament, time, and all interactions thereof included as fixed effects. Bull was the

experimental unit. Specific comparisons were made using the PDIFF option in SAS, with $P < 0.05$ considered significant. Data are presented as the least squares means ± the standard error of the mean.

RESULTS

Rectal temperature

In the 2-h period before LPS administration, there was a treatment x temperament interaction *(*$P < 0.001$) with Temperamental PNS bulls having the greatest RT. Pre-LPS RT was greater *(*$P < 0.001$) in PNS (38.81 ± 0.04°C) compared with Control (38.55 ± 0.04°C) bulls. Temperamental bulls (39.00 ± 0.05°C) had greater *(*$P < 0.001$) pre-LPS RT than Intermediate (38.46 ± 0.05 °C) and Calm bulls (38.58 ± 0.04°C). There was a pre-LPS effect of time *(*$P < 0.001$), with a slight elevation (0.20°C and 0.15°C change in PNS and Control bulls, respectively) in RT during the 1-h period prior to LPS administration. There was no *(*$P \geq 0.10$) pre-LPS treatment x time (Figure 2-1), temperament x time, or treatment x temperament x time interaction. There was a post-LPS treatment x time interaction (Figure 2-1), with all bulls appearing to produce a biphasic response to LPS. The PNS bulls had a reduced peak RT compared to Controls and slightly greater RT values in what appears to be the secondary response to LPS. There was a post-LPS treatment x temperament interaction *(*$P < 0.001$; Figure 2-1) for RT, with Temperamental PNS bulls having the greatest RT. Post-LPS RT was greater *(*$P < 0.001$) in PNS (39.01 ± 0.02°C) compared with Control bulls (38.90 ± 0.02°C). Temperamental bulls (39.05 ± 0.02°C) had greater *(*$P < 0.001$) post-LPS RT than

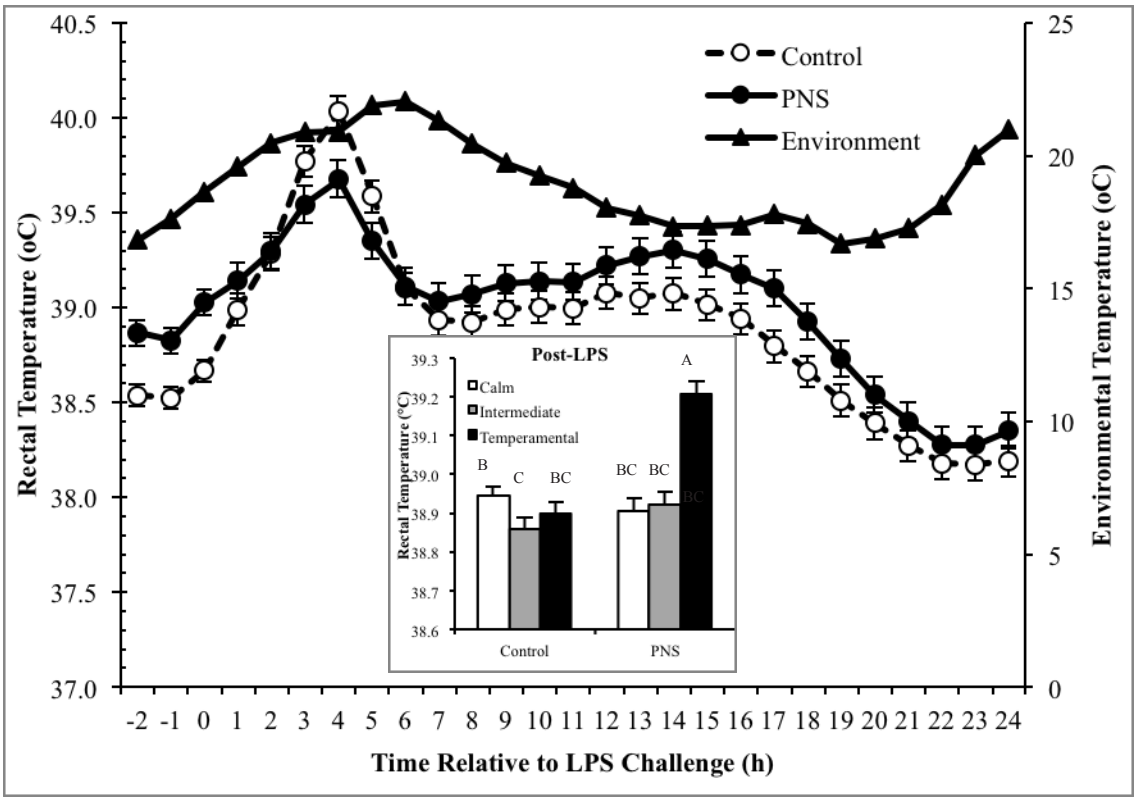

Figure 2-1. Treatment x time interaction for rectal temperature (RT) prior to *(P* = 0.921) and in response to *(P* = 0.026) an endotoxin (lipopolysaccharide, LPS; 0.5 µg/kg body weight) in prenatally stressed (PNS; n=16) and Control (n=16) bulls. Simple means for environmental temperature are included for each time point. The figure inset represents the treatment x temperament interaction differences in post-LPS RT *(P* < 0.001). Data are presented as LSM ± SEM. Columns with different superscripts differed by P ≤ 0.05

Intermediate (38.89 ± 0.02°C) and Calm bulls (38.93 ± 0.02°C). There was a post-LPS effect of time *(P* = 0.019). The RT increased in response to LPS in a biphasic manner, reaching peak values within 4 h and decreasing until 7 h. The RT increased between 7 and 14 h followed by a steady decrease back to basal (achieved around 19 h following LPS administration) and then hypothermic temperatures. There was no *(P* ≥ 0.10) post-LPS temperament x time or treatment x temperament x time interaction.

Sickness behavior score

There was no difference in SBS between PNS and Control treatment groups observed during the pre-LPS period. There was a post-LPS treatment x temperament interaction *(P* < 0.001; Figure 2-1), with Calm PNS bulls having the greatest SBS and Temperamental Control bulls having the lowest SBS. There was no difference *(P* = 0.123) in SBS between PNS (2.18 ± 0.02) and Control (2.15 ± 0.02) treatment groups observed in the post-LPS period *(P* > 0.1). Calm bulls (2.27 ± 0.02) had the greatest *(P* < 0.001) post-LPS SBS, followed by Intermediate bulls (2.15 ± 0.02), and Temperamental bulls (2.07 ± 0.02) with the least. There was a post-LPS effect of time *(P* < 0.001), with bulls experiencing an increase in SBS within 30 min after LPS administration. There was no *(P* ≥ 0.10) post-LPS effect of treatment, treatment x time (Figure 2-2), temperament x time, or treatment x temperament x time.

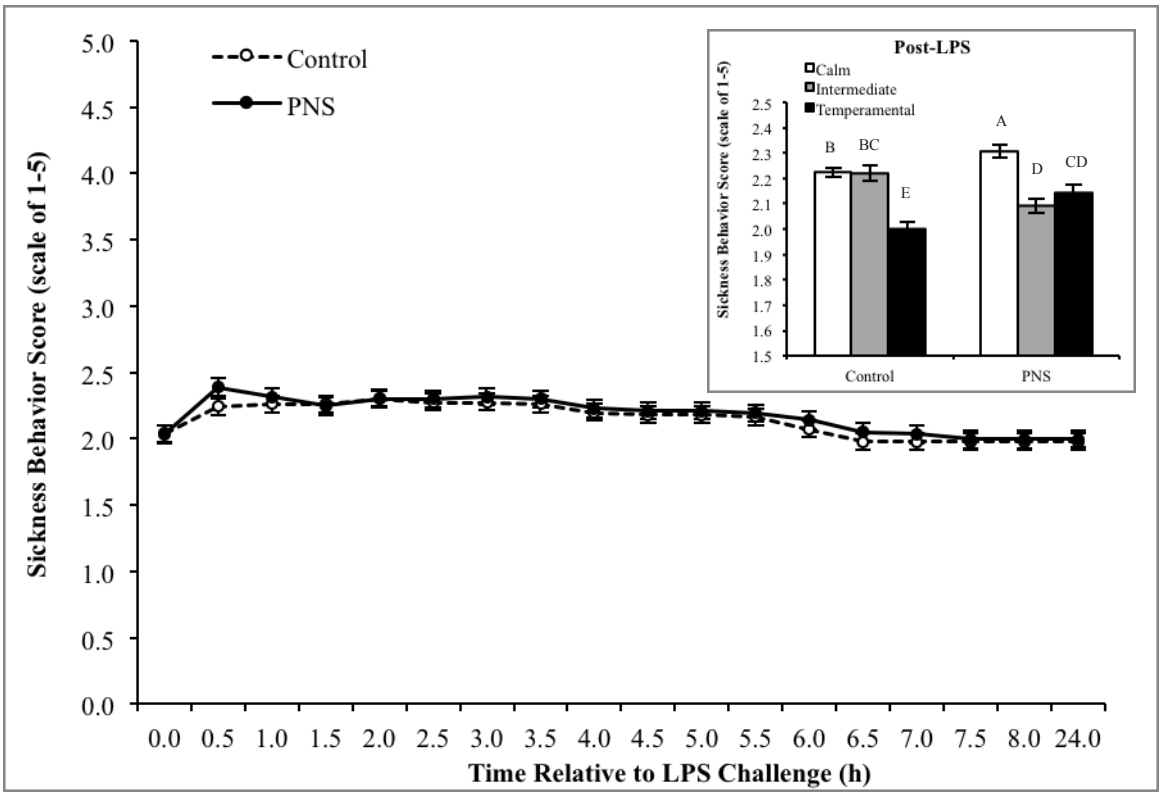

Figure 2-2. Treatment x time interaction for sickness behavior score (SBS) response to *(P* = 0.999) an endotoxin (lipopolysaccharide, LPS; 0.5 µg/kg body weight) in prenatally stressed (PNS; n=16) and Control (n=16) bulls. Bulls were scored on a scale of 1 (active or agitated), showing the least amount of sickness behavior, to 5 (lying on side with labored breathing), showing the greatest amount of sickness behavior. The figure inset represents treatment x temperament interaction differences in post-LPS SBS *(P* < 0.001). Data are presented as LSM ± SEM. Columns with different superscripts differed by P ≤ 0.05.

Serum cortisol concentrations

There was no difference *(P* = 0.417) in serum concentrations of cortisol between PNS (7.15 ± 0.47 ng/mL) and Control (6.63 ± 0.44 ng/mL) treatment groups observed in the pre-LPS period. There was a pre-LPS effect of time *(P* < 0.001), with cortisol decreasing over time prior to LPS administration but increasing in the 30-min period prior to LPS administration. There was no *(P* ≥ 0.10) pre-LPS effect of temperament, treatment x time (Figure 2-3), treatment x temperament, temperament x time, or treatment x temperament x time. There was a post-LPS treatment x temperament interaction *(P* = 0.068; Figure 2-3) for cortisol. However, there was no difference *(P* = 0.176) in serum concentrations of cortisol between PNS (25.99 ± 0.57 ng/mL) and Control (27.03 ± 0.52 ng/mL) treatment groups observed in the post-LPS period. Temperamental bulls (23.48 ± 0.71 ng/mL) had the lowest *(P* < 0.001) post-LPS concentrations of cortisol compared to Intermediate (28.02 ± 0.76 ng/mL) and Calm bulls (27.56 ± 0.56 ng/mL). In response to LPS administration, cortisol concentrations changed overtime *(P* < 0.001), i.e., increased within 0.5 h and reached peak concentrations within 4.5 h. There was no *(P* ≥ 0.10) post-LPS effect of treatment x time (Figure 2-3), temperament x time, or treatment x temperament x time.

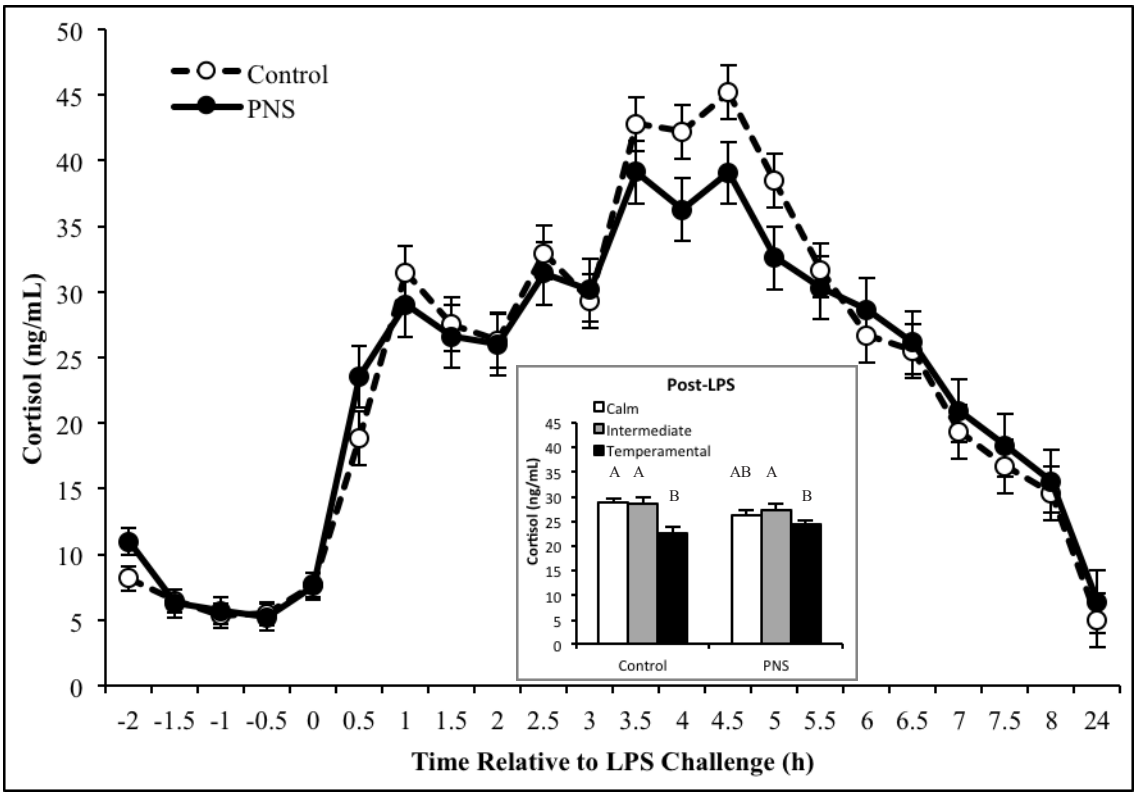

Figure 2-3. Treatment x time interaction for serum cortisol prior to *(P* = 0.482) and in response to *(P* = 0.534) an endotoxin (lipopolysaccharide, LPS; 0.5 µg/kg body weight) in prenatally stressed (PNS; n=16) and Control (n=16) bulls. The figure inset represents treatment x temperament interaction differences in post-LPS serum cortisol concentrations *(P* = 0.068). Data are presented as LSM ± SEM. Columns with different superscripts differed by P ≤ 0.05.

Serum cytokine concentrations

Pre-LPS serum concentrations of IFN-γ were reduced *(P* < 0.001) in PNS (4.15 ± 1.78 pg/mL) compared to Control bulls (13.03 ± 1.79 pg/mL). For IFN-γ, there was no *(P* ≥ 0.10) effect of temperament, time, treatment x time (Figure 2-4A), treatment x temperament, temperament x time, or treatment x temperament x time prior to LPS administration. There was a post-LPS treatment x temperament interaction *(P* < 0.001), with Intermediate PNS bulls having the greatest concentrations of IFN-γ (Figure 2-4A). Post-LPS serum concentrations of IFN-γ were greater *(P* = 0.005) in PNS (46.37 ± 2.65 pg/mL) than Control bulls (35.57 ± 2.77 pg/mL). Intermediate bulls (55.06 ± 3.39 pg/mL) had the greatest *(P* < 0.001) post-LPS concentrations of IFN-γ compared to Temperamental (33.12 ± 3.79 pg/mL) and Calm bulls (34.73 ± 2.68 pg/mL). There was a post-LPS effect of time on IFN-γ concentrations *(P* < 0.001). Peak concentrations of IFN-γ occurred 3.5 h after LPS administration and subsequently decreased over time. There was no *(P* ≥ 0.10) post-LPS effect of treatment x time (Figure 2-4A), temperament x time, or treatment x temperament x time.

Pre-LPS serum concentrations of TNF-α were greater *(P* = 0.029) in PNS bulls (103.78 ± 20.40 pg/mL) than Control bulls (40.33 ± 21.13 pg/mL). There was no *(P* ≥ 0.10) pre-LPS effect of temperament, time, treatment x time (Figure 2-4B), treatment x temperament, temperament x time, or treatment x temperament x time. There was a post-LPS treatment x temperament x time interaction *(P* = 0.004) and a post-LPS treatment x time interaction *(P* = 0.038; Figure 2-4B) with PNS bulls having greater peak TNF-α concentrations than Controls. There was a post-LPS temperament x time interaction *(P* =

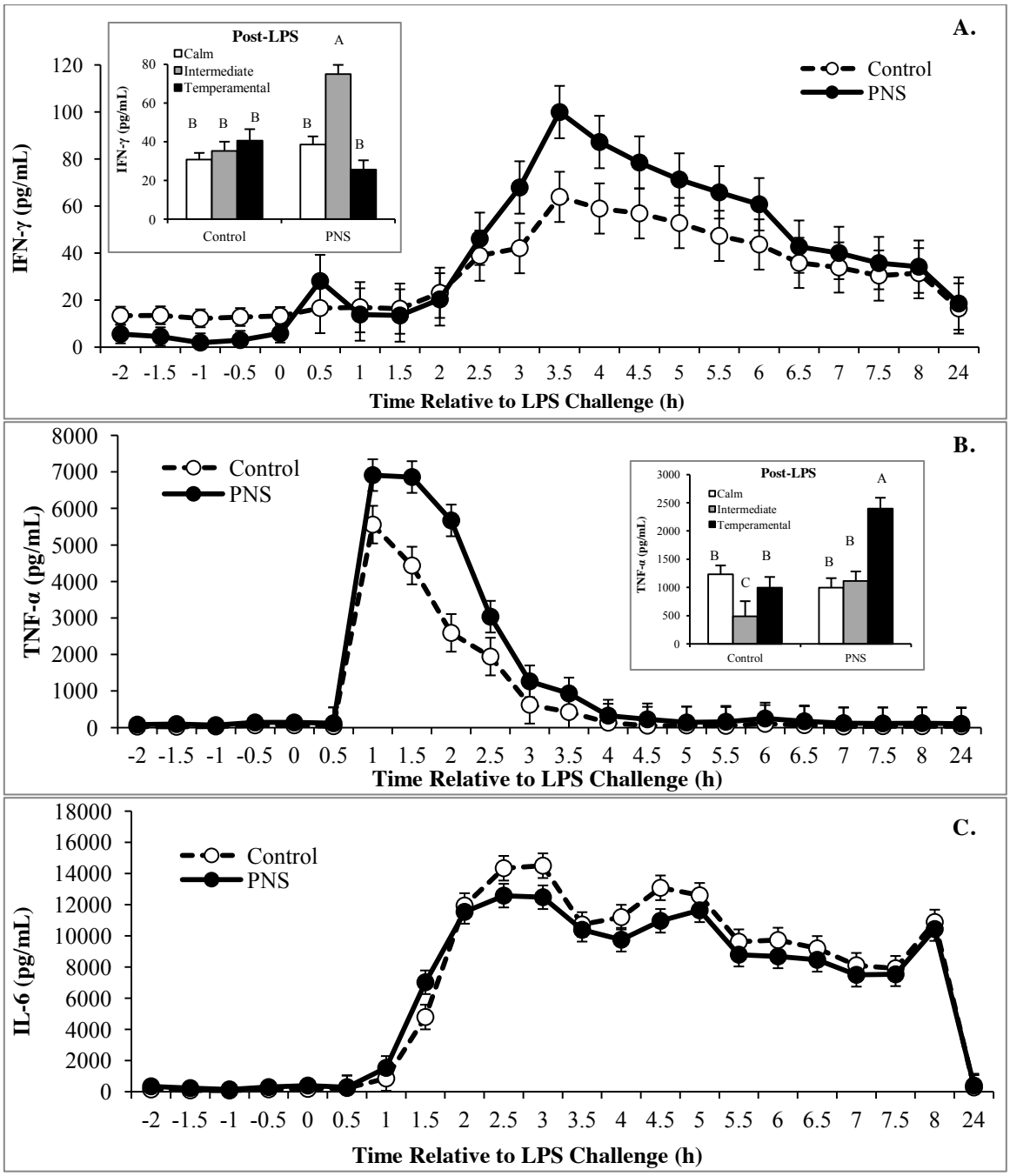

Figure 2-4. Treatment x time interaction for serum interferon-gamma (IFN-γ; Figure A), tumor necrosis factor-alpha (TNF-α; Figure B), and interleukin-6 (IL-6; Figure C) prior to (*P* = 0.995, 0.994, 0.962; respectively) and in response to (*P* = 0.883, 0.038, 0.588; respectively) an endotoxin (lipopolysaccharide, LPS; 0.5 µg/kg body weight) in prenatally stressed (PNS; n=16) and Control (n=16) bulls. The figure insets for Figures A and B represent treatment x temperament interaction differences in post-LPS serum IFN-γ (*P* < 0.001) and TNF-α (*P* < 0.001) concentrations, respectively. Data are presented as LSM ± SEM. Columns with different superscripts differed by P ≤ 0.05.

0.058), with Temperamental bulls having the greatest peak concentrations of TNF-α compared to Intermediate and Calm bulls. Greater concentrations of TNF-α were maintained in Temperamental bulls between 1 and 3 h following LPS. There was a post-LPS treatment x temperament interaction *(P* < 0.001), with Temperamental PNS bulls having the greatest post-LPS concentrations of TNF-α (Figure 2-4B). The PNS bulls (1481.12 ± 102.23 pg/mL) had greater *(P* < 0.001) post-LPS serum concentrations of TNF-α than Control bulls (905.01 ± 121.40 pg/mL). Post-LPS concentrations of TNF-α were greatest *(P* < 0.001) in Temperamental bulls (1696.25 ± 134.49 pg/mL), followed by Calm bulls (1083.33 ± 115.17 pg/mL), and lowest in Intermediate bulls (799.62 ± 159.13 pg/mL). Concentrations of TNF-α changed over time *(P* < 0.001) in response to LPS administration. Peak TNF-α concentrations were reached within 1 h after LPS administration, and basal concentrations were reached within 5 h after LPS administration.

Pre-LPS serum concentrations of IL-6 were greater *(P* = 0.017) in PNS bulls (288.48 ± 45.29 pg/mL) than Control bulls (133.98 ± 46.90 pg/mL). There was no *(P* ≥ 0.10) pre-LPS effect of temperament, time, treatment x time (Figure 2-4C), treatment x temperament, temperament x time, or treatment x temperament x time. Post-LPS serum concentrations of IL-6 were lower *(P* = 0.037) in PNS bulls (7803.01 ± 178.95 pg/mL) compared to Control bulls (8352.78 ± 197.95 pg/mL). Concentrations of IL-6 changed over time *(P* < 0.001) in response to LPS administration. Bulls exhibited a biphasic IL-6 response to LPS administration, with bulls reaching initial peak concentrations within 3 h after LPS administration and secondary peak concentrations within 4.5 to 5 h after LPS

administration. There was no ($P \geq 0.10$) post-LPS effect of temperament, treatment x time (Figure 2-4C), treatment x temperament, temperament x time, or treatment x temperament x time.

Complete blood cell counts

There was a pre-LPS treatment x temperament interaction ($P = 0.086$) for WBC counts, with the greatest WBC counts in Calm Control bulls. Pre-LPS WBC counts were greatest ($P = 0.005$) in Calm bulls compared to Intermediate and Temperamental bulls (Table 2-4). There were no other effects ($P \geq 0.10$) on pre-LPS WBC counts (Table 2-4). There was a post-LPS treatment x temperament interaction ($P = 0.037$) for WBC counts, with Calm Control and Temperamental PNS bulls having the greatest WBC counts and Temperamental Control bulls having the least WBC counts. Post-LPS WBC counts were greater ($P = 0.035$) in Calm bulls compared to Intermediate bulls, but not Temperamental bulls (Table 2-5). Post-LPS WBC counts were affected by time ($P < 0.001$). Within 2 h after LPS administration, WBC counts decreased. Basal WBC counts were reached within 24 h after LPS administration. There were no other effects ($P \geq 0.10$) on post-LPS WBC counts (Table 2-5).

Table 2-4. Summary of hematology variables measured in bulls prior to an LPS challenge*[1, 2, 3]

Variable ($10^3/\mu L$)	Trt Means		Temperament Means			P-value*				
	Control	PNS	C	I	T	Trt	Temp	Time	Trt x Temp	Trt x Time
WBC	9.38 ± 0.45	10.30 ± 0.44	11.20 ± 0.49[a]	8.83 ± 0.54[b]	9.49 ± 0.61[b]	0.15	<0.01	0.94	0.09	0.90
Neutrophil	2.90 ± 0.16	3.17 ± 0.16	3.15 ± 0.19	2.99 ± 0.19	2.97 ± 0.22	0.25	0.75	0.93	0.01	0.99
Lymphocyte	5.10 ± 0.29	5.44 ± 0.28	6.07 ± 0.31[a]	4.40 ± 0.34[b]	5.34 ± 0.39[ab]	0.40	<0.01	0.90	0.77	0.54
Monocyte	1.12 ± 0.10	1.58 ± 0.10	1.60 ± 0.12[a]	1.33 ± 0.12[ab]	1.11 ± 0.14[b]	<0.01	0.02	0.35	0.03	0.58
Eosinophil	0.055 ± 0.004	0.036 ± 0.005	0.049 ± 0.005	0.044 ± 0.005	0.045 ± 0.006	<0.01	0.80	0.81	0.90	0.63
Basophil	0.017 ± 0.002	0.009 ± 0.002	0.012 ± 0.003	0.009 ± 0.003	0.018 ± 0.003	0.02	0.14	0.05	0.78	0.83

re were no interactions for temperament (Temp) x time or treatment (Trt) x temperament x time ($P \geq 0.10$).
ı compare Control and prenatally stressed (PNS) treatment groups and are presented as least squares means (± SE).
ı are represented for bulls with Calm (C; TS < 1.78), Intermediate (I; TS > 1.78 and < 2.9), and Temperamental (T; TS > 2.9) temperament scores.
ımns with different superscripts differed by $P \leq 0.05$.

Table 2-5. Summary of hematology variables measured in bulls in response to an LPS challenge*[1, 2, 3]

Variable ($10^3/\mu L$)	Trt Means		Temperament Means			P-value*				
	Control	PNS	C	I	T	Trt	Temp	Time	Trt x Temp	Trt x Time
WBC	4.70 ± 0.18	5.01 ± 0.18	5.27 ± 0.20[a]	4.53 ± 0.21[b]	4.77 ± 0.26[ab]	0.23	0.03	<0.01	0.04	0.98
Neutrophil	0.64 ± 0.05	0.65 ± 0.05	0.71 ± 0.05	0.62 ± 0.06	0.61 ± 0.07	0.97	0.38	<0.01	0.14	0.82
Lymphocyte	3.34 ± 0.15	3.53 ± 0.15	3.68 ± 0.16[a]	3.15 ± 0.18[b]	3.48 ± 0.21[ab]	0.35	0.08	<0.01	0.10	1.00
Monocyte	0.68 ± 0.05	0.75 ± 0.05	0.81 ± 0.05[a]	0.69 ± 0.06[ab]	0.64 ± 0.07[b]	0.28	0.08	<0.01	0.47	0.12
Eosinophil	0.033 ± 0.002	0.031 ± 0.002	0.034 ± 0.002	0.031 ± 0.002	0.030 ± 0.002	0.31	0.27	<0.01	0.84	<0.01
Basophil	0.0028 ± 0.0004	0.0014 ± 0.0004	0.0019 ± 0.0005	0.0019 ± 0.0005	0.0019 ± 0.0005	0.03	0.79	<0.01	0.92	<0.01

*There were no interactions for temperament (Temp) x time or treatment (Trt) x temperament x time ($P \geq 0.10$).
[1]Data compare Control and prenatally stressed (PNS) treatment groups and are presented as least squares means (± SE).
[2]Data are represented for bulls with Calm (C; TS < 1.78), Intermediate (I; TS > 1.78 and < 2.9), and Temperamental (T; TS > 2.9) temperament scores.
[3]Columns with different superscripts differed by $P \leq 0.05$.

There was a pre-LPS treatment x temperament interaction ($P = 0.011$) for neutrophils, with Calm Control and Temperamental PNS bulls having the greatest neutrophil counts and Temperamental Control and Calm PNS bulls having the least neutrophil counts. There were no other effects ($P \geq 0.10$) on pre-LPS neutrophil counts (Table 2-4). Similar to WBC, there was a post-LPS effect of time ($P < 0.001$). Neutrophil counts decreased within 2 h after LPS administration and remained decreased throughout the remainder of the study (through 24 h). There were no other effects ($P \geq 0.10$) on post-LPS neutrophil counts (Table 2-5).

Pre-LPS lymphocytes were greater ($P = 0.003$) in Calm bulls compared to Intermediate bulls but not Temperamental bulls (Table 2-4). There were no other effects ($P \geq 0.10$) on pre-LPS lymphocyte counts (Table 2-4). There was a post-LPS treatment x temperament interaction (P=0.097), with Calm Control and Temperamental PNS bulls having the greatest lymphocyte counts and Temperamental Control and Intermediate PNS bulls having the lowest lymphocyte counts. Post-LPS lymphocytes were greater ($P = 0.081$) in Calm bulls compared to Intermediate bulls but not Temperamental bulls. Post-LPS lymphocyte counts were affected by time ($P < 0.001$). Lymphocytes decreased within 2 h after LPS administration but exceeded basal concentrations within 24 h after LPS administration. There were no other effects ($P \geq 0.10$) on post-LPS lymphocyte counts (Table 2-5).

Monocytes were affected by a pre-LPS treatment x temperament interaction ($P = 0.034$), with Calm PNS bulls having the greatest monocyte counts and Temperamental Control bulls having the least monocyte counts. Pre-LPS monocytes were greater ($P =$

0.002) in PNS bulls compared to Control bulls (Table 2-4). Pre-LPS monocytes were greater ($P = 0.024$) in Calm bulls compared to Temperamental bulls but not Intermediate bulls (Table 2-4). There were no other effects ($P \geq 0.10$) on pre-LPS monocyte counts (Table 2-4). Post-LPS monocyte counts were greater ($P = 0.084$) in Calm compared to Temperamental but not Intermediate bulls (Table 2-5). Post-LPS monocytes were affected by time ($P < 0.001$). Monocytes decreased within 2 h after LPS administration and reached basal concentrations within 24 h after LPS administration. There were no other effects ($P \geq 0.10$) on post-LPS monocyte counts (Table 2-5).

Pre-LPS eosinophils were reduced ($P = 0.004$) in PNS bulls compared to Control bulls (Table 2-4). There were no other effects ($P \geq 0.10$) on pre-LPS eosinophil counts (Table 2-4). There was a post-LPS treatment x time interaction ($P = 0.009$). The PNS bulls had reduced eosinophil counts at 0 h but reached similar eosinophil counts compared to Controls 2 h after LPS administration. Post-LPS eosinophils were affected by time ($P < 0.001$). Eosinophil counts decreased within 2 h after LPS administration followed by a steady increase, which peaked at 6 h and reached the lowest concentrations at 24 h after LPS administration. There were no other effects ($P \geq 0.10$) on post-LPS eosinophil counts (Table 2-5).

The PNS bulls had reduced ($P = 0.023$) pre-LPS basophils compared to Control bulls (Table 2-4). Pre-LPS basophils decreased over time ($P = 0.049$). There were no other effects ($P \geq 0.10$) on pre-LPS basophil counts (Table 2-4). There was a post-LPS treatment x time interaction ($P < 0.001$). The PNS bulls had reduced basophil counts at time zero but reached counts similar to Control bulls 2 h after LPS administration. The

PNS bulls had reduced ($P = 0.030$) post-LPS basophils compared to Control bulls (Table 2-5). There was a post-LPS effect of time ($P < 0.001$). Basophil counts decreased within 2 h after LPS administration and remaining decreased throughout the remainder of the study (through 24 h after LPS administration). There were no other effects ($P \geq 0.10$) on post-LPS basophil counts (Table 2-5).

DISCUSSION

This study primarily assessed whether prenatal stress influenced postnatal innate immune response to an endotoxin challenge in Brahman bulls of known temperament classification. In response to LPS, prenatally stressed bull calves exhibited increased rectal temperatures, increased serum IFN-γ and TNF-α, as well as decreased serum IL-6. Limited hematological alterations were observed in PNS compared with Control bull calves. Temperament influenced sickness behavior score, rectal temperature, cytokine, and immune cell response to LPS. Collectively, these findings are indicative of an altered physiological response to an acute immune challenge in prenatally stressed bulls. The following paragraphs discuss the effects of prenatal transportation stress on postnatal innate immune response in weaned Brahman bull calves, as well as the interaction with temperament on those results.

The literature available regarding prenatal stress and immune function pertains primarily to rodent models (Kay et al., 1998; Götz et al., 2007), and the majority of such studies suggest suppressed immune function due to prenatal stress. For example, rats whose dams were exposed to weekly noise and light stress throughout gestation had

decreased circulating and splenic lymphocytes in response to pokeweed mitogen at 2 months of age (Kay et al., 1998). Adult male rats whose dams underwent gestational confrontation stress (daily 2-h exposure to another female rat for a 2-month period) exhibited alterations in populations of neutrophils, monocytes, T cells, NK cells, and lymphocyte proliferation (Götz et al., 2007). Furthermore, prenatally stressed animals have been reported to exhibit anxious behaviors and more excitable temperaments (Coulon et al., 2011; Fride and Weinstock, 1988), including the population of prenatally stressed calves from which calves in the current study were derived (Littlejohn et al., 2016). For example, 6 month old rats whose dams were exposed to noise and light stress throughout gestation were more anxious than Control rats as demonstrated by less time spent in open areas of a maze (Fride and Weinstock, 1988). Lambs whose dams were handled roughly on a daily basis the last 35 d of gestation approached a human evaluator slower and fled a greater distance when startled (Coulon et al., 2011). These associations of prenatal stress and temperament are important to note, because temperament and stress response influence innate immune response (Burdick et al., 2010a; Carobrez et al., 2002). In the present study, prenatal transportation was used as a stressor to evaluate differences in innate immune response of calves. Differential responses in RT, IFN-γ, TNF-α, IL-6, and basophils to an endotoxin challenge in weaned Control and PNS bulls suggest an impact of prenatal environment on postnatal innate immune function.

Rectal temperature

Rectal temperature is an indicator of health status and inflammation in cattle (Burdick et al., 2010a; Carroll et al., 2009; Jacobsen et al., 2005). An increase in body temperature is characteristic of the response to an LPS challenge in cattle (Jacobsen et al., 2005). The slight elevation (0.20°C and 0.15°C change in PNS and Control bulls, respectively) in RT recorded immediately prior to LPS administration may have been due to a minor stress response of the animals to the temporarily increased frequency of humans entering and exiting the cattle housing facility. Overall, the RT response of Brahman bulls to LPS in the present study was similar to that reported by Burdick et al. (2010a). These results included a biphasic response to LPS, the length of time to reach peak RT and to return to basal RT, and a subsequent decline to sub-basal temperatures.

Temperamental calves had increased RT compared to Intermediate and Calm calves prior to and following LPS administration. Burdick et al. (2010a) reported increased RT prior to and following the initial peak response to LPS administration, but a blunted, smaller increase to peak RT in response to LPS in Temperamental bulls relative to Calm and Intermediate bulls. That decreased response might have been due to increased basal concentrations of cortisol in Temperamental cattle observed by Burdick et al., (2010a), among others (Curley et al., 2008). In concurrence, numerically increased basal concentrations of cortisol were observed in the current study and a significant elevation in basal concentrations of cortisol have been reported (Littlejohn et al., 2016) in PNS compared with Control calves in the preweaning analysis of the entire population from which the bulls in this study were derived. From that population, PNS calves were

reported to be more temperamental than Control calves (Littlejohn et al., 2016). If PNS calves in the present study had a predisposition for alterations in temperament and HPA axis activity as suggested by Littlejohn et al. (2016), prenatally stressed bull calves might be expected to exhibit a similar response to Temperamental bull calves. This might further explain, in part, why PNS bulls exhibited an increased RT prior to and following the initial peak response to LPS administration, but a blunted, smaller increase to peak RT in response to LPS in Temperamental bulls relative to Control bulls. Furthermore, these associations of PNS and temperament serve to illuminate the treatment x temperament interaction in the present study both prior to and following LPS administration in which Temperamental PNS bulls had the greatest RT. Limited research is available describing the effects of prenatal stress on rectal or core body temperature in response to an immune challenge. Fever response to LPS was greater in 8-week-old female pigs whose dams were administered cortisol during early and late gestation compared to Controls (de Groot et al., 2007). The febrile response to IL-1β was reduced in monkeys whose dams were administered ACTH during pregnancy (Reyes and Coe; 1997). It is well established that an increase in body temperature is necessary for pathogen clearance (Hasday et al., 2000). The greater rectal temperature response observed in PNS bulls suggests that these bulls may require a greater allocation of resources to subdue an infectious agent. This supposition is supported by greater circulating concentrations of post-LPS IFN-γ and TNF-α observed in PNS bulls, though IL-6 secretion was reduced in PNS bulls. Production of TNF-α, IL-1β, and IL-6 has been

reported to be involved in the fever response to increase body temperature (Dinarello, 1996).

Sickness behavior score

Sickness behavior can be defined as coordinated behavioral and physiological changes that occur in response to an infection due to the secretion of pro-inflammatory cytokines (Dantzer et al., 1996). These behavioral changes might include weakness, malaise, listlessness, inability to concentrate, depression, lethargy, lack of attention to surroundings, and decreased intake of food and water (Dantzer et al., 1996). Increased sickness behavior has been reported in response to LPS in rodents, pigs, and cattle (Avitsur and Sheridan, 2009; Burdick et al., 2010a; de Groot et al., 2007; Lay et al., 2011). Sickness behavior scores in response to LPS were similar to previously reported observations in Brahman bulls (Burdick et al., 2010a). In the present study, there was no difference in SBS between PNS and Control bulls prior to LPS administration. Limited literature is available describing SBS following an endotoxin challenge in prenatally stressed animals. Mice that underwent neonatal maternal separation had altered sickness behavior in the form of decreased food consumption compared with Controls in response to LPS administration (Avitsur and Sheridan, 2009). Pigs whose dams were handled roughly during gestation had lower SBS than pigs whose dams were either administered ACTH during gestation or were maintained as Controls (Lay et al., 2011). Pigs whose dams were administered cortisol during pregnancy recovered from an endotoxin challenge more quickly (had shortened periods of sickness behavior) compared with

Controls (de Groot et al., 2007). Though the present study demonstrated no differences in SBS between prenatal treatment groups in response to LPS administration, a treatment x temperament interaction occurred with the greatest SBS observed in Calm PNS bulls and the least SBS observed in Temperamental Control bulls. This coincides with the tendency for a post-LPS treatment x temperament interaction for cortisol, with Temperamental Control bulls having the lowest circulating concentrations of cortisol. Additionally, Calm bulls had the greatest post-LPS SBS, followed by Intermediate bulls, and Temperamental bulls with the least SBS. Behavior and stress response has been reported to influence sickness behavior following an endotoxin challenge (Burdick et al., 2010a; Cheng et al., 2004). Burdick et al. (2010a) reported similar results to this study, with Calm Brahman bulls having greater SBS compared with Intermediate and Temperamental Brahman bulls in response to an endotoxin challenge. Chickens with a genetic predisposition to be more productive and more likely to survive in a group environment have altered behavior and stress response, which resulted in differential sickness behavior response to LPS administration (Cheng et al., 2004). Furthermore, glucocorticoids have been reported to attenuate sickness behavior in response to LPS and E. coli challenges in rodents (Goujon et al., 1995; Hanaa-Mansour et al., 2016). Sickness behavior is induced by pro-inflammatory cytokines such as IL-1, IL-6, TNF-α, and interferons (Dantzer, 2001). Therefore, differences in SBS might have been expected due to the differences observed in cytokine concentrations between PNS and Control bulls. Perhaps differences in serum concentrations of cytokines may not have been sufficient to induce phenotypically detectible differences in SBS of PNS bulls.

However, sickness behavior response is regulated, in part, by the influence of the HPA axis and temperament on cytokine response. Both HPA axis function and temperament are influenced by prenatal stress, potentially resulting in the observed interaction affecting sickness behavior score.

Cortisol

Cortisol is known for its negative role in regulation of the immune system. However, cortisol is necessary in response to a pathogen to prevent a hyper-inflammatory state caused by increased concentrations of pro-inflammatory cytokines. Cortisol is an indicator of stress in cattle (Lefcourt and Elsasser, 1995). Therefore, the slight increase in cortisol 30 min prior to LPS administration might be explained by a slightly increased stress response to humans entering the facility to prepare for the challenge. This is in concordance with simultaneous elevations in RT during the challenge. Prior to and in response to administration of LPS, there were no differences in serum cortisol concentrations between treatment groups. This is consistent with reports in pigs whose dams were repeatedly exposed to a social stressor (housing with unfamiliar gilts) during late gestation (Couret et al., 2009). Furthermore, Temperamental bulls in the present study had the lowest post-LPS concentrations of cortisol compared to Intermediate and Calm bulls. These data agree with reports from Burdick et al. (2014) in which Temperamental Brahman bulls exhibited increased basal concentrations of cortisol but decreased response of cortisol to LPS compared to their Calm and Intermediate counterparts. Perhaps the reduced response of cortisol to LPS in

Temperamental bulls was due, in part, to elevated basal concentrations of circulating cortisol reported in temperamental cattle (Curley et al., 2008).

Cytokines

In the current study, pre-LPS serum concentrations of IFN-γ were reduced in PNS compared with Control bulls, while TNF-α and IL-6 were greater in PNS bulls compared with Control bulls. Similar to previous studies in steers (Burdick et al., 2011), the innate immune response to an endotoxin challenge resulted in the secretion of proinflammatory cytokines within 1–3 h. The PNS bulls had increased post-LPS concentrations of IFN-γ and TNF-α, but decreased concentrations of IL-6. There is contrasting literature regarding cytokine activity in response to an immune challenge in prenatally stressed animals. For example, increased circulating IFN-γ was reported in response to phytohemagglutinin-A in rats whose dams underwent restraint stress during gestation (Vanbesien-Mailliot et al., 2007). Additionally, pigs (across sex classes) whose dams underwent restraint stress during gestation had increased circulating TNF-α and IL-6 in response to LPS administration compared with Controls (Collier et al., 2011). Furthermore, rats whose dams underwent restraint and light stress from d 14 of gestation to partition exhibited depressive-like behavior, increased basal mRNA expression of hippocampal IL-1β, and increased LPS-stimulated hippocampal IL-1β, TNF-α, and IL-6 (Szczesny et al., 2014). Alternatively, monkeys whose dams were administered ACTH on day 120 through 133 of gestation had decreased circulating concentrations of IL-6 (Reyes and Coe, 1997). Increased post-LPS TNF-α and IFN-γ in PNS bulls supports the

concept of an enhanced inflammatory process and coincides with increased post-LPS rectal temperatures. However, post LPS IL-6 was reduced in PNS bulls. This might be due, in part, to differences in properties of IL-6 compared to other pro-inflammatory cytokines such as TNF-α and IFN-γ. Because IL-6 functions to stimulate release of acute phase proteins, as well as the subsequent adaptive immune response, it often plays a protective role by acting as an anti-inflammatory cytokine. Additionally, IL-6 can have suppressive effects on TNF-α, which has been shown to be greater in IL-6 deficient mice (Fattori et al., 1994; Di Santo et al., 1997). This may explain, in part, why PNS bulls had greater RT, IFN-γ, and TNF-α but lower IL-6 in response to LPS. Temperament influenced the pro-inflammatory cytokines, IFN-γ and TNF-α, but not IL-6. Intermediate cattle exhibited the greatest concentrations of IFN-γ and Temperamental cattle exhibited the greatest circulating concentrations of TNF-α in response to LPS administration. Much of the literature suggests increased peripheral concentrations of proinflammatory cytokines in individuals with more excitable or anxious temperaments in response to an endotoxin challenge. TNF-α production was increased in LPS-stimulated whole blood from anxious women compared with nonanxious women (Arranz et al., 2007). There was no difference in TNF-α production by LPS-stimulated whole blood from cattle with differing temperaments (Hulbert et al., 2011). Overall, prenatal treatment and temperament had strong influences on TNF-α, IFN-γ, and IL-6 secretion both prior to and in response to LPS administration, which may have played a role in RT and innate immune response in this study.

Hematology

Changes in circulating populations of immune cells in response to an endotoxin challenge are representative of innate immune response to a pathogen in cattle (Burdick et al., 2011). In the present study, PNS influenced basal hematological parameters, but few differences were observed between PNS and Control bulls in response to LPS. Pre-LPS circulating concentrations of monocytes were greater in PNS bulls, but eosinophil and basophil concentrations were reduced in PNS bulls compared with Controls. In response to LPS, basophils were reduced in PNS compared with Control bulls. Monocytes and monocyte-derived-macrophages are primary producers of TNF-α in response to LPS (Gessani et al., 1993). Therefore, increased monocyte counts may have been responsible, in part, for the greater TNF-α concentrations in PNS bull calves. There is contrasting literature regarding effects of prenatal stress on populations of immune cells. Piglets whose dams were exposed to social stress (housing with unknown gilts) during late gestation had decreased basal white blood cells and lymphocytes but increased lymphocyte proliferation in response to Concanavalin A (Couret et al., 2009). Male rats whose dams were exposed to hanging stress (daily 2-h periods from day 14 of gestation to term) had decreased basal leukocytes and lymphocytes but increased basal neutrophils and eosinophils (Llorente et al., 2002). Leukocyte counts in response to LPS were not different in weaned pigs whose dams were exposed to restraint stress daily between day 84 and 112 of gestation compared with Controls (Collier et al., 2011). Variations in the timing and type of prenatal stressor as well as differences in species of the previously listed studies might be responsible for contradicting differences in

hematology results. In the present study, temperament affected circulating immune cell populations, with pre-LPS WBC, lymphocyte, and monocyte and post-LPS WBC populations being greatest in Calm bulls. This is in contrast to reports in Brahman bulls in which there was no difference in basal leukocyte counts between Temperamental and Control bulls (Hulbert et al., 2011). However, mice with high anxiety exhibited decreased lymphocytes but no difference in granulocytes or monocytes (Rammal et al., 2010). In cattle, lymphocytes are the predominant group of leukocytes (Roland et al., 2014). Differing pre-LPS WBC and lymphocyte counts between temperament groups might suggest altered basal immune cell activity and capacity to respond to a pathological insult in Calm compared to Intermediate and Temperamental calves. Overall, there were minimal differences in hematology variables due to prenatal stress.

CONCLUSION

In response to LPS, prenatally stressed bull calves exhibited increased rectal temperatures, which may have been due to increased concentrations of IFN-γ and TNF-α, but decreased IL-6. Limited hematological alterations were observed in PNS compared with Control bull calves. As expected, temperament influenced sickness behavior score, rectal temperature, cytokine, and immune cell response to LPS in this population of Brahman bulls. Altered physiological response to an acute immune challenge was observed in weaned prenatally stressed bulls, suggesting an impact on the body's capacity to respond to an immunological insult. Differences in circulating

cytokines between treatment groups suggest that future studies should assess circulating acute phase proteins to better understand the acute phase response.

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CHAPTER III

PRENATAL TRANSPORTATION STRESS ALTERS GENOME-WIDE DNA METHYLATION IN SUCKLING BRAHMAN BULL CALVES

INTRODUCTION

Environmental stimuli or stressors incurred by a gestating dam impact postnatal phenotype of the offspring. During periods in which a dam experiences a physiological response to a stressor characterized by increased circulating concentrations of cortisol, the placental barrier enzyme 11-Beta HSD is suppressed. Because 11-BHSD functions to convert cortisol to a less active form before crossing the placenta to enter fetal circulation, decreased 11-BHSD results in increased exposure of a fetus to corticosteroids (Benediktsson et al., 1997). The means by which altered prenatal environment impacts postnatal outcome is in part through epigenetic modifications (Szyf, 2012). Such modifications include DNA methylation, a covalent modification in which a methyl group is added to the 5' position of a cytosine nucleotide (Wyatt, 1950; Razin and Riggs et al., 1980). This chemical modification to DNA plays a crucial role in regulating the unique functions between different cell types, in spite of the fact that each cell type has an identical genome (Razin and Riggs, 1980). Increased DNA methylation in promoter regions of a gene has been shown to result in repressed transcription and gene expression (Levine et al., 1991; Tate and Bird, 1993), while increased DNA methylation in gene bodies has been shown to result in increased gene expression (Hellman and Chess, 2007). However, the consistency of these relationships is unclear.

Nevertheless, methylation status of DNA can provide insight into how prenatal environment alters postnatal phenotype. The phenotype of beef cattle is of economic importance with regard to health, behavior, and production traits. This study sought to understand how environmental stressors incurred by gestating dams could impact the economically relevant traits in beef cattle, by evaluating the influence of prenatal transportation stress on genome-wide DNA methylation in their offspring.

MATERIALS AND METHODS

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

Animal Procedures

Mature Brahman cows were assigned to one of two treatment groups (transported = 48; control = 48) according to age, parity, and temperament (Littlejohn et al., 2016). Transported cows were hauled for a 2-h duration on 60 ± 5, 80 ± 5, 100 ± 5, 120 ± 5, and 140 ± 5 d of gestation (Price et al., 2015). Control cows were maintained in the same manner as stressed cows with the exception of being transported. The two groups were housed in the same pasture (at the Texas A & M AgriLife Research Center in Overton, TX) and fed the same diet (Littlejohn et al., 2016). From these cows, 26 male and 18 female calves (Control group) were born to control dams, while 20 male and 21 female calves (Prenatally stressed, **PNS**, group) were born to transported dams. Male calves

were maintained as bulls throughout the study. Each calf was restrained manually at 28 d of age for less than 5 min to obtain samples. One 10-mL vacuum tubes (BD, Franklin Lakes, NJ) containing EDTA were used to collect blood samples via jugular venipuncture using a sterile 18-gauge needle. For selection purposes, bull calves were stratified into groups based on the following criteria: 1) Adequate DNA availability, 2) Treatment group, and 3) Sire. From these bull calves 7 PNS and 7 Control calves were randomly selected for methylation assessment.

Sample Analysis

Processing of Blood Samples. Blood samples were centrifuged at 2,671 x g for 30 min at 6°C. White blood cells were isolated and transferred into 2-mL DNase- and RNase-free microcentrifuge tubes. Samples were repeatedly washed in red blood cell lysis buffer solution to produce a clean cell pellet and stored at -80°C until they were thawed for DNA extraction.

DNA Extraction. Phenol-chloroform extraction procedures were used to isolate DNA from each white blood cell pellet for methylation analysis. The following description summarizes extraction procedures. Samples were placed on ice between steps. White blood cell pellets were homogenized in extraction buffer (100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.5), to which 10 mg/mL proteinase K and 20% SDS was added for proteinase K digestion and incubated at 55°C for 2 h. Samples were then extracted two separate times with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and two separate times with an equal volume of 1-bromo-3-

chloropropane (**BCP**; substituted for chloroform). After each extraction, samples underwent centrifugation for 5 min at 8,000 x g. Samples were precipitated with 10% 3 M sodium acetate (pH 5.2) and 1 volume isopropanol, then subsequently centrifuged for 5 min at 13,000 x g. Pelleted DNA was then rinsed with 70% ethanol, centrifuged at 13,000 x g for 5 min, air-dried, rinsed with 95-100% ethanol, centrifuged, air-dried, and suspended in 150-200 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Purified DNA was stored at -80°C until selected samples were transported to Zymo Research Corp (Irvine, CA) for DNA methylation analysis.

Library Construction for DNA Methylation Analysis. A method of reduced representation bisulfite sequencing, Methyl-MiniSeq, was used to assess differential DNA methylation in samples from PNS compared with control bull calves. Libraries were prepared from 200-500 ng of genomic DNA digested with 60 units of TaqαI and 30 units of MspI (NEB) sequentially and then extracted with Zymo Research (ZR) DNA Clean & Concentrator-5 kit (Cat#: D4003). Fragments were ligated to pre-annealed adapters containing 5'-methyl-cytosine instead of cytosine according to Illumina's specified guidelines (www.illumina.com). Adaptor-ligated fragments of 150–250 bp and 250–350 bp in size were recovered from a 2.5% NuSieve 1:1 agarose gel (ZymocleanTM Gel DNA Recovery Kit, ZR Cat#: D4001). The fragments were then bisulfite-treated using the EZ DNA Methylation-Lightning Kit (ZR, Cat#: D5020). Preparative-scale PCR was performed and the resulting products were purified (DNA Clean & Concentrator - ZR, Cat#D4005) for sequencing on an Illumina HiSeq.

Methyl-MiniSeq Sequence Alignments and Data Analysis. Sequence reads from bisulfite-treated EpiQuest libraries were identified using standard Illumina base-calling software and then analyzed using a Zymo Research proprietary analysis pipeline, which is written in Python and used Bismark (http://www.bioinformatics.babraham.ac.uk/projects/bismark/) to perform the alignment. Index files were constructed using the bismark_genome_preparation command and the entire reference genome. The --non_directional parameter was applied while running Bismark. All other parameters were set to default. Filled-in nucleotides were trimmed off when doing methylation calling. The methylation level of each sampled cytosine was estimated as the number of reads reporting a C, divided by the total number of reads reporting a C or T. Fisher's exact test or t-test was performed for each CpG site which has at least five reads coverage, and promoter, gene body and CpG island annotations were added for each CpG included in the comparison.

Prediction of Biological Pathways Altered by Prenatal Stress. An enrichment analysis was executed (Analyzed on November 9, 2017) using Ingenuity Pathway Analysis software (IPA; Redwood City, CA) to assess biological pathways altered in PNS compared to control bull calves. Methylation ratio was defined as the measured number of cytosines divided by the total number of cytosines covered at that site. Each methylation difference was calculated by subtracting the average control methylation ratio at a site from the average PNS methylation ratio at a site. Only differentially methylated regions *(*$P \leq 0.05$) located in the promoter and $\geq 10\%$ differentially methylated compared to controls were utilized in the enrichment analysis. For the

purpose of predicting alterations to biological pathways, hypermethylation in gene promoters was assumed to result in increased gene activity, while hypomethylation in gene promoters was assumed to result in decreased gene activity.

RESULTS AND DISCUSSION

Control of gene activity by DNA methylation is a primary regulator of cell specific functions (Razin and Riggs, 1980). Additionally, DNA methylation can be altered by external stimuli, which can result in alterations in gene expression and phenotype (Feinberg, 2010; Szyf, 2012). The epigenome is most sensitive to change during embryogenesis and perinatal development (Reik, 2007). Early life development is critical for economically relevant traits in beef cattle (Alford et al., 2007; Brickell et al., 2009). Therefore, this study sought to evaluate the influence of a prenatal transportation stressor on postnatal alterations to the epigenome in cattle and how those alterations might affect biological systems throughout life. DNA Methylation was assessed in white blood cells (**WBC**) of PNS and Control bull calves. Relatively low variation in DNA methylation has been reported between leukocyte types in humans, suggesting WBC to be an acceptable population of cells for DNA methylation analysis (Heiss and Brenner et al., 2017). Furthermore, prenatal and early life stressors have been reported to alter DNA methylation in various immune cells (Cao-Lei et al., 2014; Provençal et al., 2012). Additionally, previously reported alterations to behavior and stress response phenotypes in the larger population from which calves in this study were derived suggested potential alterations in neural function. Because white blood cells have been reported to be an

acceptable surrogate for brain tissue DNA methylation, white blood cells were identified as a suitable option. The following sections evaluate and discuss differential DNA methylation and potentially altered biological pathways in PNS compared with Control bull calves.

Genome-wide DNA Methylation in PNS and Control Bull Calves

CpG Site Coverage. Sequence depths of unique CpG sites were 6,392,121 (8 times) for samples from control and 6,358,081 (8 times) for samples from PNS bull calves on average. Samples from control and PNS calves had an average total read number of 38,534,348 and 34,894,063 read pairs, respectively. Both control and PNS samples had an average mapping efficiency of 33% and bisulfite conversion rate of 99%. Genome-wide coverage of DNA methylation across CpG sites using the same method, reduced representation bisulfite sequencing (Methyl-MiniSeq^{TM}), was slightly lower in this cattle study compared to rodent studies (Barua et al., 2014).

Table 3-1. Summary of genome-wide distribution of differential DNA methylation (HYPER=Hypermethylation, HYPO=Hypomethylation) across CpG, CHG, and CHH sites in prenatally stressed (PNS) compared with control bull calves[1, 2]

Genomic Region	Affected Regions (N)	Percent of Total Regions Affected	HYPER Regions (N)	Percent of Affected Regions with HYPER	HYPO Regions (N)	Percent of Affected Regions with HYPO	HYPER Regions within CpG Islands (N)	Percent of HYPER Regions within CpG Islands	HYPO Regions within CpG Islands (N)	Percent of HYPO Regions within CpG Islands
CpG Sites										
Promoter	1,205	7.5%	543	3.37%	662	4.10%	307	4.14%	364	4.17%
Intron	3,103	19.2%	1,386	8.59%	1,717	10.65%	303	4.09%	354	4.06%
Exon	1,260	7.8%	602	3.73%	658	4.08%	364	4.91%	383	4.39%
Intergenic	10,560	65.5%	4,876	30.23%	5,684	35.24%	1,025	13.84%	1,146	13.14%
Total	16,128	100.0%	7,407	45.93%	8,721	54.07%	1,999	26.99%	2,247	25.77%
CHG Sites										
Promoter	10	4.4%	3	1.33%	7	3.10%	1	0.93%	2	1.69%
Intron	55	24.3%	28	12.39%	27	11.95%	1	0.93%	2	1.69%
Exon	15	6.6%	9	3.98%	6	2.65%	3	2.78%	2	1.69%
Intergenic	146	64.6%	68	30.09%	78	34.51%	5	4.63%	7	5.93%
Total	226	100.0%	108	47.79%	118	52.21%	10	9.26%	13	11.02%
CHH Sites										
Promoter	14	3.6%	6	1.53%	8	2.05%	2	0.96%	1	0.55%
Intron	121	30.9%	65	16.62%	56	14.32%	4	1.91%	5	2.75%
Exon	12	3.1%	6	1.53%	6	1.53%	2	0.96%	1	0.55%
Intergenic	244	62.4%	132	33.76%	112	28.64%	10	4.78%	5	2.75%
Total	391	100.00%	209	53.45%	182	46.55%	18	8.61%	12	6.59%

[1]Affected regions were considered $P \leq 0.05$.
[2]In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.

CpG Sites. A summary of genome-wide distribution of differential DNA methylation in CpG sites across promoters, introns, exons, and intergenic regions in PNS compared with control bull calves is located in Table 3-1. Briefly, there were 16,128 CpG sites (52.76% of which were located within CpG islands) that were differentially methylated in PNS compared to control bull calves *(*$P \leq 0.05$). The majority of differentially methylated CpG sites were found in intergenic regions (65.5%), followed by introns (19.2%), exons (7.8%), and promoters (7.5%). Of those affected sites, 45.93% were hypermethylated and 54.07% were hypomethylated. DNA methylation primarily occurs within CpG contexts; however, there have been increasing reports of DNA methylation in CHG and CHH contexts, especially in embryonic and early life. Furthermore, increased reports of differential methylation have been reported in CHG and CHH contexts due to prenatal programming (Barua et al., 2014).

CHG Sites. A summary of genome-wide distribution of differential DNA methylation in CHG sites across promoters, introns, exons, and intergenic regions in PNS compared with control bull calves is located in Table 3-1. Briefly, there were 226 CHG sites (20.28% of which were located within CpG islands) that were differentially methylated in PNS compared to control bull calves *(*$P \leq 0.05$). The majority of differentially methylated CHG sites were found in intergenic regions (64.6%), followed by introns (24.3%), exons (6.6%), and promoters (4.4%). Of those affected sites, 47.79% were hypermethylated and 52.21% were hypomethylated.

CHH Sites. A summary of genome-wide distribution of differential DNA methylation in CHH sites across promoters, introns, exons, and intergenic regions in

PNS compared with control bull calves is located in Table 3-1. Briefly, there were 391 CHH sites (15.20% of which were located within CpG islands) that were differentially methylated in PNS compared to control bull calves *(*$P \leq 0.05$). The majority of differentially methylated CHH sites were found in intergenic regions (62.4%), followed by introns (30.9%), promoters (3.6%), and exons (3.1%). Of those affected sites, 53.45% were hypermethylated and 46.55% were hypomethylated.

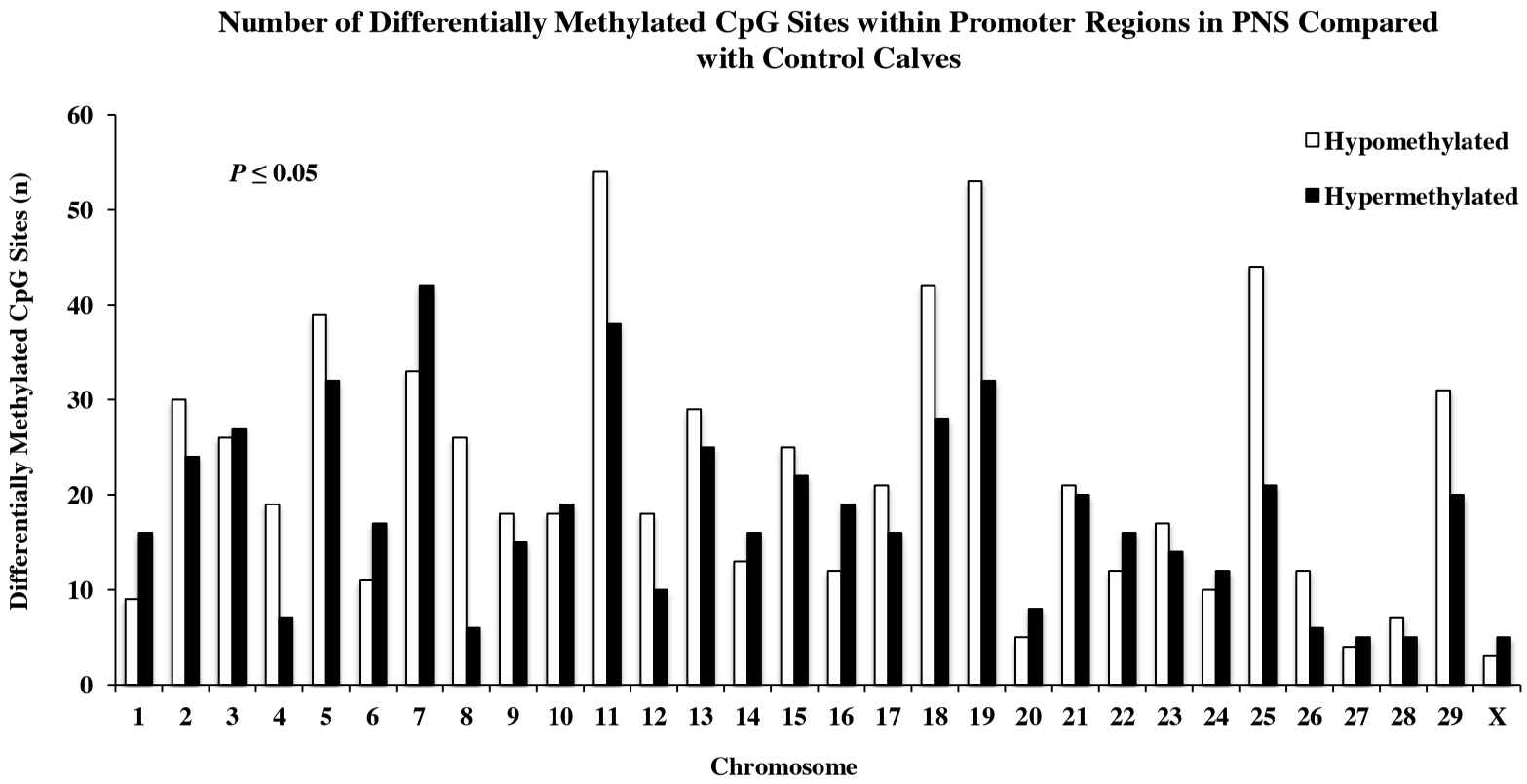

Figure 3-1. Comparison of hypermethylated (black bars) and hypomethylated (white bars) CpG sites within promoter regions in prenatally stressed (PNS) compared with Control bull calves.

Differential DNA methylation in CpG Sites

Promoter Regions. There were 1,205 differentially methylated CpG sites within promoter regions in PNS compared with Control bull calves (Table 3-1). A greater percentage of these CpG sites were hypomethylated (54.07%) compared to hypermethylated (45.93%; Table 3-1). Richetto et al. (2016) reported a similar occurrence in mice that were exposed to a prenatal viral challenge on gestational d 9 or 17, with 61% and 64% of differentially methylated regions being hypomethylated and 36% and 39% being hypermethylated, respectively. Differential methylation within promoter regions in PNS compared with control bulls was distributed throughout the genome, with all chromosomes having both hypomethylated and hypermethylated CpG sites (Figure 3-1). The number of hypermethylated compared with hypomethylated CpG sites within promoter regions in PNS compared with Control bulls is represented in Figure 3-1. Increased DNA methylation within gene promoter regions has been reported to cause suppressed transcription and gene expression (Levine et al., 1991; Tate and Bird, 1993). This suggests that hypermethylated gene promoter regions might have downregulated gene expression and hypomethylated gene promoter regions might have upregulated gene expression in PNS calves compared with controls.

Table 3-2. Strongly hypermethylated (methylation difference ≥ 0.33) CpG sites located within promoter regions in PNS compared with Control calves[1]

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	*P* value
chr7[†]	CCDC105	9178140	9178141	-	7.86	8.71	0.36	0.003097
chr11[†*]	MRPL41	105596122	105596123	-	8.57	7.00	0.35	0.003315
chr12[†]	PCDH17	5200175	5200176	-	11.14	7.29	0.34	0.0004446
chr21[*]	MIR655	67587446	67587447	+	8.57	9.43	0.45	0.008982
chr23	NQO2	50497771	50497772	-	6.71	5.86	0.39	0.002682
chr23[†]	NQO2	50497904	50497905	-	6.71	5.86	0.42	0.02622

[1]In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.
[†]DNA methylation was located within a CPG island.
[*]DNA methylation was exclusively located within the promoter region.

In agreement with this study's overall findings, there was a greater percentage of strongly hypomethylated (methylation difference ≤ -0.33) compared to strongly hypermethylated (methylation difference ≥ 0.33) CpG sites within promoter regions *(P* ≤ 0.05) in PNS bull calves compared with Controls. There were 6 strongly hypermethylated CpG sites located within promoter regions in PNS compared with Control calves (Table 3-2). Among these were 2 CpG sites, which were located within the promoter region of the NQO2 gene. This hypermethylated gene was also involved in the upregulation of the "NRF2-mediated Oxidative Stress Response" pathway (Table 3-3). Furthermore, a base pair deletion in the promoter region of this gene has been associated with schizophrenia in humans (Harada et al., 2003), suggesting the potential for physiological and behavioral alterations due to differences in neurotransmitter pathways.

Table 3-3. Top (most significant) 60 of 104 canonical pathways significantly altered in prenatally stressed compared with control bull calves[1]

Rank	Ingenuity Canonical Pathways	-log (p-value)	Ratio	Z-score	Differentially Methylated Genes ($P \leq 0.05$)
1	cAMP-mediated signaling	6.18	0.108	1	HTR6,CAMK1,GNAS,APLNR,HTR5A,PTGER3,OPRM1,CHRM4,CREB3L3,CREBBP,ADCY6,DRD5,CHRM1,ADCY9,LTB4R,VIPR1,DRD1,PDE1B,OPRK1,ADCY1,S1PR1,PDE11A,ADCY8,GNAL,PKIG
2	G-Protein Coupled Receptor Signaling	6.02	0.0986	NaN	HTR5A,PTGER3,CHRM4,CHRM1,FGFR3,LTB4R,DRD1,PRKCE,ADCY8,PDE11A,HTR6,APLNR,GNAS,OPRM1,CREBBP,CREB3L3,ADCY6,DRD5,ADRA1D,ADCY9,VIPR1,ADCY1,OPRK1,PDE1B,PIK3R6,S1PR1,GNAL,ADRA1A
3	Axonal Guidance Signaling	5.19	0.0786	NaN	FZD10,MMP7,NTN4,BMP2,LIMK2,NFATC1,SEMA4C,PRKCZ,NTN1,LIMK1,FGFR3,PLCD3,ITGA3,RHOD,ADAM19,SMO,PRKCE,ADAM23,ERBB2,ADAMTS5,ADAMTS4,EPHA7,GNAS,EPHA1,BMP8A,WNT2B,RAC1,TUBA4A,EPHA10,WNT10A,PIK3R6,BMP7,SEMA3C,WNT1,GNAL,NTN3
4	Human Embryonic Stem Cell Pluripotency	4.74	0.115	NaN	FZD10,NODAL,GNAS,BMP2,BMP8A,DVL1,WNT2B,BMPR2,SOX2,FGFR3,FGF4,WNT10A,S1PR1,PIK3R6,SMO,BMP7,WNT1
5	Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency	4.6	0.123	1	FZD10,T,BMP2,BMP8A,DVL1,WNT2B,CDX2,BMPR2,SOX2,FGFR3,WNT10A,PIK3R6,SMO,BMP7,WNT1
6	Gαs Signaling	4.33	0.123	1.069	HTR6,GNAS,HTR5A,CREB3L3,CREBBP,ADCY6,DRD5,CHRM1,ADCY9,VIPR1,DRD1,OPRK1,ADCY1,ADCY8
7	Dopamine-DARPP32 Feedback in cAMP Signaling	4.23	0.101	1.886	PPP1R14C,GNAS,PPP2R5D,CREBBP,CREB3L3,ADCY6,CSNK1D,DRD5,PRKCZ,ADCY9,PLCD3,CACNA1E,PRKG1,DRD1,ADCY1,KCNJ9,PRKCE,ADCY8
8	GABA Receptor Signaling	4.23	0.147	NaN	GABRQ,ADCY9,SLC6A11,GAD2,GNAS,GABRA4,ADCY1,ADCY6,ADCY8,AP2A2,SLC6A12
9	CDK5 Signaling	4.19	0.126	1.732	PPP1R14C,GNAS,EGR1,PPP2R5D,ADCY6,DRD5,MAPK9,ADCY9,ITGA3,DRD1,ADCY1,ADCY8,GNAL
10	Factors Promoting Cardiogenesis in Vertebrates	4.05	0.13	NaN	TBX5,NPPB,FZD10,NODAL,BMP2,BMP8A,DVL1,SMO,BMPR2,PRKCE,BMP7,PRKCZ
11	Endothelin-1 Signaling	3.92	0.0927	0.229	PLD2,GNAS,EDNRB,PLA2R1,ADCY6,MAPK9,SHE,PRKCZ,FGFR3,ADCY9,PLCD3,LCAT,PLA2G4B,CASQ1,ADCY1,PIK3R6,PRKCE,ADCY8,GNAL
12	GPCR-Mediated Integration of Enteroendocrine Signaling Exemplified by an L Cell	3.92	0.136	NaN	PLCD3,ADCY9,GNAS,VIPR1,ADCY1,NPY2R,ADCY6,SST,ADCY8,ADCYAP1,PYY

Table 3-3. Continued

Rank	Ingenuity Canonical Pathways	-log (p-value)	Ratio	Z-score	Differentially Methylated Genes ($P \leq 0.05$)
13	Gap Junction Signaling	3.84	0.0913	NaN	GNAS,GRIK3,GJB3,TUBA4A,ADCY6,CSNK1D,PRKCZ,FGFR3,ADCY9,PLCD3,PRKG1,DRD1,NPR1,ADCY1,PIK3R6,PRKCE,ADCY8,ACTA1,GRIK1
14	Sphingosine-1-phosphate Signaling	3.74	0.109	-1.941	RHOC,ADCY6,RAC1,SMPD1,FGFR3,ADCY9,PLCD3,ACER3,RHOD,CASQ1,ADCY1,S1PR1,PIK3R6,ADCY8
15	Molecular Mechanisms of Cancer	3.66	0.0721	NaN	FZD10,CDK3,BMP2,BMPR2,CDKN2B,PRKCZ,FGFR3,ITGA3,RHOD,SMO,PRKCE,ADCY8,GNAS,RHOC,BMP8A,WNT2B,CREBBP,DVL1,RAC1,ADCY6,MAPK9,ADCY9,WNT10A,ADCY1,PIK3R6,BMP7,CDKN1B,GNAL,WNT1
16	Phospholipase C Signaling	3.27	0.0803	0	PLD2,GNAS,RHOC,CREBBP,CREB3L3,RAC1,ADCY6,HDAC10,PRKCZ,NFATC1,ADCY9,PLCD3,LCK,ITGA3,HDAC3,RHOD,PLA2G4B,ADCY1,PRKCE,ADCY8
17	Colorectal Cancer Metastasis Signaling	3.18	0.0791	1.342	FZD10,MMP7,GNAS,PTGER3,RHOC,DVL1,WNT2B,RAC1,ADCY6,MAPK9,FGFR3,ADCY9,MMP23B,WNT10A,RHOD,ADCY1,PIK3R6,SMO,ADCY8,WNT1
18	Agrin Interactions at Neuromuscular Junction	3.13	0.129	0	ITGA3,DVL1,RAC1,MAPK9,ERBB3,DAG1,ERBB2,CTTN,ACTA1
19	Huntington's Disease Signaling	3.12	0.0781	-0.302	NEUROD1,PSMF1,CREBBP,CREB3L3,MAPK9,HDAC10,HIP1,VTI1B,AP2A2,GPAA1,PRKCZ,ATP5E,FGFR3,DNAJC5,HDAC3,CACNA1B,PENK,CASQ1,PIK3R6,PRKCE
20	Melanocyte Development and Pigmentation Signaling	3.11	0.11	2.53	FGFR3,ADCY9,GNAS,PAX3,CREBBP,CREB3L3,ADCY1,ADCY6,PIK3R6,POMC,ADCY8
21	GNRH Signaling	3.07	0.0933	1.069	MAP3K11,GNAS,EGR1,CREB3L3,CREBBP,ADCY6,RAC1,MAPK9,HBEGF,PRKCZ,ADCY9,ADCY1,PRKCE,ADCY8
22	eNOS Signaling	3.07	0.0865	1.291	GNAS,CHRM4,ADCY6,CHRM1,LPAR3,PRKCZ,FGFR3,BDKRB2,ADCY9,HSP90B1,PRKG1,ADCY1,PIK3R6,PRKCE,CHRNA7,ADCY8
23	Basal Cell Carcinoma Signaling	3	0.123	0.816	FZD10,WNT10A,BMP2,BMP8A,DVL1,WNT2B,SMO,BMP7,WNT1
24	CXCR4 Signaling	2.92	0.0867	-0.535	GNAS,RHOC,EGR1,ADCY6,RAC1,MAPK9,PRKCZ,FGFR3,ADCY9,RHOD,ADCY1,PIK3R6,PRKCE,ADCY8,GNAL
25	Tec Kinase Signaling	2.92	0.0867	-0.277	GNAS,RHOC,MAPK9,PRKCZ,FGFR3,YES1,ITGA3,LCK,TXK,RHOD,TNFSF12,PIK3R6,PRKCE,GNAL,ACTA1
26	Wnt/β-catenin Signaling	2.92	0.0867	-0.535	FZD10,MMP7,PPP2R5D,CREBBP,DVL1,WNT2B,CSNK1D,BMPR2,SOX2,WNT10A,CDH3,SMO,SOX14,SOX15,WNT1
27	Leptin Signaling in Obesity	2.91	0.111	2	FGFR3,PLCD3,ADCY9,LEP,ADCY1,ADCY6,PIK3R6,GHSR,POMC,ADCY8

Table 3-3. Continued

Rank	Ingenuity Canonical Pathways	-log (p-value)	Ratio	Z-score	Differentially Methylated Genes ($P \leq 0.05$)
28	PCP Pathway	2.9	0.131	1.414	FZD10,WNT10A,DVL1,WNT2B,RAC1,SMO,MAPK9,WNT1
Rank	Ingenuity Canonical Pathways	-log (p-value)	Ratio	Z-score	Differentially Methylated Genes ($P \leq 0.05$)
29	Breast Cancer Regulation by Stathmin1	2.88	0.0806	NaN	PPP1R14C,GNAS,CAMK1,PPP2R5D,RAC1,TUBA4A,ADCY6,LIMK2,PRKCZ,LIMK1,FGFR3,ADCY9,ADCY1,PIK3R6,PRKCE,CDKN1B,ADCY8
30	Corticotropin Releasing Hormone Signaling	2.88	0.0976	1.155	ADCY9,GNAS,NPR1,CREBBP,CREB3L3,ADCY1,SMO,ADCY6,PRKCE,POMC,ADCY8,PRKCZ
31	Gαi Signaling	2.88	0.0976	-1.508	ADCY9,LTB4R,GNAS,APLNR,PTGER3,OPRM1,CHRM4,OPRK1,ADCY1,S1PR1,ADCY6,ADCY8
32	Dopamine Receptor Signaling	2.88	0.11	2.121	ADCY9,TH,PPP1R14C,DRD1,PPP2R5D,COMT,ADCY1,ADCY6,DRD5,ADCY8
33	Sperm Motility	2.82	0.0915	0.832	MAP3K11,GNAS,PLA2R1,PRKCZ,NPPB,PLCD3,PRKG1,TXK,LCAT,NPR1,PLA2G4B,PDE1B,PRKCE
34	Leukocyte Extravasation Signaling	2.75	0.0783	0.577	MMP7,RAC1,JAM2,MAPK9,CLDN7,CLDN6,PRKCZ,FGFR3,ITGA3,MMP23B,TXK,CLDN8,PIK3R6,PRKCE,CTTN,ACTA1,ACTN1
35	Osteoarthritis Pathway	2.73	0.078	0.5	FZD10,LEP,H19,BMP2,CREBBP,CREB3L3,RAC1,BMPR2,NKX32,FGFR3,ITGA3,HDAC3,IL1RL2,CASQ1,SMO,ADAMTS5,ADAMTS4
36	Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	2.69	0.0753	NaN	FZD10,BMP2,BMP8A,DVL1,WNT2B,MAPK9,BMPR2,NFATC1,FGFR3,ITGA3,IL1RL2,WNT10A,PIK3R6,SMO,BMP7,ADAMTS5,WNT1,ADAMTS4
37	Renin-Angiotensin Signaling	2.65	0.0916	1.732	FGFR3,ADCY9,ADCY1,RAC1,REN,ADCY6,PIK3R6,MAPK9,PRKCE,ADCY8,SHE,PRKCZ
38	IL-15 Production	2.58	0.179	NaN	TXK,MAP3K11,IRF3,PRKCZ,IRF1
39	Transcriptional Regulatory Network in Embryonic Stem Cells	2.57	0.13	NaN	SOX2,GBX2,LHX5,CDX2,PAX6,OTX1,SIX3
40	Adipogenesis pathway	2.54	0.0889	NaN	FGFR3,FOXC2,FZD10,HDAC3,LEP,SREBF1,BMP2,NR2F2,SMO,HDAC10,BMPR2,BMP7
41	Eicosanoid Signaling	2.53	0.106	2	ALOX15,PTGES,LTB4R,LCAT,PTGER3,PLA2R1,PLA2G4B,ALOX12,PTGDS
42	CREB Signaling in Neurons	2.44	0.0773	2.138	GNAS,CREB3L3,CREBBP,GRIK3,ADCY6,PRKCZ,FGFR3,ADCY9,PLCD3,ADCY1,PIK3R6,PRKCE,ADCY8,GNAL,GRIK1
43	Germ Cell-Sertoli Cell Junction Signaling	2.42	0.0795	NaN	MAP3K11,RHOC,RAC1,TUBA4A,MAPK9,LIMK2,LIMK1,FGFR3,ITGA3,RHOD,KEAP1,PIK3R6,ACTN1,ACTA1
44	Role of Tissue Factor in Cancer	2.34	0.088	NaN	FGFR3,YES1,LCK,ITGA3,EGR1,RAC1,PIK3R6,HBEGF,LIMK2,LIMK1,FGF5

Table 3-3. Continued

Rank	Ingenuity Canonical Pathways	-log (p-value)	Ratio	Z-score	Differentially Methylated Genes ($P \leq 0.05$)
45	Cardiomyocyte Differentiation via BMP Receptors	2.33	0.2	NaN	NPPB,BMP2,BMPR2,BMP7
46	Neuregulin Signaling	2.27	0.0968	-1.134	HSP90B1,ITGA3,HBEGF,PRKCE,ERBB3,ERRFI1,ERBB2,CDKN1B,PRKCZ
47	GPCR-Mediated Nutrient Sensing in Enteroendocrine Cells	2.27	0.0968	NaN	PLCD3,ADCY9,GNAS,ADCY1,ADCY6,PRKCE,ADCY8,PYY,PRKCZ
48	Ephrin A Signaling	2.23	0.113	NaN	EPHA7,FGFR3,EPHA10,EPHA1,RAC1,PIK3R6,LIMK1
49	Cardiac Hypertrophy Signaling	2.21	0.0694	1.291	MAP3K11,GNAS,RHOC,CREBBP,ADCY6,MAPK9,ADRA1D,FGFR3,ADCY9,PLCD3,CACNA1E,RHOD,ADCY1,PIK3R6,ADCY8,ADRA1A,GNAL
50	Glioblastoma Multiforme Signaling	2.21	0.0778	0.277	FZD10,RHOC,WNT2B,RAC1,FGFR3,PLCD3,IGF2,WNT10A,RHOD,PIK3R6,SMO,CDKN1B,WNT1
51	Regulation of the Epithelial-Mesenchymal Transition Pathway	2.15	0.0741	NaN	FZD10,SNAI2,EGR1,FGF14,DVL1,WNT2B,FGFR3,FGF4,FOXC2,WNT10A,PIK3R6,SMO,WNT1,FGF5
52	Thrombin Signaling	2.13	0.0714	-1.069	GNAS,CAMK1,RHOC,ADCY6,GATA2,PRKCZ,FGFR3,ADCY9,PLCD3,RHOD,ADCY1,PIK3R6,PRKCE,ADCY8,GNAL
53	Wnt/Ca+ pathway	2.08	0.106	-1.134	PLCD3,FZD10,DVL1,CREBBP,CREB3L3,SMO,NFATC1
54	NRF2-mediated Oxidative Stress Response	2.08	0.0725	1.134	NQO2,CREBBP,HSPB8,MAPK9,GSTT1,PRKCZ,FGFR3,DNAJC5,ERP29,SCARB1,KEAP1,PIK3R6,PRKCE,ACTA1
55	Glycine Cleavage Complex	2.02	0.231	NaN	T,GCSH,AMT
56	GP6 Signaling Pathway	2	0.0791	0.905	LAMB2,COL1A2,FGFR3,COL4A1,COL4A3,RAC1,PIK3R6,PRKCE,PRKCZ,COL15A1,COL26A1
57	Serotonin Receptor Signaling	1.98	0.113	NaN	ADCY9,HTR6,HTR5A,ADCY1,ADCY6,ADCY8
58	Role of NFAT in Cardiac Hypertrophy	1.95	0.07	NaN	GNAS,CAMK1,ADCY6,MAPK9,HDAC10,PRKCZ,FGFR3,ADCY9,PLCD3,HDAC3,ADCY1,PIK3R6,PRKCE,ADCY8
59	TGF-β Signaling	1.94	0.092	NaN	NODAL,BMP2,CREBBP,MAPK9,BMPR2,BMP7,VDR,INHBB
60	IL-8 Signaling	1.93	0.0697	0.535	ANGPT2,PLD2,GNAS,RHOC,RAC1,MAPK9,HBEGF,LIMK2,PRKCZ,LIMK1,FGFR3,RHOD,PIK3R6,PRKCE

[1]Ingenuity Pathway Analysis (IPA) software (Analyzed on November 9, 2017) was utilized to determine top canonical pathways altered in PNS bull calves. The analysis was conducted using differentially methylated (≥ a 10% difference between treatment groups) CpG sites located within promoter regions ($P \leq 0.05$).

For a broader view, the most significant 30 of 543 significantly *(P* ≤ 0.05) hypermethylated CpG sites located within promoter regions in PNS compared with control calves are listed in Table 3-4. Among these was a CpG site within the promoter region of the CDX2 gene. The CDX2 gene is involved in early embryo pluripotency, differentiation, and development (Xie et al., 2013). Therefore, it is consistent that this gene was also involved in the upregulation of the pathway, "Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency" (Table 3-3). *In vitro* hyperosmolar stress resulted in reduced CDX2 and inhibited potency in the early embryo of the mouse (Xie et al., 2013). Furthermore, bovine embryos that were exposed to heat stress exhibited reduced CDX2 gene expression (Silva et al., 2013). These studies agree with the current study, which is predictive of downregulated expression of CDX2 due to increased DNA methylation in the promoter region of CDX2 in PNS bulls compared with Controls.

Table 3-4. Top (most significant) 30 hypermethylated CpG sites located within promoter regions in PNS compared with control calves[1]

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	*P* value
chr1[†]	CLSTN2	130108179	130108180	-	11.71	13.00	0.11	0.000009504
chr1	CLDN8	5036161	5036162	+	16.57	14.29	0.13	0.0003032
chr2[†]	TMEM200B	125008770	125008771	+	12.43	9.71	0.13	0.00028
chr3[†]	SHE	16143944	16143945	+	7.14	12.29	0.18	0.0009009
chr7[†]	HNRNPM	18289571	18289572	-	9.43	8.71	0.21	0.0002804
chr7[†]	HNRNPM	18289557	18289558	-	9.43	8.71	0.22	0.0006671
chr7	SIL1	52143957	52143958	+	13.00	10.71	0.1	0.001113
chr7[†]	HNRNPM	18289508	18289509	-	9.43	8.57	0.21	0.001569
chr7[†]	HNRNPM	18289493	18289494	-	9.29	8.71	0.22	0.001877
chr7[†]	HNRNPM	18289497	18289498	-	9.43	8.71	0.22	0.001877
chr7[†]	HNRNPM	18289505	18289506	-	9.43	8.71	0.22	0.001877
chr10[*]	EIF3J	103983752	103983753	+	9.43	8.86	0.13	6.067E-07
chr10[†*]	SIX6	72952940	72952941	-	13.00	10.14	0.19	0.0001318
chr10[†]	SHC4	61503850	61503851	+	8.86	9.14	0.17	0.001072
chr10[*]	EIF3J	103983746	103983747	+	9.29	8.86	0.14	0.001454
chr10	SHC4	61504208	61504209	-	9.86	7.29	0.15	0.001506
chr10[†]	LHFPL2	9527046	9527047	+	13.00	8.86	0.11	0.001618
chr11	BRE, RBKS	71827429	71827430	-	12.14	10.86	0.3	0.0007929
chr12[*]	CDX2	32316737	32316738	-	18.86	13.00	0.25	0.0002012
chr12[†]	PCDH17	5200175	5200176	-	11.14	7.29	0.34	0.0004446
chr13[†]	BMP7	59425545	59425546	-	8.00	8.00	0.11	0.001394
chr15	MMP7	6390211	6390212	+	8.57	6.29	0.19	0.000205
chr15[†]	CHRM4	77253713	77253714	+	10.71	6.14	0.13	0.000309
chr16[†*]	MMP23B	52263865	52263866	+	10.71	8.86	0.12	0.001214
chr16[†*]	SLC45A1	45908696	45908697	-	11.57	9.00	0.11	0.001281
chr19	ITGA3	37230331	37230332	+	12.29	11.00	0.12	0.0000896
chr19[*]	CORO6	21435132	21435133	+	21.00	18.00	0.17	0.001546
chr25[†]	METRN	613852	613853	-	8.71	6.00	0.12	0.001253
chr26	ZWINT	2851210	2851211	+	10.00	11.71	0.15	0.0001167
chr28[†]	TRIM67	3519622	3519623	+	18.14	19.14	0.11	0.001037

[1]In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.
[†]DNA methylation was located within a CPG island.
[*]DNA methylation was exclusively located within the promoter region.

There were 22 strongly hypomethylated CpG sites located within promoter regions in PNS compared with Control calves (Table 3-5). Among these was a CpG site within the promoter region of the GNAS gene. In cattle and other mammals, GNAS is an imprinted gene that has been reported to play a major role in development, growth, and metabolism (Sikora et al., 2011; Plagge et al., 2004). DNA methylation of a paternally expressed transcript of this gene, GNASXL, has been reported to be positively associated with prenatal maternal stress (as quantified by increased depression, anxiety, and cortisol) in humans (Vangeel et al., 2015). This is in contrast with the hypomethylated CpG site within the promoter region of the GNAS gene in PNS bull calves in this study.

Table 3-5. Strongly hypomethylated (methylation difference ≤ -0.33) CpG sites located within promoter regions in PNS compared with control calves[1]

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	*P* value
chr1*	EPCAM	157207666	157207667	-	7.4	8.9	-0.34	0.0495
chr1†*	GNAS	154101009	154101010	-	10.6	5.4	-0.35	0.0147
chr7*	TNFRSF10D	18053077	18053078	-	10.9	16.0	-0.38	0.01531
chr8	KHNYN, CBLN3	104517901	104517902	-	11.9	9.0	-0.35	0.03365
chr9	PLA2G4B	103674611	103674612	+	14.7	6.9	-0.44	0.04478
chr11	ATF6B	103182975	103182976	+	13.7	9.4	-0.33	0.002325
chr11†	SNX19	100835712	100835713	-	8.0	11.4	-0.35	0.006669
chr13†	LOXL4	58167618	58167619	-	12.7	12.4	-0.34	0.01073
chr13†	MGRN1	58167617	58167618	+	11.3	23.0	-0.33	0.004263
chr13†	YDJC	54817882	54817883	-	6.7	10.9	-0.39	0.007604
chr17†	YDJC	73172496	73172497	-	7.3	12.0	-0.45	0.009104
chr19	ATF6B	22423681	22423682	-	14.3	14.0	-0.38	0.01513
chr20*	CSNK1D	69935936	69935937	-	9.0	9.4	-0.39	0.01086
chr21†*	GNAS	30886861	30886862	-	11.3	9.6	-0.33	0.01602
chr23†	MGRN1	51619275	51619276	-	11.0	8.6	-0.33	0.004263
chr23†	SNX19	17940352	17940353	-	9.4	11.1	-0.35	0.02976
chr23†	TMOD1	51239299	51239300	+	11.6	9.0	-0.35	0.0389
chr23*	UBXN8	48211861	48211862	-	10.9	6.3	-0.37	0.001421
chr24†	MGRN1	3104521	3104522	-	10.9	7.0	-0.33	0.004263
chr25*	CARD9	1721752	1721753	-	9.7	9.7	-0.34	0.002587
chr25	MIR99B, MIRLET7E	4471948	4471949	+	8.4	6.3	-0.38	0.005474
chr25*	TNFRSF10D	2238087	2238088	+	12.3	9.1	-0.37	0.02807

[1]In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.
†DNA methylation was located within a CPG island.
*DNA methylation was exclusively located within the promoter region.

For a broader view, the most significant 30 of 662 significantly *(P* ≤ 0.05) hypomethylated CpG sites located within promoter regions in PNS compared with control calves are listed in Table 3-6. Among these was a CpG site within the promoter region of the DRD1 gene. The DRD1 gene encodes for dopamine receptor D1, and has been associated with behavioral disorders such as psychosis and schizophrenia (Andreou et al., 2016). The DRD1 gene was involved in the following pathways in this study: "cAMP-mediated signaling, G-Protein Coupled Receptor Signaling, Gαs Signaling, Dopamine-DARPP32 Feedback in cAMP Signaling, CDK5 Signaling, Gap Junction Signaling, and Dopamine Receptor Signaling" (Table 3-3). Rat pups that were separated from their mothers for 6-h periods each day during the first 2 weeks of life had downregulated DRD1 gene expression in the nucleus accumbens (Zhu et al., 2010). Rat pups that were separated from their mothers for 3-h periods had decreased mid-brain tyrosine hydroxylase-immunoreactive dopaminergic neurons as juveniles (15 d of age) but increased numbers as adolescents (35 d of age) and adults (70 d of age; Chocyk et al., 2011). These varying results suggest that alterations to the DRD1 gene due to prenatal or early life stress are specific to life stage.

Table 3-6. Top (most significant) 30 hypomethylated CpG sites located within promoter regions in PNS compared with control calves[1]

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	*P* value
chr3†	ECHDC2	94016695	94016696	+	9.14	12.29	-0.28	0.0006247
chr4†	OSBPL3	71296952	71296953	+	17.29	15.57	-0.13	0.001308
chr7	FDX1L	16072432	16072433	+	9.43	9.57	-0.18	0.0004641
chr7†	NMRK2	21294100	21294101	+	10.00	7.43	-0.16	0.000824
chr8	MIR2887-2, MIR2887-1, MIR2904-1, MIR2904-3, MIR2904-2	59158057	59158058	+	9.71	13.57	-0.23	0.0008405
chr8†*	XKR6	8255834	8255835	-	14.57	7.43	-0.11	0.001542
chr9*	SEC63	42650623	42650624	+	14.14	9.29	-0.17	0.0002254
chr9	OPRM1	92152196	92152197	+	8.14	9.43	-0.13	0.0002767
chr9	OPRM1	92152194	92152195	+	8.14	9.43	-0.2	0.001245
chr10*	DRD1	5653417	5653418	+	9.14	7.86	-0.19	0.0003017
chr10	LTB4R	20689223	20689224	-	8.43	9.86	-0.16	0.0007834
chr11†	SIX2	27263419	27263420	+	21.00	17.00	-0.11	0.0005395
chr11†*	SURF4	104335032	104335033	-	11.29	11.14	-0.14	0.001013
chr15†	APLNR	81738036	81738037	+	25.57	24.57	-0.23	0.0004384
chr18*	EGLN2	50330819	50330820	-	8.00	6.57	-0.15	0.0007258
chr18†*	NAT14	62474955	62474956	-	11.57	10.86	-0.25	0.001256
chr18	FUZ	56625931	56625932	-	10.57	8.43	-0.3	0.001281
chr19†	PMP22	33382476	33382477	-	11.43	12.57	-0.12	0.00007101
chr19	ERBB2	40722503	40722504	+	13.14	9.57	-0.1	0.0004212
chr19*	MPDU1	27924874	27924875	-	8.14	6.57	-0.15	0.001674
chr21†*	NKX2-8	47199004	47199005	+	12.86	14.86	-0.24	0.0003029
chr21*	CHRNA7	30181352	30181353	-	11.00	7.29	-0.29	0.0005365
chr21†	CSPG4	33574762	33574763	-	7.14	6.86	-0.17	0.00072
chr22†	ITGA9	10946832	10946833	-	17.00	17.43	-0.26	0.001275
chr25†	PGP	1746496	1746497	+	18.57	17.57	-0.25	0.001152
chr26	MIR2397	49743379	49743380	+	12.43	9.57	-0.14	0.00004049
chr26	PRLHR	39220962	39220963	+	11.43	9.29	-0.22	0.00008747
chr27*	UBXN8	26008284	26008285	-	10.00	8.57	-0.37	0.001421
chr29*	TALDO1	50864154	50864155	-	8.57	8.14	-0.27	0.0001148
chr29*	AP2A2	50445180	50445181	+	10.71	7.71	-0.2	0.0004282

[1]In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.
†DNA methylation was located within a CPG island.
*DNA methylation was exclusively located within the promoter region.

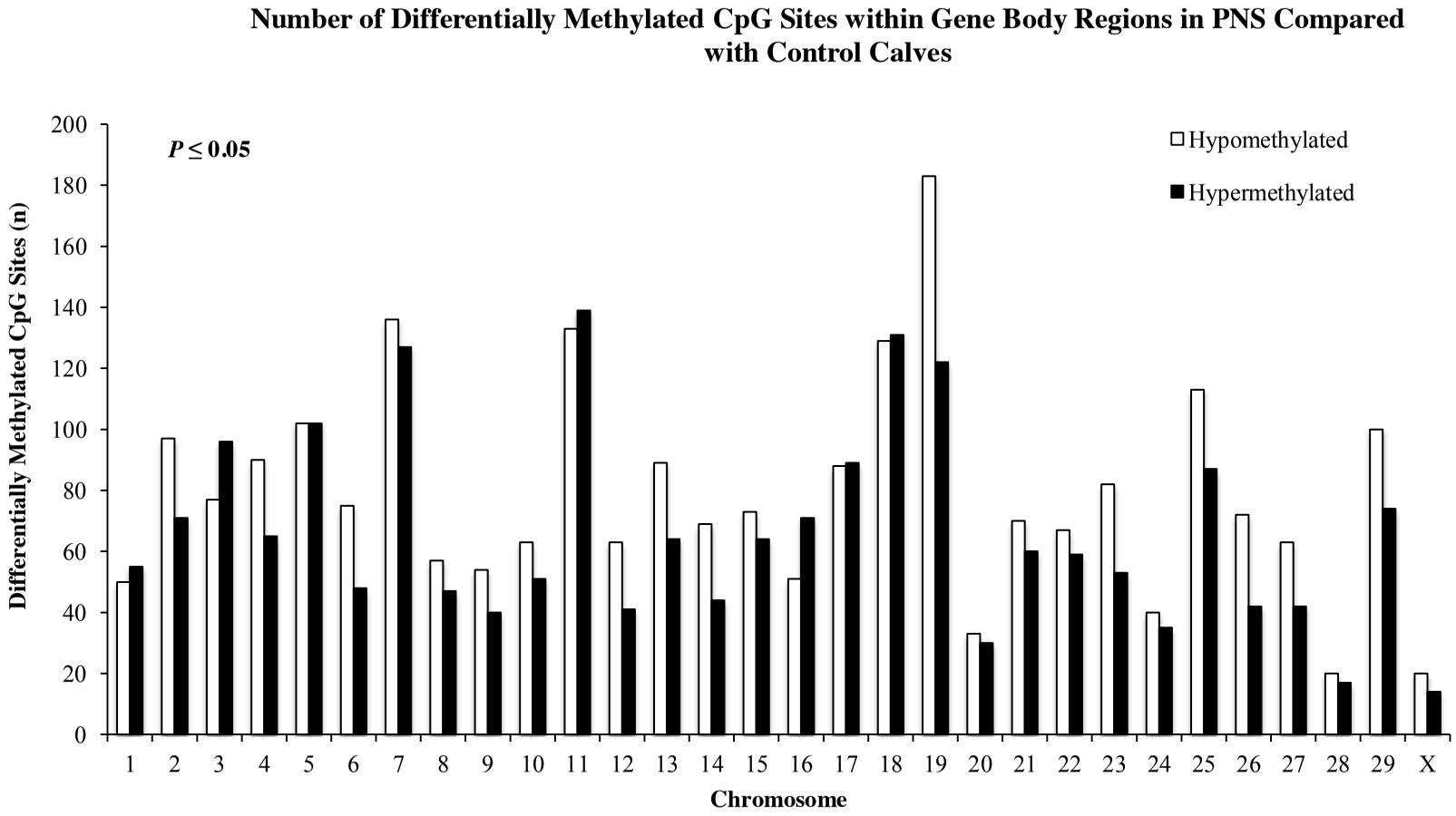

Figure 3-2. Comparison of hypermethylated (black bars) and hypomethylated (white bars) CpG sites within gene body regions in prenatally stressed (PNS) compared with Control bull calves.

Gene Body Regions. There were 4,363 differentially methylated CpG sites within gene body regions in PNS compared with Control bull calves (Table 3-1). A greater percentage of these CpG sites were hypomethylated compared to hypermethylated (Table 3-1). Differential methylation within gene body regions in PNS compared with control bulls was distributed throughout the genome, with all chromosomes having hypomethylated and hypermethylated CpG sites (Figure 3-2). The number of hypermethylated compared with hypomethylated CpG sites within gene body regions in PNS compared with Control bulls are represented in Figure 3-2. Increased DNA methylation in gene bodies may result in activated gene expression (Hellman and Chess, 2007). This suggests that hypermethylated gene body regions might have upregulated gene expression and hypomethylated gene body regions might have downregulated gene expression in PNS calves compared to Controls.

The most significant 30 of 1,988 significantly *(*$P \leq 0.05$) hypermethylated CpG sites located within gene body regions in PNS compared with control calves are listed in Table 3-7. Among these was a CpG site within the gene body region of the DIO3 gene, suggesting a potential upregulation in gene expression. The DIO3 gene is an imprinted gene in cattle, mice, and other species that encodes iodothyronine deiodinase 3, which inactivates thyroid hormones (Yang et al., 2017; Tsai et al., 2002). Thyroid hormones are involved in central nervous system development (Bernal, 2005) and behavior (Stohn et al., 2018). Male and female mice that were deficient in DIO3 exhibited increased aggression in response to an intruder (Stohn et al., 2018). Furthermore, rats whose dams consumed ethanol from day 8 to 21 of gestation had increased placental DIO3 mRNA

compared to controls (Shukla et al., 2011), suggesting the ability of the prenatal environment to shape nervous system development.

Table 3-7. Top (most significant) 30 hypermethylated CpG sites located within gene body regions in PNS compared with control calves[1]

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	*P* value
chr1§	CLDN8	5036161	5036162	+	16.57	14.29	0.13	0.0003032
chr1†‡	CLSTN2	130108179	130108180	-	11.71	13.00	0.11	0.000009504
chr1*‡	EPHB1	135353859	135353860	-	12.43	12.29	0.16	0.00003959
chr2†‡	TMEM200B	125008770	125008771	+	12.43	9.71	0.13	0.00028
chr3†*§	DMRTA2	96456243	96456244	-	19.29	15.29	0.15	0.0003634
chr3*‡	C3H1orf52	59027290	59027291	-	6.29	8.71	0.15	0.00001461
chr4*‡	NUDCD3	77653827	77653828	-	5.86	6.29	0.4	0.0004187
chr5*‡	ZNF384	104130929	104130930	+	7.71	8.14	0.15	0.0003626
chr7*§	F12	40258637	40258638	-	7.71	5.71	0.13	0.0004367
chr7†§	HNRNPM	18289571	18289572	-	9.43	8.71	0.21	0.0002804
chr7*‡	CRTC1	4435796	4435797	+	11.29	10.14	0.14	0.0004044
chr11*§	SLC8A1	22824023	22824024	+	9.57	8.86	0.29	0.0001272
chr11*‡	EHMT1	105461042	105461043	+	7.29	6.57	0.63	0.0003017
chr11*‡	RALGDS	103166859	103166860	-	7.14	6.29	0.55	0.00003935
chr11*‡	RALGDS	103166862	103166863	-	7.14	6.29	0.48	0.0001696
chr11*‡	RALGDS	103166855	103166856	-	7.14	6.29	0.55	0.0003
chr12†*‡	CARS2	89257092	89257093	-	11.71	9.00	0.2	0.0001168
chr12*‡	DOCK9	79833906	79833907	+	10.00	6.00	0.2	0.0002803
chr12†*‡	GRTP1	90628533	90628534	-	6.57	6.43	0.11	0.0004244
chr15†§	CHRM4	77253713	77253714	+	10.71	6.14	0.13	0.000309
chr15§	MMP7	6390211	6390212	+	8.57	6.29	0.19	0.000205
chr15†*‡	PHOX2A	52625075	52625076	-	6.29	6.14	0.17	0.0002289
chr19‡	ITGA3	37230331	37230332	+	12.29	11.00	0.12	0.0000896
chr21†*§	DIO3	68152290	68152291	+	14.86	14.14	0.18	0.0001115
chr22*‡	IL17RD	44515500	44515501	-	18.57	18.14	0.27	0.00007418
chr23*‡	MDGA1	11586464	11586465	-	21.14	15.43	0.17	0.0004023
chr23*‡	PHACTR1	43380907	43380908	+	10.14	9.29	0.11	0.0002705
chr26‡	ZWINT	2851210	2851211	+	10.00	11.71	0.15	0.0001167
chr28*§	C28H10orf35	26104046	26104047	+	14.00	8.57	0.15	0.0003049
chr29†*§	NRXN2	43477994	43477995	+	10.57	6.71	0.28	0.0001266

[1]In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.

†DNA methylation was located within a CpG island.

*DNA methylation was exclusively contained in the gene body region of the gene.

‡ DNA methylation was located within an intron region.

§ DNA methylation was located within an exon region.

The most significant 30 of 2,375 significantly ($P \leq 0.05$) hypomethylated CpG sites located within gene body regions in PNS compared with control calves are listed in Table 3-8. Among these was a CpG site within the gene body region of the ADCYAP1 gene, suggesting a potential downregulation in gene expression. The ADCYAP1 gene encodes for the PACAP peptide and is associated with stress disorders and posttraumatic stress disorder (Ressler et al., 2011). Mice that were deficient in PACAP exhibited behavioral abnormalities such as hyperactivity, jumping, and depression (Ishihama et al., 2010). Furthermore, mice whose dams were exposed to a posttraumatic stress disorder model (restrained for 2-h period and then forced to swim for a 20-min period with six other rats) had downregulated ADCYAP1 gene expression (Zang et al., 2016). These studies support potential influence of prenatal stress on the ADCYAP1 gene in this study and suggest altered behavioral effects due to prenatal stress.

Table 3-8. Top (most significant) 30 hypomethylated CpG sites located within gene body regions in PNS compared with control calves[1]

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	*P* value
chr2*§	CRYBA2	107655452	107655453	+	17.86	12.57	-0.28	0.0001131
chr2†*§	TMEM200B	125010636	125010637	+	9.00	7.14	-0.12	6.12E-08
chr2*‡	DPP10	68059420	68059421	+	13.43	10.43	-0.29	0.00007136
chr3*§	LRRC42	92815815	92815816	-	15.57	11.71	-0.12	0.0003827
chr3*‡	GRIK3	109641283	109641284	-	13.86	9.43	-0.15	0.00006294
chr5*§	FMNL3	30363597	30363598	+	10.14	13.00	-0.16	0.00001178
chr6†*‡	NSG1	106743364	106743365	-	11.14	8.71	-0.18	0.0001679
chr7‡	FDX1L	16072432	16072433	+	9.43	9.57	-0.18	0.0004641
chr9§	OPRM1	92152196	92152197	+	8.14	9.43	-0.13	0.0002767
chr11†*‡	GBGT1	103182975	103182976	+	9.43	9.43	-0.6	0.0001948
chr11†*‡	NR5A1	95535022	95535023	-	12.57	11.57	-0.12	0.00006977
chr13*‡	CUBN	31602752	31602753	-	24.86	31.14	-0.1	0.0002177
chr13*‡	TGIF2	66340155	66340156	+	8.86	10.14	-0.11	0.0003646
chr14†*‡	AGO2	4114895	4114896	-	7.00	6.14	-0.43	0.000509
chr14*‡	CPQ	69484191	69484192	+	9.86	9.71	-0.1	0.000005467
chr15†§	APLNR	81738036	81738037	+	25.57	24.57	-0.23	0.0004384
chr16*‡	ACOT7	47839343	47839344	+	6.43	7.00	-0.11	0.0001594
chr16*‡	RGS7	36591986	36591987	-	23.86	20.29	-0.13	0.000143
chr17*‡	SELM	72065070	72065071	-	16.14	18.14	-0.27	0.0003458
chr18*‡	CBLC	52956154	52956155	+	8.86	7.86	-0.2	0.0002327
chr19*§	ABR	22423681	22423682	-	13.71	14.00	-0.64	0.00003286
chr19‡	ERBB2	40722503	40722504	+	13.14	9.57	-0.1	0.0004212
chr19†‡	PMP22	33382476	33382477	-	11.43	12.57	-0.12	0.00007101
chr22*‡	ALDH1L1	61235727	61235728	+	17.00	12.57	-0.15	0.0000172
chr22*‡	LTF	53523469	53523470	-	11.14	8.14	-0.24	0.0003724
chr24†*‡	ADCYAP1	36118731	36118732	+	13.29	10.43	-0.16	0.0004943
chr26§	PRLHR	39220962	39220963	+	11.43	9.29	-0.22	0.00008747
chr26‡	GLRX3	49743379	49743380	+	12.43	9.57	-0.14	0.00004049
chr27*‡	MTUS1	18657599	18657600	-	10.57	11.71	-0.21	0.00008578
chr29†*‡	MOB2	50986147	50986148	-	13.86	11.29	-0.26	0.0003578

[1]In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.
†DNA methylation was located within a CpG island.
*DNA methylation was exclusively contained in the gene body region of the gene.
‡ DNA methylation was located within an intron region.
§ DNA methylation was located within an exon region.

Differential DNA methylation in CHG Sites

Promoter Regions. There were 10 differentially methylated CHG sites within promoter regions in PNS compared with Control bull calves (Table 3-1). A greater percentage of these CHG sites were hypomethylated compared to hypermethylated (Table 3-1).

The 3 significantly ($P \leq 0.05$) hypermethylated CHG sites located within promoter regions in PNS compared with Control calves are listed in Table 3-9. Among these was a CHG site within the promoter region of the CRYBB3 gene. The CRYBB3 gene is a member of the crystallin gene family, dysregulations of which have been associated with neural disorders, such as schizophrenia and Alzheimer's disease (Middleton et al., 2002; Shinohara et al., 1993). Crystallin family heat shock proteins likely have a neuroprotective function (Masilamoni et al., 2006; Ousman et al., 2007). Specifically, CRYBB3 was upregulated in 3 models of mice whose dams underwent immune activation during gestation (dams were administered human influenza virus, poly (I:C), or recombinant IL-6 during gestation; Garbett et al., 2012). Previous studies suggest differential methylation of the CRYBB3 gene might result in alterations in neural and behavioral characteristics of PNS calves.

Table 3-9. Hypermethylated CHG sites located within promoter regions in PNS compared with control calves[1]

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	*P* value
chr1	COLQ	154285093	154285094	+	9.92	7.71	0.27	0.02224
chr12[†]	FAM155A	87425041	87425042	+	8.00	5.71	0.16	0.000134
chr17[*]	CRYBB3	67543284	67543285	+	12.43	12.86	0.17	0.02543

[1]In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.
[†]DNA methylation was located within a CPG island.
[*]DNA methylation was exclusively located within the promoter region.

The 7 significant ($P \leq 0.05$) hypomethylated CHG sites located within promoter regions in PNS compared with control calves are listed in Table 3-10. Among these was a CHG site within the promoter region of the F2RL3 gene. The F2RL3 gene is hypomethylated in adult smokers and has been associated with mortality risk due to cardiovascular diseases (Breitling et al., 2012). In conjunction with altered CpG and CHH contexts within promoter regions, hypermethylated and hypomethylated CHG sites suggest various potential influences of prenatal stress on postnatal biology, including neural function.

Table 3-10. Hypomethylated CHG sites located within promoter regions in PNS compared with control calves[1]

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	*P* value
chr4[†]	AKR1B1	99033535	99033536	+	5.50	9.00	-0.1	0.00876
chr7[*]	F2RL3	6100112	6100113	-	9.13	13.14	-0.13	0.04596
chr17	HIC2	74182640	74182641	+	7.15	8.00	-0.11	0.01282
chr18	CYP2S1	50656104	50656105	+	9.63	7.43	-0.12	0.04467
chr21	FURIN	22213152	22213153	-	13.28	14.71	-0.11	0.04053
chr22	QARS	51484825	51484826	-	9.30	8.86	-0.13	0.01024
chr25[†*]	SBDS	28636530	28636531	+	9.39	10.00	-0.11	0.01421

[1]In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.
[†]DNA methylation was located within a CPG island.
[*]DNA methylation was exclusively located within the promoter region

Gene Body Regions. There were 70 differentially methylated CHG sites within gene body regions in PNS compared with Control bull calves (Table 3-1). A slightly greater percentage of these CHG sites were hypermethylated compared to hypomethylated (Table 3-1).

The most significant 30 of 37 significantly ($P \leq 0.05$) hypermethylated CHG sites located within gene body regions in PNS compared with control calves are listed in Table 3-11. Among these was a CHG site within the gene body region of the PRKCA gene. The PRKCA gene encodes for Protein Kinase C Alpha, which is a member of the serine- and threonine-specific protein kinases and is involved in various cell-signaling processes. The PRKCA gene has been associated with PTSD and memory in humans (De Quervain et al., 2012). Furthermore, rats whose dams were stressed by placing them on an elevated platform made of Plexiglass® twice each day for a 10-min period between 12 and 16 d of gestation resulted in genome-wide alterations in gene expression, including expression of PRKCA (Mychasiuk et al., 2011). Due to its influence on cell-signaling pathways, differential methylation of PRKCA has the potential to influence many biological processes in PNS calves.

Table 3-11. Top (most significant) 30 hypermethylated CHG sites located within gene body regions in PNS compared with control calves[1]

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	*P* value
chr1[‡]	COLQ	154285093	154285094	+	11.29	7.71	0.27	0.02224
chr2[*‡]	CAPZB	133916622	133916623	+	10.57	8.57	0.1	0.03793
chr4[*§]	ADCY1	76872462	76872463	-	9.43	6.43	0.11	0.01105
chr4[*‡]	LRGUK	98721906	98721907	+	29.29	29.43	0.16	0.007548
chr4[*‡]	LRGUK	98750277	98750278	+	11.57	14.57	0.17	0.03429
chr4[*‡]	SSPO	113439057	113439058	+	8.29	5.00	0.11	0.04102
chr5[*‡]	CNTN1	40172096	40172097	-	5.43	5.57	0.11	0.01133
chr5[*‡]	BTBD11	71077326	71077327	+	11.14	13.86	0.13	0.01252
chr5[*‡]	BTBD11	71077307	71077308	+	12.71	15.29	0.12	0.01786
chr5[*‡]	BTBD11	71077336	71077337	+	9.71	11.86	0.14	0.0197
chr5[*‡]	BTBD11	71077331	71077332	+	10.57	13.43	0.14	0.0197
chr5[*‡]	GTSE1	117731307	117731308	+	14.14	13.71	0.12	0.02663
chr10[*‡]	SLC8A3	82134282	82134283	+	8.14	6.57	0.11	0.008943
chr11[*‡]	ASS1	100828396	100828397	-	8.14	9.14	0.37	0.01106
chr11[†*§]	GBGT1	103182822	103182823	+	7.14	6.71	0.45	0.003044
chr12[†§]	FAM155A	87425041	87425042	+	9.14	5.71	0.16	0.000134
chr13[*‡]	PLCB1	1664698	1664699	+	9.14	7.71	0.39	0.02851
chr14[*‡]	TRAPPC9	4363947	4363948	+	9.86	7.57	0.26	0.03318
chr15[*‡]	GDPD5	55422374	55422375	+	9.29	7.00	0.2	0.002641
chr16[*‡]	RGS7	36590606	36590607	+	16.86	22.71	0.11	0.03004
chr16[*‡]	RGS7	36590615	36590616	+	10.43	11.86	0.1	0.03718
chr18[*§]	MYADM	62023403	62023404	-	11.00	6.14	0.18	0.04267
chr19[*‡]	PRKCA	63490629	63490630	+	9.57	10.71	0.1	0.02086
chr19[*‡]	TRPV2	33822975	33822976	-	8.57	5.86	0.12	0.01706
chr22[*§]	RNF123	51074315	51074316	-	9.14	6.00	0.15	0.01264
chr22[*§]	RNF123	51074297	51074298	-	9.14	6.00	0.12	0.03985
chr23[*‡]	EFHC1	24617468	24617469	+	9.71	7.71	0.11	0.01582
chr23[*‡]	GMDS	51389933	51389934	+	10.14	12.29	0.14	0.0292
chr25[*§]	INTS1	41995171	41995172	+	8.86	6.00	0.12	0.01351
chr27[†*‡]	CSGALNACT1	37970135	37970136	+	10.86	8.29	0.42	0.01612

[1]In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.
[†]DNA methylation was located within a CpG island.
[*]DNA methylation was exclusively contained in the gene body region of the gene.
[‡]DNA methylation was located within an intron region.
[§]DNA methylation was located within an exon region.

The most significant 30 of 33 significantly ($P \leq 0.05$) hypomethylated CHG sites located within gene body regions in PNS compared with control calves are listed in Table 3-12. Among these was a CHG site within the gene body region of the DGAT1 (diacylglycerol acyltransferase 1) gene, which is a protein enzyme involved in the conversion of diacylglycerol and fatty acyl CoA to triacylglycerol. This gene is known to be involved in metabolic diseases. Mice whose dams underwent daily restraint stress for a 3-h period from 8 d of gestation to birth had increased accumulation of lipids in the liver and increased DGAT1 gene expression (Maeyama et al., 2015). Differential methylation of DGAT1 suggests an influence of prenatal stress on postnatal metabolic processes. In conjunction with altered CpG and CHH contexts within gene body regions, hypermethylated and hypomethylated CHG sites suggest various potential influences of prenatal stress on postnatal biology, including neural and metabolic function.

Table 3-12. Top (most significant) 30 hypomethylated CHG sites located within gene body regions in PNS compared with control calves[1]

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	*P* value
chr1[*‡]	OXNAD1	155073206	155073207	-	13.71	14.29	-0.34	0.01431
chr3[*‡]	TRAF3IP1	118294302	118294303	-	7.57	6.57	-0.35	0.02739
chr4[†§]	AKR1B1	99033535	99033536	+	6.29	9.00	-0.1	0.00876
chr6[*§]	PDGFRA	71409734	71409735	+	12.57	8.29	-0.1	0.00002454
chr7[*‡]	NMRK2	21292863	21292864	-	9.14	7.71	-0.35	0.001535
chr11[*§]	IFITM5	107203536	107203537	+	10.00	8.57	-0.17	0.03473
chr11[*‡]	FAM102A	98633234	98633235	+	12.29	9.57	-0.22	0.01901
chr11[*‡]	GTF3C5	103098035	103098036	-	8.43	7.29	-0.11	0.03774
chr11[*‡]	POMT1	101661140	101661141	+	8.71	7.71	-0.45	0.005054
chr13[*‡]	CTNNBL1	67382273	67382274	+	11.43	9.14	-0.11	0.01673
chr14[*‡]	DGAT1	1799370	1799371	+	5.86	6.57	-0.11	0.01688
chr14[*‡]	DPYS	62412347	62412348	+	10.57	11.43	-0.12	0.01354
chr16[†*§]	CAMK1G	75614071	75614072	+	7.86	7.43	-0.39	0.006132
chr17[*‡]	ISCU, LOC533308	66689455	66689456	+	7.57	8.14	-0.11	0.01066
chr17[‡]	PRODH	74182640	74182641	+	8.14	8.00	-0.11	0.01282
chr18[*§]	GAN	8018228	8018229	+	7.43	8.14	-0.1	0.008568
chr18[†*‡]	DDX19A	1774349	1774350	+	9.29	7.14	-0.35	0.03018
chr18[*‡]	VAT1L	5066203	5066204	-	9.29	7.43	-0.27	0.03996
chr19[*‡]	BAIAP2	52216891	52216892	-	6.14	6.00	-0.1	0.01496
chr19[*‡]	EXOC7	56206159	56206160	+	7.29	5.71	-0.11	0.02192
chr19[*‡]	SAMD14	37166191	37166192	+	6.43	5.86	-0.1	0.01496
chr21[‡]	FURIN	22213152	22213153	-	15.14	14.71	-0.11	0.04053
chr21[*‡]	LOC524810	71546924	71546925	-	33.14	18.57	-0.12	0.02952
chr21[*‡]	OTUD7A	30644587	30644588	+	12.71	10.00	-0.43	0.02269
chr22[*‡]	PTPRG	39397678	39397679	-	10.00	9.00	-0.31	0.0106
chr22[‡]	QARS	51484825	51484826	-	10.57	8.86	-0.13	0.01024
chr27[†*‡]	MTMR7	18983825	18983826	-	7.29	9.86	-0.3	0.03781
chr27[*‡]	RNF122	28663566	28663567	-	15.86	18.00	-0.24	0.007464
chr29[*§]	TSSC4	49837555	49837556	+	11.14	10.14	-0.15	0.003493
chr29[*‡]	FAT3	2565099	2565100	+	16.29	11.86	-0.41	0.01684

[1]In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.

[†]DNA methylation was located within a CpG island.

*DNA methylation was exclusively contained in the gene body region of the gene.

‡ DNA methylation was located within an intron region.

§ DNA methylation was located within an exon region.

Differential DNA methylation in CHH Sites

Promoter Regions. There were 14 differentially methylated CHH sites within promoter regions in PNS compared with Control bull calves (Table 3-1). A greater percentage of these CHH sites were hypomethylated compared to hypermethylated (Table 3-1).

The 6 significantly ($P \leq 0.05$) hypermethylated CHH sites located within promoter regions in PNS compared with Control calves are listed in Table 3-13. Among these was a CHH site within the promoter region of the IER2 gene. The IER2 gene encodes the Immediate Early Response 2 gene and is involved in neural development. Prenatal exposure to arsenic has been associated with altered IER2 gene expression, with upregulation of IER2 gene expression potentially serving as a biomarker of prenatal arsenic exposure (Fry et al., 2007).

Table 3-13. Hypermethylated CHH sites located within promoter regions in PNS compared with control calves[1]

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	*P* value
chr1[*]	C1H21orf91	18719042	18719043	-	7.00	7.43	0.13	0.04541
chr1[*]	C1H21orf91	18719029	18719030	-	7.00	7.43	0.1	0.01395
chr4[†*]	ZYX	107597956	107597957	-	5.13	6.57	0.19	0.01628
chr5	LOC511240	72050111	72050112	+	11.00	9.86	0.22	0.03802
chr5[*]	POLR3B	70062294	70062295	-	15.50	14.14	0.11	0.03267
chr7[†*]	IER2	13545518	13545519	-	7.50	6.57	0.1	0.02712

[1]In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.
[†]DNA methylation was located within a CPG island.
[*]DNA methylation was exclusively located within the promoter region.

The 8 significantly ($P \leq 0.05$) hypomethylated CHH sites located within promoter regions in PNS compared with control calves are listed in Table 3-14. Among these was a CHH site within the promoter region of the IFITM1/IFITM2 genes. Patients with schizophrenia had increased IFITM1 and IFITM2 gene expression, which was likely indicative of an early environmental insult (Arion et al., 2007; Hwang et al., 2013). In conjunction with altered CpG and CHG contexts within promoter regions, hypermethylated and hypomethylated CHH sites suggest various potential influences of prenatal stress on postnatal biology, including neural and behavioral function.

Table 3-14. Hypomethylated CHH sites located within promoter regions in PNS compared with control calves[1]

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	*P* value
chr4	CPA5	94878884	94878885	+	8.54	11.29	-0.14	0.03917
chr5[†*]	SRGAP1	50119329	50119330	-	9.28	11.29	-0.12	0.0108
chr11	IFITM2, IFITM1	107192312	107192313	+	12.05	10.00	-0.22	0.006774
chr11	IFITM2, IFITM1	107192386	107192387	+	12.93	11.00	-0.19	0.01444
chr13[*]	ZSWIM1	75359296	75359297	-	7.73	8.57	-0.2	0.003527
chr15[*]	MAML2	14155574	14155575	+	6.77	7.14	-0.15	0.01866
chr15	C15H11orf74	67844693	67844694	+	10.75	13.43	-0.1	0.0426
chr18	POP4	40371128	40371129	-	10.53	14.29	-0.11	0.007739

[1]In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.
[†]DNA methylation was located within a CPG island.
[*]DNA methylation was exclusively located within the promoter region.

Gene Body Regions. There were 133 differentially methylated CHH sites within gene body regions in PNS compared with Control bull calves (Table 3-1). A slightly greater percentage of these CHH sites were hypermethylated compared to hypomethylated (Table 3-1).

The most significant 30 of 71 significantly ($P \leq 0.05$) hypermethylated CHH sites located within gene body regions in PNS compared with control calves are listed in Table 3-15. Among these was a CHH site within the gene body region of the PPARD gene. The PPARD gene plays a key role in glucose and lipid metabolism (Brunmair et al., 2006). Male and female rats whose dams were administered dexamethasone between 13 d of gestation and birth exhibited hyperinsulinemia, altered glucose and fatty acid metabolism, and females (only) had increased PPARD gene expression in skeletal muscle (Wyrwoll et al., 2008). Holstein cows fed a moderate-energy (1.47 Mcal/kg) diet compared to controls (1.24 Mcal/kg) during late gestation had lower PPARD expression compared to controls after parturition (Osorio et al., 2013).

Table 3-15. Top (most significant) 30 hypermethylated CHH sites located within gene body regions in PNS compared with control calves[1]

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	*P* value
chr1*‡	COLQ	154250800	154250801	-	14.00	10.71	0.31	0.02325
chr1*‡	SH3BP5	154100975	154100976	-	6.43	5.43	0.55	0.006741
chr3*‡	ATG16L1	113620590	113620591	-	12.71	12.00	0.14	0.001883
chr5*‡	BTBD11	71077321	71077322	+	12.00	14.14	0.14	0.006485
chr5*‡	BTBD11	71076725	71076726	-	26.00	17.14	0.11	0.009977
chr5*‡	BTBD11	71077305	71077306	+	12.71	15.29	0.14	0.01081
chr5*‡	BTBD11	71077300	71077301	+	11.57	13.57	0.13	0.01306
chr5*‡	BTBD11	71077320	71077321	+	13.00	15.29	0.11	0.01355
chr5*‡	BTBD11	71077302	71077303	+	12.29	15.00	0.12	0.01688
chr5*‡	BTBD11	71077322	71077323	+	12.00	14.29	0.13	0.01713
chr5*‡	BTBD11	71077304	71077305	+	12.86	15.14	0.12	0.01713
chr5*‡	BTBD11	71077313	71077314	+	12.86	15.14	0.12	0.01786
chr6*‡	PDE5A	7031186	7031187	-	13.57	9.71	0.25	0.00821
chr8*‡	MOB3B	16811946	16811947	+	9.57	11.14	0.35	0.01953
chr13*‡	PKIG	73718134	73718135	+	6.57	7.57	0.13	0.002584
chr13*‡	PKIG	73718143	73718144	+	6.57	7.57	0.13	0.003265
chr13*‡	PKIG	73718119	73718120	+	6.43	7.43	0.11	0.01428
chr19*§	SEZ6	20882874	20882875	+	11.00	6.86	0.41	0.02207
chr19*‡	NXN	22530169	22530170	-	10.29	9.86	0.39	0.005695
chr20*‡	DAP	62637623	62637624	+	15.71	12.14	0.14	0.02114
chr20*‡	SKIV2L2	23806050	23806051	-	14.00	15.00	0.14	0.00286
chr21*‡	FAN1	27938912	27938913	-	10.71	9.29	0.29	0.01662
chr23*‡	PHACTR1	43337982	43337983	-	10.86	11.00	0.23	0.02088
chr23*‡	PPARD	9321494	9321495	+	7.86	8.57	0.11	0.01898
chr23*‡	PRIM2	2729219	2729220	-	5.86	9.71	0.15	0.02263
chr25*§	INTS1	41995205	41995206	+	8.86	6.00	0.12	0.01351
chr25†*‡	LFNG	41308254	41308255	-	13.29	12.00	0.17	0.01471
chr26*‡	RBM20	31655067	31655068	-	11.71	8.43	0.12	0.008981
chr27*‡	RNF122	28624366	28624367	+	29.00	14.86	0.11	0.01688
chr29*‡	RCOR2	42870766	42870767	+	11.00	9.57	0.1	0.01591

[1]In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.

†DNA methylation was located within a CpG island.

*DNA methylation was exclusively contained in the gene body region of the gene.

‡ DNA methylation was located within an intron region.

§ DNA methylation was located within an exon region.

The most significant 30 of 62 significantly ($P \leq 0.05$) hypomethylated CHH sites located within gene body regions in PNS compared with control calves are listed in Table 3-16. Among these was a CHH site within the gene body region of the DPYSL2 (dihydropyrimidinase-like 2) gene. The DPYSL2 gene is a collapsin response mediator protein that is involved in neurotransmission and synapse function. Rats whose dams underwent gestational stressors (restraint stress, food deprivation, forced swimming, reversed light-dark cycles, and overcrowding stress during dark cycles) between 14 d of gestation and birth exhibited decreased DPYSL2 expression and potentially increased susceptibility to schizophrenic characteristics (Lee et al., 2015). Furthermore, rats whose dams were stressed by placing them on an elevated platform made of Plexiglass® twice each day for a 10-min period between 12 and 16 d of gestation resulted in genome-wide alterations in gene expression, including expression of DPYSL2 (Mychasiuk et al., 2011). In conjunction with altered CpG and CHG contexts within gene body regions, hypermethylated and hypomethylated CHH sites suggest various potential influences of prenatal stress on postnatal biology, including metabolic and neural function.

Table 3-16. Top (most significant) 30 hypomethylated CHH sites located within gene body regions in PNS compared with control calves[1]

Chrom	Gene	Start	End	Strand	Average Total CPG (Control)	Average Total CPG (PNS)	Methyl Diff	*P* value
chr1*‡	TBC1D5	156103383	156103384	+	12.71	11.43	-0.27	0.02232
chr2*‡	UBR4	134135665	134135666	+	9.29	7.86	-0.37	0.02694
chr3*‡	BARHL2	52695313	52695314	-	15.43	12.57	-0.15	0.01694
chr7*‡	MAP2K2	21141408	21141409	-	23.00	16.86	-0.29	0.02658
chr7*‡	PLVAP	5687715	5687716	-	12.43	8.00	-0.12	0.0146
chr7*‡	SLC12A2	27042120	27042121	+	9.00	10.57	-0.11	0.005449
chr8*‡	DPYSL2	75136546	75136547	+	7.14	8.43	-0.32	0.03065
chr8*‡	GSN	112604634	112604635	+	7.86	5.86	-0.11	0.02649
chr8*‡	TNFRSF10D	71053599	71053600	+	72.00	44.71	-0.21	0.03105
chr10*‡	LRRC16B	20910389	20910390	+	14.00	11.71	-0.12	0.01559
chr11*‡	FUBP3	100947908	100947909	+	11.43	13.71	-0.56	0.001667
chr11‡	IFITM2, IFITM1	107192312	107192313	+	13.71	10.00	-0.22	0.006774
chr11‡	IFITM2, IFITM1	107192386	107192387	+	14.71	11.00	-0.19	0.01444
chr11*‡	NACC2	103605839	103605840	-	20.71	12.29	-0.49	0.006619
chr11*‡	NEK6	95337824	95337825	+	12.86	9.57	-0.13	0.01493
chr12*‡	FARP1	79248800	79248801	-	10.29	11.43	-0.28	0.02768
chr13*‡	BCAS1	82210449	82210450	+	7.00	7.29	-0.24	0.03362
chr14*‡	ASAP1	11452197	11452198	+	12.86	9.71	-0.13	0.02803
chr14*‡	DPYS	62412342	62412343	+	10.43	11.43	-0.12	0.01296
chr14*‡	DPYS	62412355	62412356	+	10.43	11.29	-0.12	0.01296
chr14*‡	DPYS	62412345	62412346	+	10.57	11.43	-0.12	0.01354
chr15*‡	LOC509058	76786259	76786260	-	10.86	8.57	-0.43	0.01323
chr18‡§	POP4	40371128	40371129	-	12.00	14.29	-0.11	0.007739
chr18*‡	MTHFSD	12404197	12404198	-	10.14	9.29	-0.12	0.03374
chr18*‡	PEPD	44033725	44033726	-	6.86	7.29	-0.5	0.0007633
chr19*‡	RAB37	57344842	57344843	-	17.43	13.71	-0.11	0.01538
chr21*‡	AKAP6	43500188	43500189	+	14.86	12.00	-0.24	0.02095
chr21*‡	LOC524810	71577837	71577838	-	19.86	16.29	-0.17	0.02587
chr21*‡	PSTPIP1	32652323	32652324	-	6.86	6.86	-0.24	0.01042
chr27*‡	KCNU1	31979794	31979795	+	8.71	8.57	-0.27	0.03064

[1]In limited cases, multiple genes were represented within one recorded region. In such cases, the record was considered one site.
†DNA methylation was located within a CpG island.
*DNA methylation was exclusively contained in the gene body region of the gene.
‡ DNA methylation was located within an intron region.
§ DNA methylation was located within an exon region.

Biological Pathways Altered in PNS Compared with Control Bull Calves

There were 104 canonical pathways significantly altered in PNS compared with Control bull calves. The top 60 canonical pathways and the differentially methylated genes in each pathway are represented in Table 3-3. Among the pathways altered in PNS bull calves were pathways related with behavior, stress response, immune function, metabolism, reproduction, cell signaling.

Pathways Related to Behavior, Stress Response, and Neural Function. Many genes and multiple canonical pathways related to behavior, stress response, and neural function were significantly altered in PNS compared with Control bull calves. Several of these pathways involved the hypothalamic-pituitary-adrenal (HPA) axis and neurotransmitter signaling processes such as dopamine, GABA, and serotonin signaling. The "Corticotropin Releasing Hormone Signaling" pathway was activated in PNS bull calves (Table 3-3). Other studies have reported differences in methylation of genes involved in HPA axis regulation, especially at the level of the glucocorticoid receptor gene, NR3C1 (Perroud et al., 2014). Although this study did not show differences in NR3C1 methylation, it did show differences at other levels of the HPA axis, including POMC methylation. "Dopamine-DARPP32 Feedback in cAMP Signaling and Dopamine Receptor Signaling" pathways were activated in PNS bull calves (Table 3-3). Previous reports suggest an influence of prenatal stress on dopamine regulation, especially at the level of COMT (Thompson et al., 2012) and dopamine receptors (Berger et al., 2002). The COMT gene and two dopamine receptor gene subtypes (DRD1 and DRD5) were differentially methylated in PNS bull calves. The "GABA Receptor Signaling" pathway

was altered in PNS bull calves (Table 3-3). Other studies have reported differences genes involved in GABA regulation, development of GABAergic cells, and associated anxious behavior (Berger et al., 2002, Lussier and Stevens, 2016). The "Serotonin Receptor Signaling" pathway, with an emphasis on serotonin receptor subtypes, was altered in PNS bull calves (Table 3-3). Prenatal stress has been associated with differences in serotonin receptor binding, serotonin synthesis, and associated behavioral alterations (Van den Hove et al., 2006; Peters, 1986). Richetto et al. (2016) reported "Neuronal Differentiation" to be the most enriched gene ontology term in mice that were exposed to a prenatal viral challenge on gestational d 9 or 17, significant subterms of which included gamma-aminobutyric acidergic differentiation, central nervous system differentiation, noradrenergic system differentiation, and dopamine differentiation. Alterations to neurotransmitter pathways, such as dopamine, GABA, and serotonin have been associated with psychiatric disorders such as depression, anxiety, psychosis, and schizophrenia (Markham and Koenig, 2011). Furthermore, alterations in behavior, stress response, and neural function agree with increased HPA axis activity and more excitable temperaments observed in calves in the population from which bull calves in this study were derived (Littlejohn et al., 2016). Previous studies are consistent with alterations in pathways related to behavior, stress response, and neural function in PNS bull calves.

Pathways Related to Immune Function. Many genes and multiple canonical pathways related to immune function were significantly altered in PNS compared with Control bull calves. This might have been expected considering DNA methylation was assessed in immune cells. Several of these pathways included, "Leukocyte Extravasation

Signaling, IL-15 Production, and IL-8 Signaling" (Table 3-3). Prenatal stress has been shown to result in alterations to immune function as shown by hematology and cytokine alterations in swine, primates, and rodents (Couret et al., 2009; Reyes and Coe, 1997; Vanbesien-Mailliot et al., 2007). Richetto et al. (2016) reported "Leukocyte Differentiation" to be significantly altered in mice that were prenatally exposed to a viral challenge on gestational d 9 or 17. Cao-Lei et al. (2014) reported that children whose mothers were in the 1998 ice storm in Quebec during gestation had altered genome-wide DNA methylation in T cells at 13 years of age. Six of the top 10 pathways assessed by IPA that were altered in those prenatally stressed children were related to immune function. Specifically, each of those pathways was directly involved in T lymphocyte function (Cao-Lei et al., 2014). Alterations in methylation of immune function related genes and canonical pathways in PNS bull calves were related to differences in immune response to an endotoxin challenge observed in the population of calves from which bull calves in this study were derived (Burdick Sanchez et al., 2013). These alterations included several differences in leukocyte and cytokine parameters, which was in concordance with the altered pathways in this study. Previous studies as well as methylomic and phenotypic differences related to immune function in prenatally stressed bull calves suggest a potential influence of prenatal stress on overall health and immune response in bovine.

Pathways Related to Metabolism. Multiple genes and canonical pathways related to metabolic function were significantly altered in PNS compared with Control bull calves. Several of these pathways included, "Leptin Signaling in Obesity,

Adipogenesis pathway, and Glycine Cleavage Complex" (Table 3-3). Cao-Lei et al. (2014) reported the influence of the prenatal Quebec ice storm stressor on the "Type 1 Diabetes Mellitus Signaling" pathway, as predicted by differential DNA methylation of T cells at 13 years of age (assessed by IPA). Furthermore, male mice whose dams were exposed to repeated exposure to an aggressive lactating female during late gestation had increased circulating triglyceride concentrations, decreased hepatic 5α-reductase and Pparα mRNA expression, and decreased subcutaneous fat PEPCK mRNA expression (Brunton et al., 2013). Alterations to metabolism related pathways have implications for metabolic diseases, such as diabetes. However, alterations to metabolic processes in cattle might translate to differences in growth, gain, or feed efficiency, which could result in profitability differences in prenatally stressed cattle.

Pathways Related to Reproduction. Multiple genes and canonical pathways related to reproduction were significantly altered in PNS compared with Control bull calves. Several of these pathways included, "GnRH Signaling, Sperm Motility, and Germ Cell-Sertoli Cell Junction Signaling" (Table 3-3). The GnRH signaling pathway has been reported to be altered in zebrafish that were exposed to dexamethasone during the larval stages of development, in which GnRH and Kisspeptin genes were differentially methylated and expressed (Khor et al., 2016). Prenatal environment is known to program postnatal reproductive characteristics in males (Zambrano et al., 2014). For example, prenatal betamethasone exposure in male rats resulted in decreased sperm production and quality (Piffer et al., 2009). Alterations in reproductive endocrinology, specifically GNRH signaling, agree with phenotypic differences

observed in the population from which bull calves in this study were derived (Littlejohn et al., 2017). It is clear that differences exist in reproductive endocrinology and gamete development due to prenatal stress. However, more research is required to better understand the implications of these alterations on reproduction.

Pathways Related to Cell Signaling. Many genes and canonical pathways related to cell signaling were significantly altered in PNS compared with Control bull calves. Some of these altered general cell signaling pathways included, "cAMP-mediated signaling, G-Protein Coupled Receptor Signaling, Gαs Signaling, Phospholipase C Signaling, Tec Kinase Signaling, Gαi Signaling, and TGF-β Signaling" (Table 3-3). Consistently, Cao-Lei et al. (2014) also reported an influence of the prenatal Quebec ice storm stressor on the "Phospholipase C Signaling" pathway, as predicted by differential DNA methylation of T cells at 13 years of age (assessed by IPA). Furthermore, Massart et al. (2016) reported that rhesus monkeys undergoing early life maternal separation stress and a lack of maternal-rearing had altered genome-wide DNA methylation in CD3+ T cells from d 14 to 2 y of age. From that study, five of the top 6 canonical pathways (assessed by IPA) altered in prenatally stressed monkeys were also significantly differentially methylated in prenatally stressed bull calves in this study. Two of those mutually altered pathways were related to cell signaling: "G-protein coupled receptor signaling and cAMP-mediated cell signaling." Alterations to cell signaling pathways in previous studies and in PNS bull calves, suggests a potential influence of prenatal stress on many biological processes through cell signaling processes employed across many cell types.

Other Pathways. While multiple other canonical pathways were altered in PNS bulls, two areas seemed to stand out both in this and previous studies: cancer and cell pluripotency.

Cao-Lei et al. (2014) reported the influence of the prenatal Quebec ice storm stressor on the "Molecular Mechanisms of Cancer" pathway, as predicted by differential DNA methylation of T cells at 13 years of age (assessed by IPA). The "Molecular Mechanisms of Cancer" pathway was also significantly altered in PNS bull calves. The following cancer pathways were additionally altered (several of which were activated; Table 3-3) in PNS calves: "Colorectal Cancer Metastasis Signaling, Basal Cell Carcinoma Signaling, Breast Cancer Regulation by Stathmin1, Role of Tissue Factor in Cancer, and Glioblastoma Multiforme Signaling." Furthermore, prenatal stress associated with maternal bereavement has been associated with an increased risk of specific types of cancer in children (Li et al., 2012). Alterations to cancer-related pathways might suggest potential long-term health concerns associated with cow longevity in beef cattle herds.

Massart et al. (2016) reported that rhesus monkeys undergoing early life maternal separation stress and a lack of maternal-rearing shared two mutually altered pathways related to pluripotency of cells with PNS bulls: "Human Embryonic Stem Cell Pluripotency and Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency." Because DNA methylation is a primary regulator of cell specific functions (Razin and Riggs, 1980), predicted alterations to pluripotency of cells is logical and suggests developmental programming of various cell types.

Genome-wide Overview of Differentially Methylated Regions

Overall, genome-wide distribution of differential DNA methylation (hypermethylation and hypomethylation) in prenatally stressed (PNS) compared with Control bull calves were similar to previous reports in humans and nonhuman primates (Cao-Lei et al., 2014; Massart et al., 2016). Heat maps in Figure 3-3 and 3-4 compare the most significant 100 methylation ratios (specific to each individual animal) that were hypermethylated and hypomethylated, respectively, in prenatally stressed (PNS) compared with Control bull calves. Hierarchical cluster analysis was performed with regard to prenatal treatment. The dendrograms above and to the left of the heat maps represent this clustering.

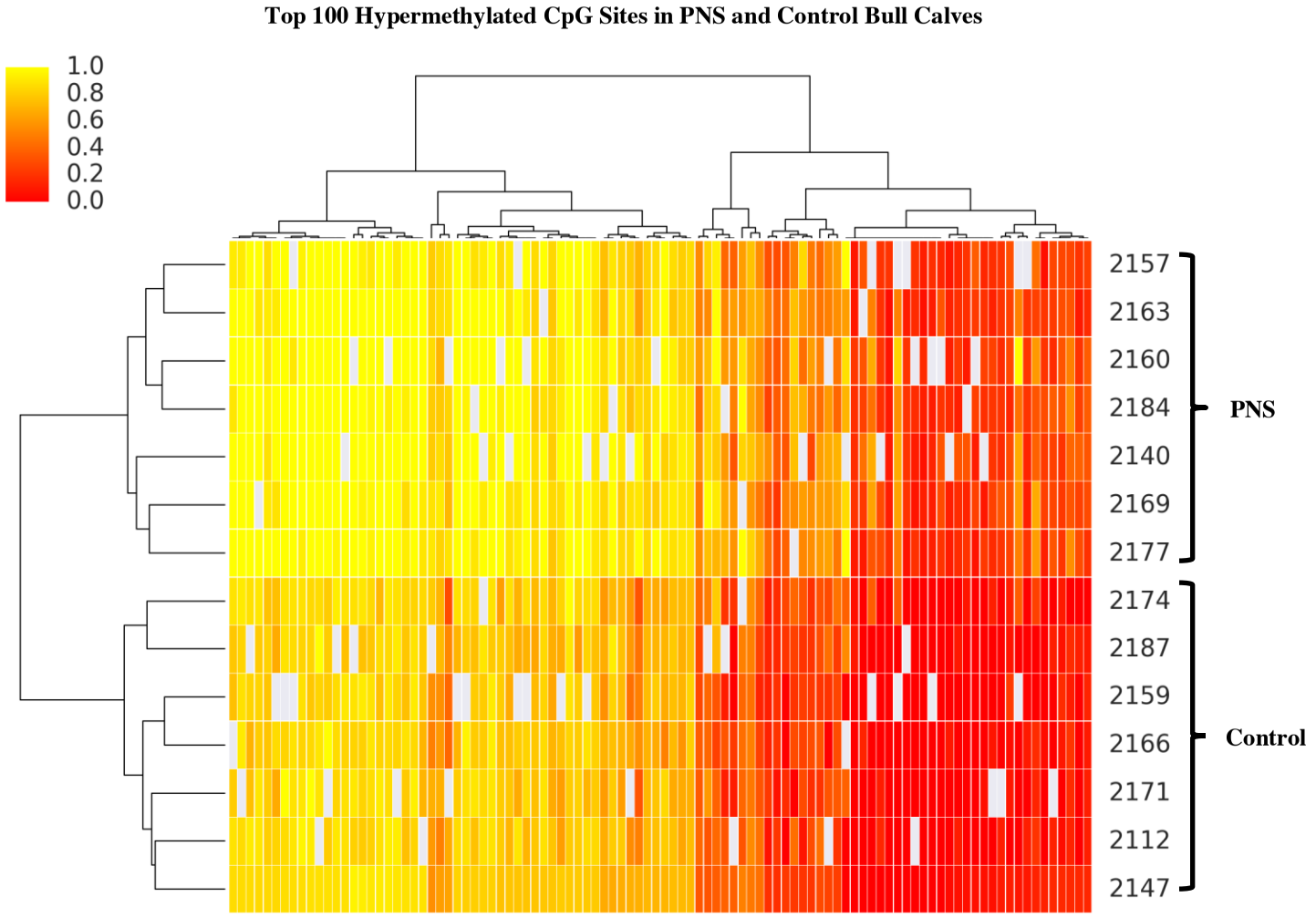

Figure 3-3. Comparison of the top 100 methylation ratios that were hypermethylated in prenatally stressed (PNS) compared with Control bull calves.

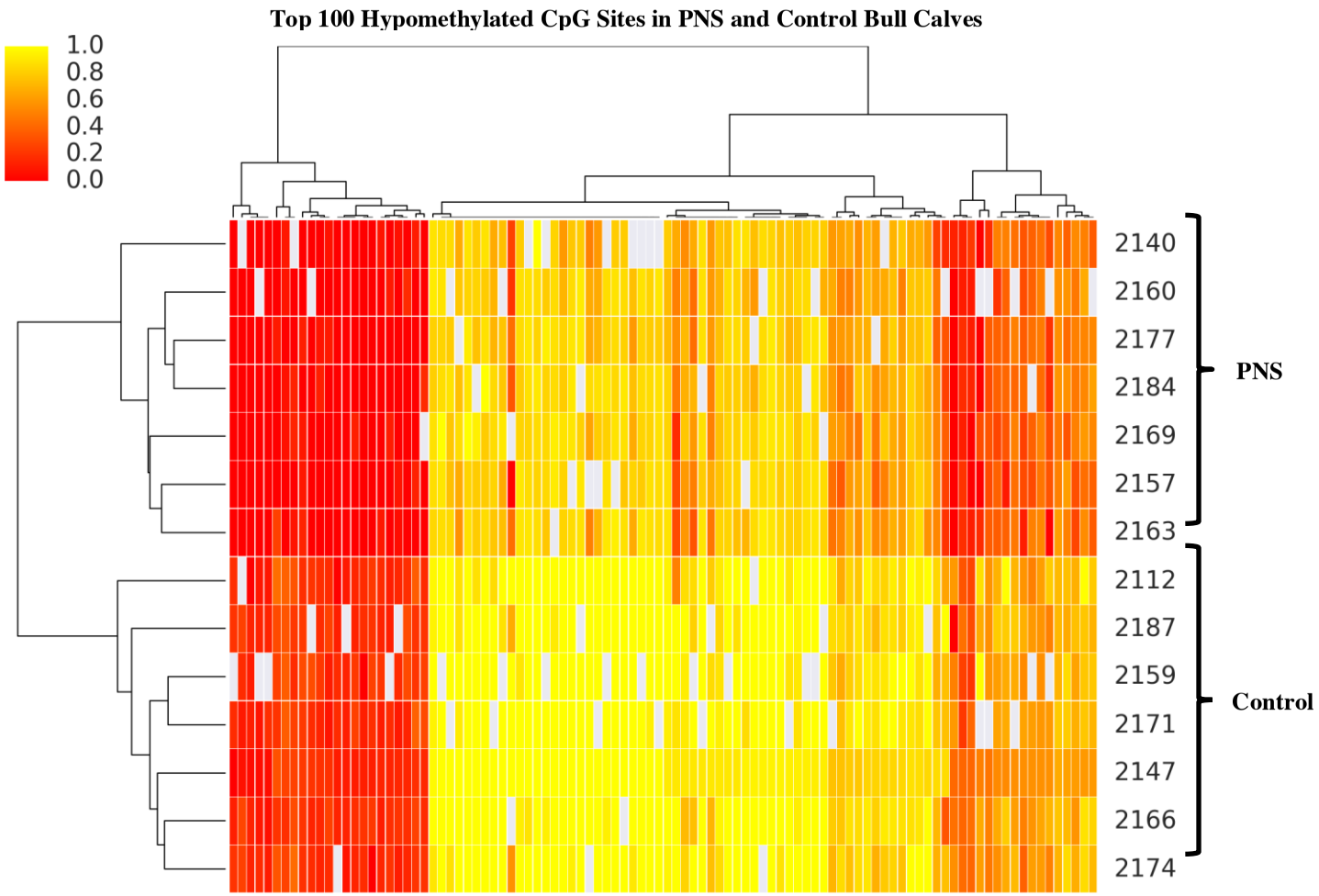

Figure 3-4. Comparison of the top 100 methylation ratios that were hypomethylated in prenatally stressed (PNS) compared with Control bull calves.

Genome-wide chromosome distribution of differentially methylated CpG sites with regard to significance, $-\log_{10}$(p-value), is represented in the form of a Manhattan plot in Figure 3-5. Fourteen CpG sites surpassed the $-\log_{10}$(p-value) threshold of 5. Of these sites, 1 was located exclusively within a promoter region (EIF3J), 1 was located within a promoter and gene body region (CLSTN2), 2 were located exclusively within a gene body region (TMEM200B and CPQ), and 10 were located within an intergenic region. These data reveal a uniform distribution of significant differentially methylated CpG sites in PNS calves, suggesting an influence of prenatal environment on gene function.

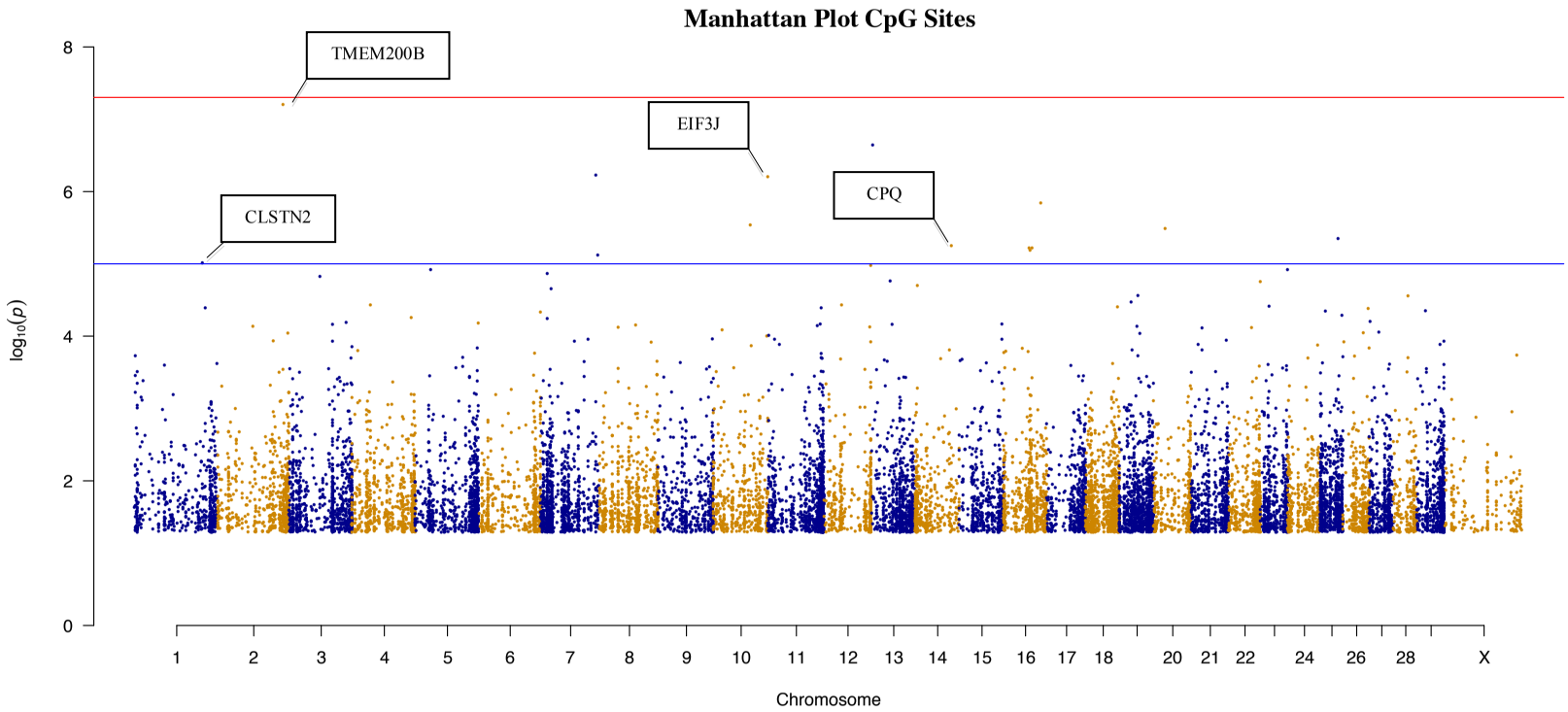

Figure 3-5. Manhattan plot of $-\log_{10}$(p-values) for all differentially methylated CpG sites across the genome (all region types).

Genome-wide chromosomal distributions of DNA methylation ratios are represented in Figure 3-6, which consisted of CpG sites within all region types with a minimum sequence read depth of 5 times. Although there was a greater percentage of significantly hypomethylated than hypermethylated CpG sites in PNS compared with control bull calves (Table 3-1), the genome-wide illustration of all analyzed CpG sites in PNS and Control calves suggests the opposite relationship (Figure 3-6). Overall, these data show uniform genome-wide distribution of differential methylation across each chromosome in PNS compared with Control bull calves.

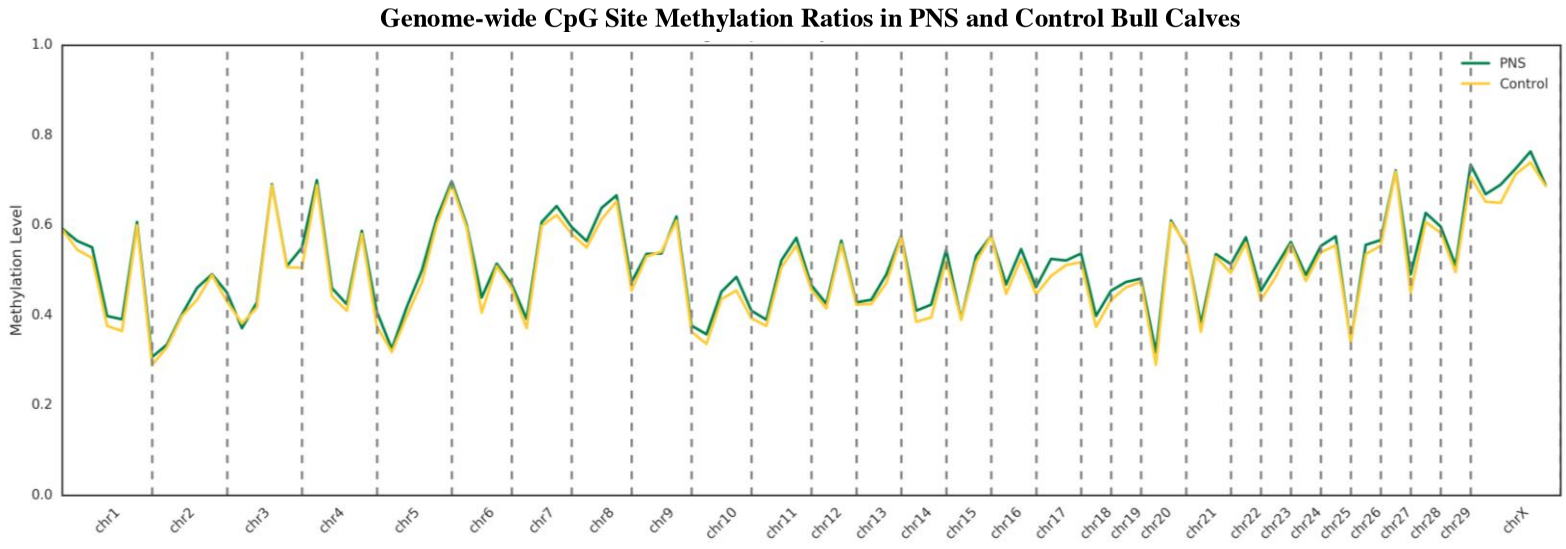

Figure 3-6. Comparison of genome-wide PNS (green line) and Control (yellow line) CpG site methylation ratios (all region types).

CONCLUSIONS

To our knowledge, these data are the first reports of a genome-wide assessment of DNA methylation in the prenatally stressed bovine. Overall, these data seem to show consistency and similarities with data from models of prenatal stress in humans and nonhuman primates (Cao-Lei et al., 2014; Massart et al., 2016). Prenatal transportation stress in cattle altered genome-wide DNA methylation profiles, which were predicted by IPA to impact pathways related to immune function, behavior, stress response, neural function, metabolism, reproduction, cell signaling, and other biological processes. Alterations in behavior, stress response, immune function, and reproductive endocrinology are related with phenotypic differences observed in the population of calves from which bull calves in this study were derived (Littlejohn et al., 2016; Burdick Sanchez et al., 2013; Littlejohn et al., 2017). Future studies should assess associations of differential DNA methylation with altered phenotype of prenatally stressed calves.

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CHAPTER IV

USE OF RANDOM REGRESSION TO ESTIMATE GENETIC PARAMETERS OF TEMPERAMENT ACROSS AN AGE CONTINUUM IN A CROSSBRED CATTLE POPULATION[1]

INTRODUCTION

Repeated record acquisition permits partitioning of phenotype in ways that may be beneficial for estimation and refinement of genetic merit. When regression coefficients are modeled as random effects, they impose a covariance structure. Random regression analyses facilitate assessment of traits along a continuous gradient, often time, although other interesting gradients have been explored, including temperature-humidity indices in dairy cattle (Santana et al., 2017), race length in equine (Negro Rama et al., 2016), or weight in beef cattle as the continuum (Speidel et al., 2016). In such analyses, additive genetic covariance values are smoothed across a quantitative dimension (such as time) using a covariance function. Random regression models assess deviations around a phenotypic trajectory across the continuum (Schaeffer, 2016). Repeated temperament assessment in cattle is accomplished easily in conjunction with husbandry or data collection activities. Temperament is both an economically and logistically important trait at most phases of beef production. This may be because temperamental cattle have been reported to have reduced growth rates (Tulloh, 1961; Voisinet et al., 1997; Cafe et al., 2011), compromised immune function (Fell et al., 1999), increased difficulty to manage, and inferior carcass characteristics (King et al., 2006; Cafe et al., 2011).

Temperament is highly heritable in beef cattle (Schmidt et al., 2014; Riley et al., 2014). It is of special interest in *Bos indicus* purebreds and crossbreds, because *Bos indicus* influenced cattle are considered to be more temperamental compared to British breeds. The objective of this study was to assess genetic parameters across an age continuum in crossbred cattle with repeated records using random regression and compare with those from repeated records analyses. A secondary objective was to evaluate temperament measures of dams as covariates in analyses of calf temperament traits.

MATERIALS AND METHODS

All animal procedures were in compliance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching and were approved by the Mississippi State University Animal Care and Use Committee (IACUC #08-049 and #13-010).

Animals

Records were utilized from a population of mostly Brahman-British crossbred cattle with known breed composition. This herd is located at the Mississippi Agricultural and Forestry Experiment Station in Raymond, MS. *Bos taurus* breed types represented in this population included Angus, Hereford, Red Angus, Gelbvieh, Maine-Anjou, Charolais, and Limousin. *Bos indicus* breedtypes represented in this population included Brahman and Gyr. There were 4,891 animals in the 5-generation pedigree including 118 sires and 1,292 dams with progeny that had records. Calves in the population with at least one record consisted of males (n = 2,063) and females (n = 1,958), born in the fall

(n = 1,245) and spring (n = 2,776) between the years 2002 and 2015. Age at weaning was 217.4 d of age on average, and ranged from 83 to 311 d of age.

Temperament Evaluation

Three measures of calf temperament, pen score (**PS**; a subjective measurement; Hammond et al., 1996), exit velocity (**EV**; an objective measurement; Burrow et al., 1988), and an overall temperament score (**TS**; Curley et al., 2006) were assessed at weaning. The PS was recorded prior to restraining animals for other measurements. To determine PS, an experienced observer visually evaluated (scored on a scale of 1 to 5; Table 4-1) individual calves in groups of 3 to 5 within a pen. The same experienced observer scored all calves. Subsequently, each group of calves was herded into a separate adjacent pen, where they remained until PS was recorded for all calves. After pen scoring, calves were walked into an enclosed handling facility and individually entered a squeeze chute for restraint. Exit velocity was defined as the rate, measured in m/s, at which an animal traversed 1.83 m upon exiting the squeeze chute using an infrared beam sensor system (FarmTek Inc., North Wylie, TX). Temperament score was defined as the numerical average of PS and EV, as previously reported (Curley et al., 2006; King et al., 2006).

Table 4-1. Descriptions of subjective pen score classifications (Hammond et al., 1996)[1]

Score	Description
1	Calves walked slowly and were not excited by evaluator
2	Calves ran along fences and kept distance from evaluator
3	Calves heads were high, avoided the evaluator, and ran when approached by the evaluator but stopped before hitting fences
4	Calves stayed at the back of the group with their heads high, were very aware of humans, and often ran into fences
5	Calves were very excited or aggressive, ran into fences, and ran over anything in their path

[1]Pen score, a subjective measure of temperament, in which reactions of an individual calf to a constant experienced evaluator within a pen of 3 to 5 calves are scored on a scale of 1 to 5 (1 = calm, 5 = excitable).

Statistical Analyses

All analyses were completed using ASReml (Gilmour et al., 2009). Fixed effects were determined using repeated measures analyses, in which the random components modeled were additive genetic (modeled as animal) and permanent environmental effects. Fixed effects investigated included 5 dam age categories (2, 3, 4, 5-to-10, and older than 10 yr of age), which were slight modifications of those detailed in national genetic evaluation guidelines (Cundiff et al., 2016). Calf sex included 2 categories, male and female. There were 27 contemporary groups (148.3 calves on average per group; ranging from 79 to 243 calves) consisting of combinations of year and season of birth. Calves and dams in the research herd consisted primarily of 2- or 3-breed crosses of many breeds and purebreds of a few breeds (e.g., there were 3,877 crossbred and 144 purebred calves with at least one record; 3,560 calves had crossbred dams and 461 had purebred dams). All except 289 calves and 598 dams had some proportion *Bos indicus* background. Many parameterizations of breed type of calves and their dams were investigated. Results are reported for what appeared to be the parameterization to best adjust for effects. This included two 2-level fixed effects representing purebred and crossbred calves, and purebred and crossbred dams. The proportions of *Bos indicus* in calves and their dams were modeled as linear covariates (entire parameter space represented; averages for calves and dams were 0.179 and 0.195, respectively). Calf age in days at the time of record was included as a linear covariate.

Estimates of additive genetic variance and permanent environmental variance, and as proportions of the phenotypic variance, were obtained from these repeated

measures single-trait analyses for comparison to results from random regression models.

Additional covariates were investigated after final models were determined. These included regression of calf temperament on dam temperament (PS, EV, and TS recorded when they were weaned as calves) and on calf weight at time of record. These were modeled only to assess significance and correspondingly obtain least squares constants as estimates of the regression coefficient. They were not included in any final model, that is, any model that was used for estimation of genetic parameters.

Random regression models followed a strategy similar to that described by Speidel et al. (2016). For each trait, random regression modeling employed the fixed effects determined from the repeated measures models, with the exception of calf age. The maximum number of records for individual animals in these data was 5. Those roughly corresponded to 1 mo before weaning, weaning (approximately 7 mo of age), 1 mo after weaning, 2 mo after weaning, and 1 yr of age. Therefore, the maximum possible polynomial regression supported by these data would be of order 4. Order is one less than the number of covariates estimated, and consists of the covariate raised to each power; for example, an order of 4 indicates age (in this case) raised to these powers: 0, 1, 2, 3, and 4.

Two random regressions of traits on orthogonal normalized Legendre polynomials (first suggested for this kind of use by Kirkpatrick et al., 1990) for age at the time of record were modeled for additive genetic and permanent environmental effects. Model building was initiated with the order of the additive genetic random regression equal to 1 (that is, an intercept and one linear covariate fitted). Speidel et al.

(2016) reported that inclusion of the permanent environmental random regression component models the covariance of residuals, and was preferred for random regression of days of age on weight rather than distinct residual error categories associated with measurement times. In the present study, a corresponding fixed regression of traits on orthogonal Legendre polynomials for age at the time of record was then modeled so that it was equal to the order of the random additive genetic regression (Gilmour, 2009, as cited by Speidel et al., 2016), and the order of this fixed regression was increased until the partial *F*-statistic (Gilmour, 2009, as cited by Speidel et al., 2016) of the highest coefficient was not significant.

After the fixed regression order was determined, while maintaining the order of the polynomial random regression for permanent environmental effects, the order of the polynomial random regression for additive genetic effects was increased by 1. Log-likelihood tests of the additional order (2 times the absolute difference of log-likelihoods of nested models as a χ^2 with degrees of freedom equal to the difference in number of parameters estimated) were then conducted until the next higher order was not significant.

Subsequently, the order of the permanent environmental polynomial random regression was increased by 1. The order of the additive genetic polynomial random regression was reset to 1. All of the procedures for determination of the fixed and random regression orders described above were repeated for the higher order permanent environmental random regression. The addition of the higher order random regressions in each case was tested using log-likelihood ratio tests for random regression of either

effect. Final models were concluded when the addition of terms was not significant. The maximum permissible order of any regression was 4.

The numbers of animals and records analyzed are reported in Table 4-2. Subsets of data were used for random regression analyses. That is, all records from animals that had fewer than the order of the polynomial modeled + 1 were removed from analyses. For example, in a linear random regression analysis, all animals that had less than 2 records were removed from the data.

Table 4-2. Summary statistics from random regression models for temperament traits

	Linear[1]			Quadratic[2]		
	PS[3]	EV	TS	PS	EV	TS
Total records	9,833	10,101	9,800	9,833	10,101	9,800
Total animals	3,923	4,005	3,907	3,923	4,005	3,907
Records removed	1,756	1,721	1,745	3,070	3,109	3,055
Animals removed	1,756	1,721	1,745	2,413	2,415	2,400
Records analyzed	8,077	8,380	8,055	6,763	6,992	6,745
Animals with records	2,167	2,284	2,162	1,510	1,590	1,507
Mean	2.83	2.61	2.709	2.84	2.59	2.705
SD	1.013	1.108	0.92	1.002	1.104	0.911
Minimum	1	0.22	0.61	1	0.22	0.61
Maximum	5	6.674	6.527	5	6.671	6.118
Age (d) at time of record						
Mean	242.61	243.34	242.67	250.7	251.06	250.74
SD	67.198	68.611	67.216	69.099	70.535	69.095
Minimum	83	83	83	83	83	83
Maximum	454	454	454	454	454	454

[1]All records of animals with less than 2 records were removed for linear random regression analyses.
[2]All records of animals with less than 3 records were removed for quadratic random regression analyses.
[3]PS = pen score, a subjective measure of temperament, in which reactions of an individual calf to a constant experienced evaluator within a pen of 3 to 5 calves are scored on a scale of 1 to 5 (1 = calm, 5 = excitable). EV = exit velocity, an objective measure of temperament, defined as the rate (m/s) in which a calf travels 1.83 m upon immediately exiting a squeeze chute. TS = temperament score, numerical average of pen score and exit velocity.

RESULTS AND DISCUSSION

Record summaries for the random regression analyses in Table 4-2 are limited to polynomials of orders 1 and 2, because increasing the random regression polynomials to order 3 was never appropriate, as log-likelihood values decreased.

Fixed Effects

Fixed effect components of models were determined using the repeated records model. Although the primary intent of their inclusion was to adjust for those effects in these data, Tables 4-3 and 4-4 summarize that information as modeled. Probability values of these effects were not always identical in random regression analyses, but were similar. No fixed effect moved from inclusion to non-inclusion model status (or vice-versa) in repeated records and random regression analyses. Contemporary group (calves grouped within the same birth year and season) was a highly significant component of all models *(*$P < 0.001$; Table 4-3). Such differences are expected, due to known differences in calf temperament attributed to variability from year to year and season to season. The covariate for proportion of *Bos indicus* in the calf indicated a strong positive relationship (regression coefficients were 0.41, 0.85, and 0.57, respectively; $P < 0.01$; Table 4-3) with each calf temperament trait (greater proportions of *Bos indicus* were associated with worse (larger) measures of temperament). This was consistent with all earlier comparisons of Brahman and Brahman-cross breed types with British breeds (e.g., Fordyce et al., 1982; Riley et al., 2010, Chase et al., 2017). Alternatively, the relationship of proportion of *Bos indicus* in dams was not influential for any calf

Table 4-3. Probability values for *F* statistic of fixed effects and least squares constants solutions of certain regression coefficients: repeated records analyses of temperament traits[1, 2, 3, 4, 5, 6]

Effect	Calf Traits		
	Pen score	Exit velocity	Temperament score
Dam age	< 0.001	< 0.001	< 0.001
Calf contemporary group	< 0.001	< 0.001	< 0.001
Calf sex	< 0.001	< 0.001	< 0.001
Proportion *Bos indicus* in calf	0.009 0.41 ± 0.20	< 0.001 0.85 ± 0.21	0.001 0.57 ± 0.18
Proportion *Bos indicus* in dam	0.305	0.539	0.877
Calf age, d	< 0.001 –0.001 ± 0.0002	< 0.001 –0.002 ± 0.0001	< 0.001 –0.002 ± 0.0001
Purebred vs. crossbred calf	0.033	0.666	0.154
Purebred vs. crossbred dam	0.098	0.066	0.229

[1]Dam age was modeled in categories, as recommended by the Beef Improvement Federation (2016): 2-, 3-, 4-, 5- to 10-yr-olds, and cows older than 10.
[2]Contemporary groups were comprised of calves born in the same year and season.
[3]Sex: males and females.
[4]Proportion *Bos indicus* in calves and dams, and calf age were modeled as linear regressions.
[5]Because of the large numbers of breeds represented in the research population, breed type in both calves and dams was investigated as 2-level fixed effects.
[6]PS = pen score, a subjective measure of temperament, in which reactions of an individual calf to a constant experienced evaluator within a pen of 3 to 5 calves are scored on a scale of 1 to 5 (1 = calm, 5 = excitable). EV = exit velocity, an objective measure of temperament, defined as the rate (m/s) in which a calf travels 1.83 m upon immediately exiting a squeeze chute. TS = temperament score, numerical average of pen score and exit velocity.

Table 4-4. Means for significant fixed effects (repeated measures analyses)[1, 2]

	Calf Traits		
	Pen score	Exit velocity	Temperament score
Dam age			
2-yr-olds	2.51 ± 0.08[b]	2.60 ± 0.08[c]	2.58 ± 0.06[b]
3-yr-olds	2.62 ± 0.08[b]	2.53 ± 0.08[c]	2.60 ± 0.07[b]
4-yr-olds	2.78 ± 0.08[b]	2.76 ± 0.08[b]	2.80 ± 0.07[a]
5- to 9-yr-olds	2.82 ± 0.07[a]	2.86 ± 0.06[a]	2.86 ± 0.05[a]
10 yr or older	2.81 ± 0.07[a]	2.85 ± 0.07[ab]	2.85 ± 0.06[a]
Calf sex			
Female	2.83 ± 0.07[a]	2.83 ± 0.06[a]	2.85 ± 0.05[a]
Male	2.59 ± 0.07[b]	2.61 ± 0.06[b]	2.62 ± 0.05[b]
Crossbred vs Purebred			
Crossbred calves	2.80 ± 0.06[a]	---	---
Purebred calves	2.62 ± 0.09[b]	---	---
Crossbred dams	---	2.67 ± 0.06[b]	---
Purebred dams	---	2.77 ± 0.08[a]	---

[a,b,c]Within traits and effects (separated by empty lines in table), means that do not share a common superscript differ *(P* < 0.05).

[1]Because of the large numbers of breeds represented in the research population, breed type in both calves and dams was investigated as 2-level fixed effects.

[2]Pen score, a subjective measure of temperament, in which reactions of an individual calf to a constant experienced evaluator within a pen of 3 to 5 calves are scored on a scale of 1 to 5 (1 = calm, 5 = excitable). Exit velocity, an objective measure of temperament, defined as the rate (m/s) in which a calf travels 1.83 m upon immediately exiting a squeeze chute. Temperament score, numerical average of pen score and exit velocity.

temperament trait *(P* > 0.29; Table 4-3) in this study. The Brahman maternal effect was influential on temperament of heifers produced in a diallel mating design (Riley et al., 2010). These results for modeling *Bos indicus* may be due to sire contribution of *Bos indicus* influence in calves in these data. Because of potential collinearity, these two covariates were confirmed independently, that is, without the presence of the other in the model, and similar significance levels were observed. Increasing calf age in days was associated with lower values (calmer temperaments) for all temperament traits *(P* < 0.001; Table 4-3). Burdick et al. (2011) reported increased EV as suckling Brahman calves aged to weaning. Later, Schmidt et al. (2014) reported increased EV until weaning followed by a slight decrease with increasing ages. Such results may be due to an inverse relationship of PS, EV, or TS with BW in cattle; that is, the greater body mass in heavier cattle may cause them to exit the squeeze chute at a slower rate; this was first noted by Elzo et al. (2009). A similar inverse relationship of EV with BW was observed by Riley et al. (2010) in heifers between the ages of 7 and 19 mo, but the significance of that covariate may have been due to the pregnant status of most of the heifers in that study in latter months. Dam age *(P* < 0.001 for all traits, Table 4-3) categories indicated that calves born to older cows often had greater, more excitable, temperaments relative to those born to younger dams (Table 4-4). These results are in contrast to reports among purebred Brahman cattle that calves born to very young cows had greater PS (Schmidt et al., 2014). Riley et al. (2014) reported no effect of dam age on temperament measures approximately 1 mo after weaning in crossbred calves. No differences in calf PS were noted due to age of their dams, but those calves born to cows between 6 and 10 yr of age

had slower exit velocities (Chase et al., 2017). All temperament variables in the present study were larger in females relative to males (Table 4-3 and 4-4), which is consistent with the vast majority of literature on temperament in cattle (e.g., Voisinet et al., 1997; Riley et al., 2014; Chase et al., 2017), although an effect of calf gender was not detected in temperament traits of Brahman calves (Schmidt et al., 2014). Crossbred calves in the present study had larger ($P = 0.03$) PS than purebreds (Tables 4-3 and 4-4). This may be due to the majority of the purebreds in these data being British and the majority of crossbreds being *Bos indicus*-influenced; however, no interactions of the terms representing breed type were detected ($P > 0.3$). Differences attributable to purebred vs. crossbred cows were not included in final models ($P > 0.16$) of any trait (Table 4-3).

Table 4-5. Probability values of *F* statistics and least squares constants estimates ± SE (when $P < 0.1$) of regression coefficients of calf temperament on dam's temperament and calf temperament on calf weight[1, 2]

	Calf Traits					
Effect	PS	Est ± SE	EV	Est ± SE	TS	Est ± SE
Dam's PS	0.243	---	0.011	0.11 ± 0.04	0.053	0.07 ± 0.04
Dam's EV	0.022	0.05 ± 0.02	0.238	---	0.043	0.09 ± 0.04
Dam's TS	0.268	---	0.005	0.14 ± 0.05	0.079	0.03 ± 0.02
Calf weight	< 0.001	–0.001 ± 0.0001	< 0.001	–0.001 ± 0.0001	< 0.001	–0.001 ± 0.0008

[1]*Post hoc* evaluations of these variables in final repeated measures analyses.
[2]PS = pen score, a subjective measure of temperament, in which reactions of an individual calf to a constant experienced evaluator within a pen of 3 to 5 calves are scored on a scale of 1 to 5 (1 = calm, 5 = excitable). EV = exit velocity, an objective measure of temperament, defined as the rate (m/s) in which a calf travels 1.83 m upon immediately exiting a squeeze chute. TS = temperament score, numerical average of pen score and exit velocity.

Regressions of Calf Temperament on Dam Temperament

Post hoc evaluations of dam temperament traits (measured when they were calves at weaning) and calf BW (at the time of record) as covariates were significant for most calf temperament traits (Table 4-5). Significant regression coefficient estimates for dam temperament ranged from 0.05 ± 0.02 (calf PS regressed on dam EV) to 0.14 ± 0.05 m/s (calf EV regressed on dam TS). No measure of dam temperament explained significant variation in the corresponding temperament measure in calves; however, there was a tendency *(P* = 0.08) for dams with increased TS to have calves with increased TS (Table 4-5). Stronger associations were expected, given the high heritability of these traits. Calf BW was highly significant and indicated lower (calmer) PS, EV, and TS values associated with heavier BW (Table 4-5). Body weight may explain much of the same variation as age in days, as discussed in the previous paragraph; similar results have been reported for growing heifers (Elzo et al., 2009; Riley et al., 2010).

Table 4-6. Genetic parameter of temperament estimates[1, 2, 3]

	Pen Score		Exit velocity		Temperament score	
Effect	All	Subset	All	Subset	All	Subset
σ_a^2	0.260	0.335	0.306	0.325	0.258	0.312
σ_c^2	0.333	0.299	0.302	0.331	0.262	0.267
σ_p^2	0.997	0.997	1.01	1.059	0.731	0.792
h^2	0.28 ± 0.03	0.34 ± 0.04	0.31 ± 0.03	0.31 ± 0.04	0.35 ± 0.03	0.39 ± 0.04
c^2	0.34 ± 0.03	0.30 ± 0.04	0.30 ± 0.03	0.31 ± 0.03	0.36 ± 0.03	0.34 ± 0.04

[1] σ_a^2, σ_c^2, σ_p^2, h^2, c^2 represent estimates of additive genetic variance, permanent environmental variance, phenotypic variance, narrow sense heritability, and permanent environmental effects, respectively, as a proportion of the phenotypic variance.
[2] All: parameters were estimated from repeated records analyses using all data. Subset: For comparison to random regression results, parameters were estimated from repeated records analyses that included only the data for each trait used in the quadratic random regression analyses (animals with less than 3 records were not included).
[3] Pen score, a subjective measure of temperament, in which reactions of an individual calf to a constant experienced evaluator within a pen of 3 to 5 calves are scored on a scale of 1 to 5 (1 = calm, 5 = excitable). Exit velocity, an objective measure of temperament, defined as the rate (m/s) in which a calf travels 1.83 m upon immediately exiting a squeeze chute. Temperament score, numerical average of pen score and exit velocity.

Estimates of Genetic Parameters - Repeated Measures

Estimates of genetic parameters for the 3 temperament traits from repeated measures analyses are shown in Table 4-6 from all data and including only the subsets of data used in random regression analyses, that is, only from cattle that had 3 or more records. The estimates of heritability were lower than those reported for Brahman (Schmidt et al., 2014) or Nellore-Angus crosses (Riley et al., 2014), but were similar to most of the reviewed reports of Adamczyk et al. (2013). The magnitudes of the permanent environmental variance as a proportion of the phenotypic variance **(c^2)** were similar to narrow sense heritability estimates. Prayaga and Henshall (2005) estimated a similar value (0.25) for permanent environmental variance as a proportion of the phenotypic variance in evaluation of flight time (equivalent to the denominator of EV; that is, elapsed time in seconds).

Random Regression

Pen Score. The sequence of modeling steps began with estimation of a fixed regression on Legendre polynomials of age, while the random regressions representing additive genetic and permanent environmental effects were set at an order of 1 (intercept and linear covariate). The order of the fixed regression on days of age was supported to an order of 2 *($P < 0.001$)*, that is, fitting an intercept, linear and quadratic coefficients, but no higher *($P = 0.99$)*. Then, while holding the fixed regression at that order (2), increasing the orders of first the additive genetic random regression and then the permanent environmental random regression to 2 were highly significant, and within

addition of each of those random regressions, attempts to increase the order of the fixed regression on days of age to 3 were not supported *(P* > 0.73). Increasing the random regressions to order 3 (intercept, linear, quadratic, and cubic covariates) was not merited, as the log-likelihood values decreased for each.

Table 4-7. Estimates of residual variance and variances of coefficients of regression on Legendre polynomials of age for temperament traits[1, 2, 3]

	Pen score			EV			TS		
Component	Estimate	SE	h^2	Estimate	SE	h^2	Estimate	SE	h^2
Residual	0.272	0.0068	---	0.322	0.0075	---	0.155	0.0039	---
Permanent environmental									
Intercept	0.599	0.0883	0.403	0.788	0.0769	0.500	0.554	0.0720	0.447
Cov (intercept, linear)	–0.051	0.0474		0.029	0.0365	---	0.052	0.0329	
Linear	0.319	0.0515	0.412	0.127	0.0325	0.180	0.151	0.0301	0.323
Cov (intercept, quadratic)	–0.009	0.0392	---				–0.047	0.0268	---
Cov (linear, quadratic)	–0.123	0.0328	---				–0.024	0.0192	---
Quadratic	0.066	0.0324	0.170				0.014	0.0193	0.064
Additive genetic									
Intercept	0.617	0.1170	0.415	0.467	0.0960	0.296	0.531	0.0963	0.428
Cov (intercept, linear)	–0.005	0.0572	---	0.002	0.0477	---	–0.031	0.0418	---
Linear	0.183	0.0533	0.237	0.257	0.0450	0.364	0.162	0.0356	0.347
Cov (intercept, quadratic)	0.011	0.0424	---	–0.016	0.0271	---	0.030	0.0307	---
Cov (linear, quadratic)	–0.089	0.0314	---	–0.130	0.0228	---	–0.088	0.0211	---
Quadratic	0.050	0.0262	0.128	0.077	0.0216	0.194	0.051	0.0173	0.231

[1]Estimates for pen score and temperament score are from a quadratic random regression model on Legendre polynomials for both permanent environmental and additive genetic effects. Estimates for exit velocity were from a model that included a linear random regression of permanent environmental effects on Legendre polynomials of age and a quadratic random regression of additive genetic effects on Legendre polynomials of age.

[2]Estimates of h^2 are heritabilities of the coefficients of the random regressions (Schaeffer, 2016).

[3]Pen score, a subjective measure of temperament, in which reactions of an individual calf to a constant experienced evaluator within a pen of 3 to 5 calves are scored on a scale of 1 to 5 (1 = calm, 5 = excitable). Exit velocity, an objective measure of temperament, defined as the rate (m/s) in which a calf travels 1.83 m upon immediately exiting a squeeze chute. Temperament score, numerical average of pen score and exit velocity

Estimates of variances from the best model (quadratic fixed and random regressions) are shown in Table 4-7. The SE for covariance terms of the intercept with either linear or quadratic estimates of both the additive genetic and permanent environmental random regressions were large. There were low correlations of coefficients of the intercept with either the linear or quadratic terms of either the permanent environmental or the additive genetic polynomials (ranged from –0.12 to 0.06). This differed from results of early implementations of this methodology in growth of cattle (Meyer, 2001). However, the estimates of the correlation of the linear and quadratic coefficients in this study were strong and negative (–0.93 and –0.85 for additive genetic and permanent environmental regressions, respectively). The estimates of variance for the intercept were large, which was noted in other work (Meyer, 2001). For the additive genetic and permanent environmental random regressions, estimates of heritability for the coefficients (Schaeffer, 2016) in the present study were moderate to large for the intercept and the linear terms, but smaller for the quadratic terms. Plots of curves representing variances, narrow sense heritability (estimates of heritability for traits, not coefficients of the random regression), and permanent environmental variance as a proportion of the phenotypic variance across age in days within the limits of the project data (method previously demonstrated by Kirkpatrick et al., 1990) are shown in Figure 4-1. These plots suggest that variances and estimates of h^2 and c^2 are largest at younger ages and decrease with age. Others have observed larger genetic variances on the extremes of the data ranges, which could be a consequence of using Legendre polynomials in the random regression (Schaeffer and Jamrozik, 2008; Schaeffer, 2016).

However, multiple types of covariance functions will generate such fluctuations of parameters at those extremes (Mookpram et al., 2017). Erratic estimates at covariate boundaries may intensify with higher order polynomials (Meyer, 1999). In the present study, the curve suggests that variances increase slightly after 300 d of age. Estimates of h^2 and c^2 suggest large repeatability of this trait, and therefore additional records may have lower value for prediction purposes. The plots of these parameters against calf age suggest that h^2 and c^2 are approximately the same, especially with increasing calf age.

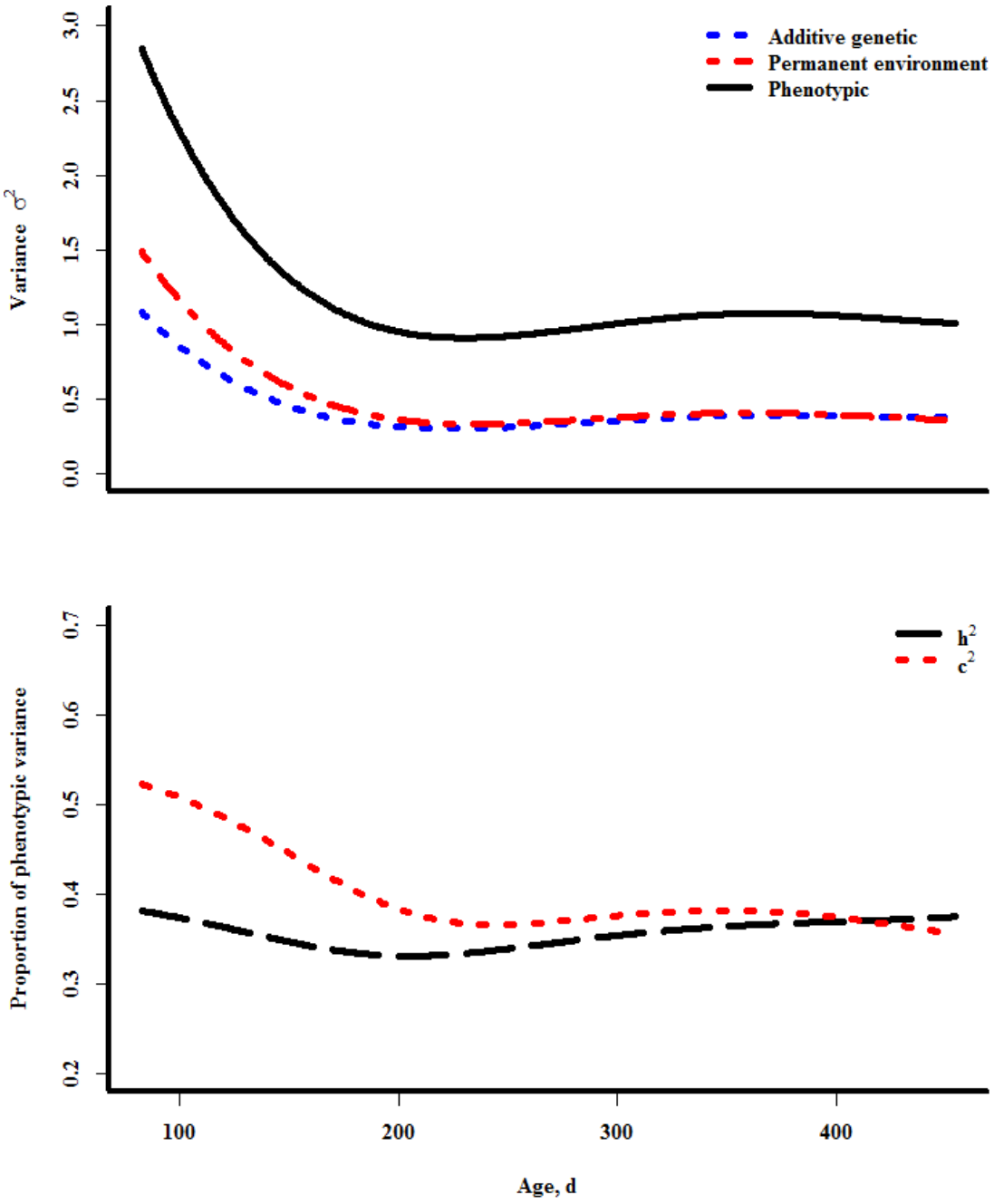

Figure 4-1. Plots of variance components for pen score across age (upper) and plots of heritability in the narrow sense and permanent environmental variance as a proportion of the phenotypic variance; all results from quadratic random regression models.

Exit Velocity. Beginning with linear random regressions (order 1) on days of age for permanent environmental effects and for additive genetic effects, the fixed linear regression on age was highly significant (Table 4-7). However, increasing the order of this fixed regression to 2 was not supported *(P* = 0.94). Then a quadratic additive genetic random regression on age was fitted for the component, while maintaining a linear permanent environmental random regression. Comparison of log-likelihood values of the random regression models strongly favored quadratic additive genetic random regression *(P* < 0.001). Increasing the order of the permanent environmental random regression was not supported, because the log-likelihood of this model decreased relative to that of the linear permanent environmental/quadratic additive genetic model. Increasing the order of the fixed regression on days of age was not merited *(P* = 0.91). Equal orders of two random regressions is not unusual; modeling of mature cow weight by Arango et al. (2004) required a larger order for the permanent environmental random covariate than for the additive genetic random covariate. Cubic random regressions for additive genetic effects resulted in decreases in log-likelihood values. Estimates of random regression coefficients and their heritabilities are presented in Table 4-7. These estimates of heritability were larger in magnitude for the intercept in the permanent environmental random regression, but not in the additive genetic random regression. Only the covariance of the linear and quadratic terms of the additive genetic regression appeared to differ from 0.

Curves representing variances and parameter estimates across age represented in these data are shown in Figure 4-2. The greatest values for the additive genetic and

phenotypic variances for EV were at younger ages, and the permanent environmental component appeared to slightly increase at older ages. Most notable in these curves was the increasing permanent environmental variance as a proportion of phenotypic variance across ages, accompanied by an ultimate decrease in estimates of heritability. Similar trends for additive genetic and permanent environmental variances as a proportion of phenotypic variance were reported for BW in pigs (Huisman et al., 2002). Arango et al. (2004) reported decreasing influence of permanent environmental effects and increasing additive genetic effects for mature cow body weights over time. Additive genetic and permanent environmental variances both increased in random regression analyses of observed days to weight in fed cattle (Speidel et al., 2016), and estimates of heritability for BW in quail increased with age (Karami et al., 2017). Again, the present study indicated increasing c^2 and decreasing h^2 over time, suggesting an increasing influence of environmental and decreasing relative influence of additive genetic effects on EV with increasing age.

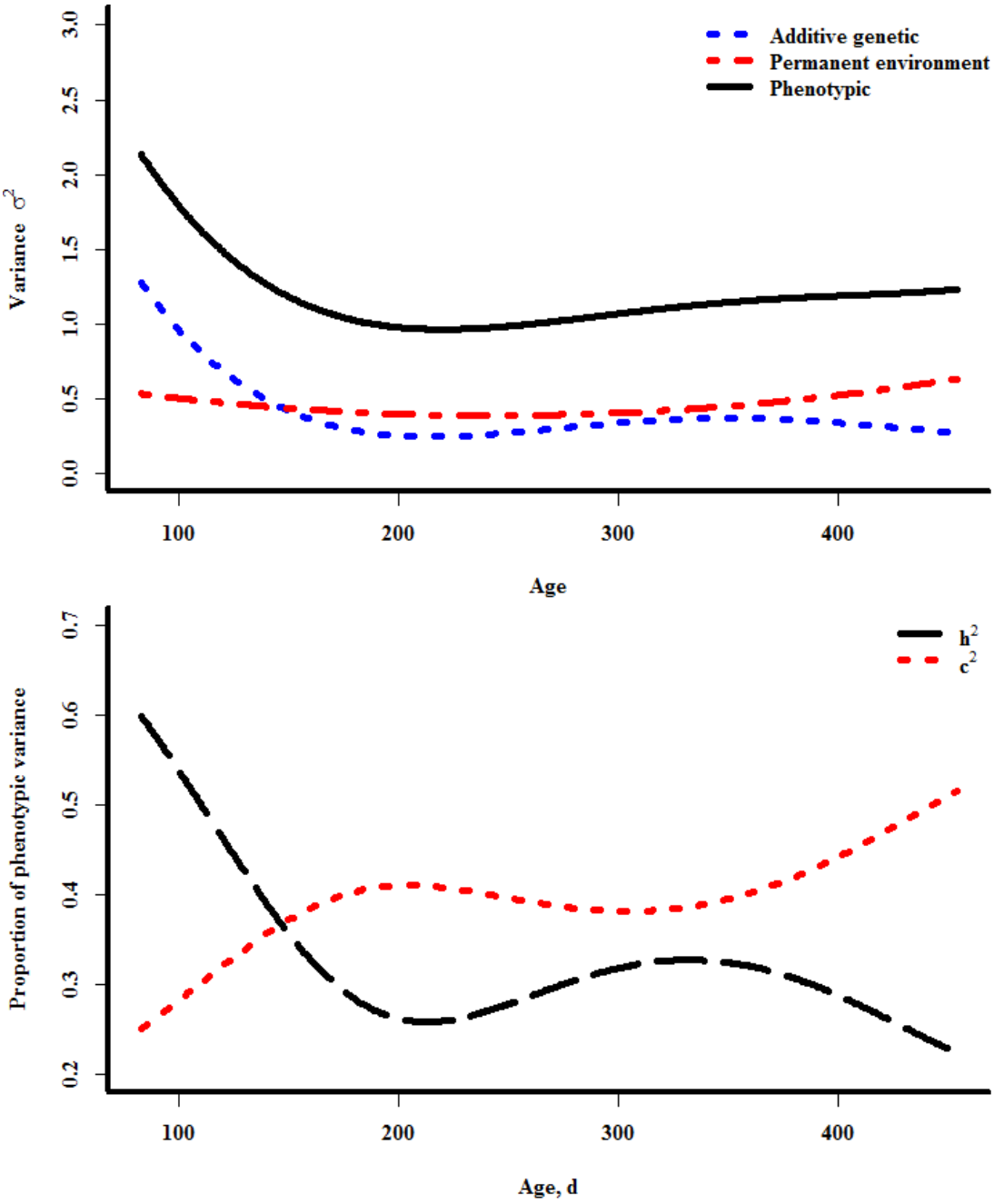

Figure 4-2. Plots of variance components for exit velocity across age (upper) and plots of heritability in the narrow sense and permanent environmental variance as a proportion of the phenotypic variance; results from models with quadratic additive genetic random regression and linear permanent environmental random regression.

Temperament Score. Within linear random regressions on Legendre polynomials of age for additive genetic and permanent environmental effects, the order of the fixed regression on days of age was best modeled as order 2 *(P* < 0.001). The cubic term was not appropriate *(P* = 0.57). Subsequently, quadratic random regressions on age were highly significant for additive genetic and permanent environmental effects. Within those regressions (order 2 for both additive genetic and permanent environmental random regressions), the quadratic fixed regression on age was preferred *(P* = 0.02), as a cubic fixed regression was not significant *(P* > 0.2). Log-likelihood values were not significantly improved by increasing the order of either random regression beyond 2. Estimates of variance of coefficients and corresponding heritabilities are shown in Table 4-7. Although a function of both PS and EV, results for TS more closely resembled those of PS. Major characteristics include 1) larger estimates of heritability for intercept terms, 2) covariances of regression terms that do not appear to differ from 0, and 3) a somewhat larger heritability estimate of the quadratic additive genetic coefficient relative to the quadratic permanent environmental coefficient. Curves in Figure 4-3 show larger additive genetic, permanent environmental, and phenotypic variances at the younger compared to the older ages in these data. Similar to results for EV, the permanent environmental component as a proportion of phenotypic variance appears to show relative increased influence on phenotype at older ages. Perhaps to a greater extent than PS, estimates of heritability were high at younger ages and decreased as calf age increased. These results suggest that environmental influence at least partially overrides

additive genetic influence over time, maybe as a consequence of exposure to compounding environmental stimuli.

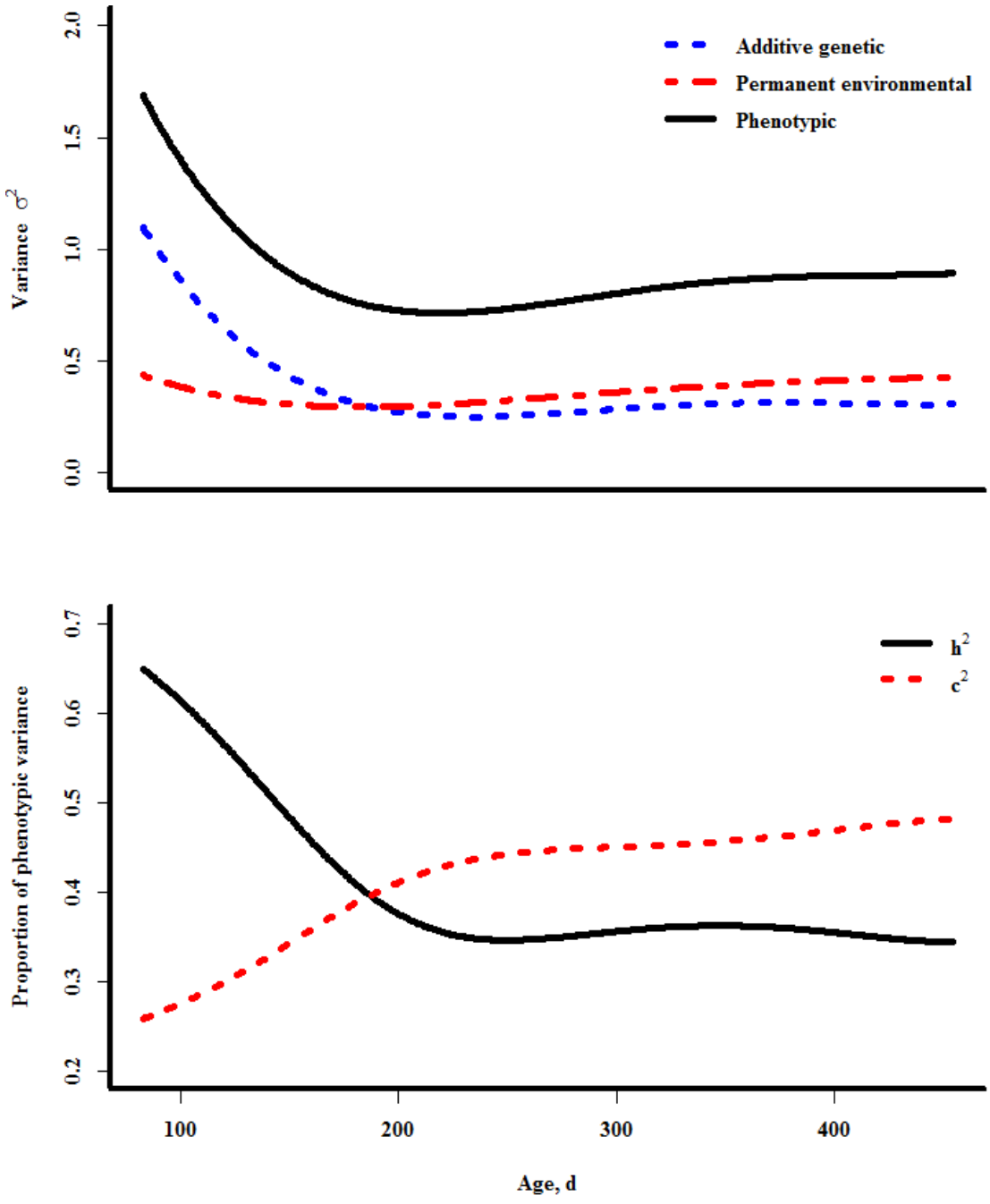

Figure 4-3. Plots of variance components for temperament score across age (upper) and plots of heritability in the narrow sense and permanent environmental variance as a proportion of the phenotypic variance; all results from quadratic random regression models.

Dynamics of components of variance for calf temperament accompanying age

Changes in the variance components and associated parameters could occur as a result of increased trait variance. However, that does not seem likely, as raw variances of records that correspond roughly with records taken at 28 d before weaning, weaning, 28 and 56 d postweaning, and 1 yr of age increased substantially only for EV (Table 4-8). The permanent environmental effects from analyses of EV and TS appear to accumulate across time, particularly with repeated exposure to data collection. This seems reasonable when considering that additive genetic and permanent environmental effects appear to be equally influential early in life, becoming less equal over time as permanent environmental effects accumulate. This is less apparent in the results from analyses of PS, in which both components contribute almost equally across the ages present in the data. Such acclimation to conditions seems natural from day to day observations of cattle exposed to repeated processing. Peixoto et al. (2016) reported a negative relationship for behavioral reactivity (mechanical measurement of movement while confined in a box scale) with age, that is, less movement was associated with older ages in *Bos indicus* (Guzerat) dairy cows. Neither EV nor chute score (subjective score representing amount of movement when confined to a scale box) decreased with monthly measurements on heifers (Riley et al., 2010). Exit velocity increased in calves from weaning to 24 h post-weaning, followed by a decrease at 72 h post-weaning, and PS from the same calves improved at each of those measurement times (Chase et al., 2017). In that work, transported steers had lower EV at 4 wk post-transport than measurements at the time of shipment up to 2 wk after (Chase et al., 2017). The content or characterization of the

Table 4-8. Estimates of variance (s^2) for temperament traits by time of record[1]

	s^2		
	Pen score	Exit velocity	Temperament score
Weaning minus 28 d	0.995	1.208	0.882
Weaning	0.965	1.097	0.774
Weaning + 28 d	0.965	1.144	0.785
Weaning + 56 d	0.976	1.298	0.832
Yearling	1.016	1.397	0.894

[1]Pen score, a subjective measure of temperament, in which reactions of an individual calf to a constant experienced evaluator within a pen of 3 to 5 calves are scored on a scale of 1 to 5 (1 = calm, 5 = excitable). Exit velocity, an objective measure of temperament, defined as the rate (m/s) in which a calf travels 1.83 m upon immediately exiting a squeeze chute. Temperament score, numerical average of pen score and exit velocity.

repeated exposure or handling might affect acclimation in different ways, for example, rough, traumatic handling could be detrimental (Petherick et al., 2009; Peixoto et al., 2016) and appear to have an effect opposite of acclimation. In such cases, the partitioning of variance might yield unexpected results.

We would hesitate to suggest that random regression analyses would be the best method for predicting merit for temperament traits. Temperament is very different relative to other traits evaluated with repeated measures or random regression analyses, such as weight or lactation. Particularly, BW across some time dimension could be influenced by activation or inactivation of distinct genes or groups of genes—that seems less likely for temperament traits. They may have a distinct genetic basis, possibly with maternal additive genetic influence on phenotypes recorded preweaning or in the proximity of weaning (although maternal additive genetic effects were not detected in preliminary analyses of the records at and prior to weaning). An alternative is that maternal presence contributes to accumulation of permanent environmental effects, and those become substantial across time. Maternal permanent environmental effects were detected in some preliminary analyses of these traits; sometimes such effects act as a variance term that could accommodate a combination of maternal additive genetic and maternal permanent environmental effects, especially in smaller data sets. Genes influencing temperament may act additively and constant across time (the heavy influence and heritability of the intercept terms in the present study may support this notion) but permanent environment accrues and becomes the more important component of variance. Since temperament records are easily accumulated, an estimate of producing

ability (Lush, 1945; all genetic components and permanent environment, as described by Falconer and Mackay, 1996) might be an effective predictor of merit. Otherwise, a single evaluation at a young age may be appropriate for estimation of breeding value, although the higher additive genetic variances at younger ages may be a consequence of modeling. A final caveat for interpretation here is that if performance is worse for those cattle with poor temperaments, as is commonly assumed (e.g., Tulloh, 1961; Voisinet et al., 1997; King et al., 2006; Cafe et al., 2011), it could be expected that there would be negative selective pressure on their presence with increasing age. This might result in less phenotypic variance for temperament at older ages, and be more of an issue for records in mature cows measured at various times much later in life.

The breed type component of this population may influence these results in unknown ways. The majority of cattle with records were *Bos taurus-Bos indicus* crosses. Although heterosis may be important (yet unfavorable) for temperament traits (Chase et al., 2017), crossbred Brahman have also been reported to have similar temperament measurements as Brahman cattle (Riley et al., 2010; Schmidt et al., 2014; Chase et al., 2017). There was no convenient simple parameterization of breed type of calves and their dams in these data (and of course the two are functions of each other). When one of the breeds involved in temperament evaluation is Brahman, it becomes especially important. Most of the introduction of Brahman ancestry in animals in this project was more subtle, as through the use of Brangus bulls. These factors could have influenced breed type effects on temperament in unrevealed manners.

CONCLUSIONS

Estimates of heritability were similar to those previously reported for temperament in beef cattle, which supports assertions that selection programs could be effectively implemented for these traits in beef cattle breeding programs. To our knowledge, this is the first report of genetic parameters of temperament in beef cattle estimated across time (as a calf ages) with random regression analyses. Random regression analyses of temperament in cattle seemed to emphasize the increasing influence of permanent environmental effects and the decreasing influence of additive genetic effects across time as the bovine matures. This suggests inherited temperament traits are predictable and observable in early life, but may become gradually overridden as calves age, maybe due to accumulating exposure to environmental stimuli.

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CHAPTER V

CONCLUSIONS

Understanding how phenotypic variance might be attributable to epigenetic and genetic control provides insight to the improvement of economically relevant traits in beef cattle. As described herein, unique models of stress and genetic analysis of a large pedigreed herd were coupled with modern genomic techniques and statistical methods to increase the knowledge base regarding genetic factors that influence physiologic processes of economic relevance in the beef industry. The previous chapters evaluated the influence of prenatal transportation stress on epigenetic regulation of physiological functions and estimated genetic parameters of temperament across an age continuum in cattle. Prenatal transportation stress in cattle altered genome-wide DNA methylation profiles, which were predicted to impact pathways related to immune function, behavior, stress response, neural function, metabolism, reproduction, and cell signaling. Alterations in behavior, stress response, immune function, and reproductive endocrinology were related with phenotypic differences observed in the population of calves from which bull calves in this study were derived (Littlejohn et al., 2016; Burdick Sanchez et al., 2013; Littlejohn et al., 2017). Altered innate immune response to an endotoxin challenge suggested an impact of prenatal stress on the body's capacity to respond to an immunological insult. Temperament and biological systems known to regulate temperament were among the most noteworthy to show epigenetic and

phenotypic alterations due to prenatal stress. To our knowledge, these data are the first reports of a genome-wide assessment of DNA methylation in prenatally stressed calves.

Random regression analyses of temperament in a large pedigreed herd of beef cattle appeared to emphasize the increasing influence of permanent environmental effects and the decreasing influence of additive genetic effects across time as the bovine matures. This suggests inherited temperament traits are predictable and observable in early life, but may become gradually overridden as calves age, maybe due to accumulating exposure to environmental stimuli. To our knowledge, this is the first report of genetic parameters of temperament in beef cattle estimated across time with random regression analyses.

These studies revealed methylomic and phenotypic alterations associated with prenatal stress. However, fundamental concepts of the central dogma of biology suggest that the influence of prenatal stress at the RNA level should be assessed. Therefore, future studies should assess the association of DNA methylation and gene expression, as well as how it relates to phenotype of prenatally stressed calves. DNA methylation is understood to be a stable modification. However, the degree of this stability with regard to changes in DNA methylation as a result of developmental programming is far from understood. This study assessed the influence of prenatal stress in young calves. Future studies should assess the plasticity of methylomic differences, as well as the plasticity of the interaction of methylomic and transcriptomic differences.

Most studies assessing the epigenetic control of developmental programming have focused primarily on DNA methylation, though other types of epigenetic

modifications are known to play a regulatory roll in that programming. Therefore, future studies should strive to better understand how all types of epigenetic modifications work in concert to alter the epigenome, transcriptome, and phenotype. Because the primary function of epigenetic modification is to program cell specific function, the epigenome between cell types is diverse. This raises concerns when evaluating surrogate tissues and tissues with nonhomogeneous cell populations. Future studies should evaluate methylomic differences between cell types and tissue types in prenatally stressed animals.

Ultimately, prenatal programming as regulated by methylomic changes appeared to account for some percentage of total phenotypic variation in calves. This has illuminated the inadequacy of the traditional model of phenotypic variation that states phenotypic variation consists of some combination of genotypic and environmental variance. This model of prenatal stress suggests that developmental programming as regulated by epigenetic control could explain a percentage of the total phenotypic variation. Understanding the mechanisms controlling prenatal programming brings the scientific community a step closer to improving the traditional model of phenotypic variation. A more precise model of factors contributing to phenotypic variation will enable more accurate prediction and selective control of the phenotypic expression of economically relevant traits in beef cattle.