

ENDOGENOUS CORTISOL, LUTEINIZING HORMONE AND TESTOSTERONE  
SECRETION, AND GNRH-INDUCED LUTEINIZING HORMONE AND  
TESTOSTERONE SECRETION IN PRENATALLY STRESSED SEXUALLY  
MATURE BRAHMAN BULLS

A Thesis

by

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## ABSTRACT

The purpose of this experiment was to determine the effects of prenatal transportation stress (PNS) on LH, testosterone, and cortisol secretion before and after GnRH stimulation in sexually mature Brahman bulls. Forty-eight Brahman cows were exposed to a transportation event at 5 stages of gestation (and 48 cows were non-transported controls). Bulls from these cows were electroejaculated every 2 wk beginning at a scrotal circumference of 24 cm through sexual maturity (i.e., 500 million sperm/ejaculate). The initial 11 control and 12 PNS bulls to reach sexual maturity were selected for endocrine evaluation. Within 7-21 d after reaching sexual maturity, bulls were fitted with jugular cannulas, and blood samples were collected at 15-min intervals for 6 h. Exogenous GnRH was then administered intravenously (10 ng/kg BW) and blood collection continued at 15-min intervals for an additional 8 h. Concentrations of LH, testosterone, and cortisol in serum were determined. Amplitude and maximum concentration of a detectable LH pulse and testosterone response, baseline concentration, average concentration in the h prior to GnRH administration, and area under the curve were calculated for LH and testosterone in the 4-h period immediately preceding and 6-h period immediately following GnRH administration. Cortisol in the h prior to GnRH administration and area under the curve were calculated for the 4-h period immediately preceding GnRH administration. Duration of the GnRH-induced LH release was determined. More PNS (10 of 11) than control (3 of 12) bulls exhibited an LH pulse prior to GnRH administration ( $P < 0.01$ ). More PNS bulls exhibited an endogenous

testosterone response to endogenous LH secretion (9 of 11;  $P=0.02$ ) relative to control bulls (4 of 12). In the h preceding GnRH administration, testosterone was greater ( $P=0.0064$ ) in PNS compared to control bulls, and cortisol was lower in PNS compared to control bulls. No other characteristic associated with the release of LH, testosterone, or cortisol secretion prior to GnRH administration differed between groups ( $P>0.1$ ). Bulls responded similarly to exogenous GnRH, except duration of GnRH-induced LH release which was greater ( $P=0.02$ ) in PNS ( $268\pm 18$  min) relative to control ( $207\pm 16$  min) bulls. Prenatal stress affected postnatal secretion of LH, testosterone, and cortisol in sexually mature Brahman bulls.

## DEDICATION

This thesis is dedicated to my parents, Danny and Shellie Littlejohn. They are both my greatest role models. My parents have truly shown me how I want to live my life. Their example, morals, and work ethic have taught me to have high expectations of myself and that I can do absolutely anything I set my mind to. Without that mindset and their unconditional love and support, I would not be where I am today.

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## NOMENCLATURE

AcH	Acetylcholine
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
AUC	Area under the curve
BW	Body weight
cAMP	Cyclic adenosine monophosphate
CPM	Counts per minute
CRH	Corticotrophin-releasing hormone
CV	Coefficient of variation
E	Epinephrine
EV	Exit velocity
GR	Glucocorticoid receptor
HPA	Hypothalamic-pituitary-adrenal
HPG	Hypothalamic-pituitary-gonadal
HPT	Hypothalamic-pituitary-testicular
LH	Luteinizing hormone
MR	Mineralocorticoid receptor
NE	Norepinephrine
oLH	Ovine luteinizing hormone
PEPCK	Phosphoenolpyruvate carboxykinas

POMC	Proopiomelanocortin
PNS	Prenatal stress
PS	Pen score
PVN	Paraventricular nucleus
SAM	Sympathetic-adrenal-medullary axis
TS	Temperament score
VP	Arginine vasopressin

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## CHAPTER I

### INTRODUCTION AND LITERATURE REVIEW

#### **Introduction**

The United States of America has a vast demand for beef derived meat products, with the world trade of animal derived meat products becoming a larger and growing industry (USDA, 2013). This demand for beef necessitates continuous replenishment of efficient herds of cattle. Therefore, it is imperative to produce beef derived products as efficiently as possible. This involves every level of production, starting with the cow-calf industry. Therefore, optimizing fertility and reproductive efficiency in beef cattle is a vital concern of the beef industry. Factors that might alter the secretion of fertility-related hormones may affect reproduction and potentially affect productivity and profitability. Stress is one factor that can affect fertility-related hormones in beef cattle (Dobson and Smith, 1995, 2000). For example, transportation is known to delay the onset of LH surge in both cows and sheep (Nanda et al., 1989; Smart et al., 1994). 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). Specifically, transportation caused increased rectal temperature as well as circulating concentrations of cortisol and glucose (Price et al., 2012). Prenatal stress is known to affect development of the fetus and lead to adverse health issues later in life (Barker et al., 2002). A prenatal stressor, such as transportation, may affect the progeny of dams undergoing this stress during gestation. Pregnant Brahman cows were transported at 60,

80, 100, 120, and 140 d of gestation. The progeny were studied through weaning, revealing that prenatal transportation stress was associated with altered temperament, circulating cortisol concentrations, and growth rates (Littlejohn et al., 2013; Price, 2013).

### **Physiology of Stress**

Stress is 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 within acceptable ranges of physiological variables (Cannon, 1929). The concept and importance of homeostasis in living organisms has long been known. Claude Bernard was the first to report ideas that would lead to the understanding of homeostasis. In 1878, Bernard stated, “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” (Bernard, 1878). Hans Selye led the way in the early understanding of the concepts of stress which he introduced to the literature in 1936 and defined stress as “the nonspecific response of the body to any demand upon it” (Selye, 1936; Selye, 1974). Selye described a process of events occurring in response to a stressor. These events compose the “General Adaptation Syndrome”. The “General Adaptation Syndrome” consists of an initial “alarm reaction” followed by a period of adaptation to the stress, ending with a final state of exhaustion (Selye, 1974). Selye reported that when injected with various tissue extracts or formalin, rats exhibited larger adrenal glands and degenerated lymphoid tissue in the thymus, spleen, and lymph nodes,

and bleeding gastrointestinal ulcers (Selye, 1952). Every individual reacts differently to stress, thus biological responses to stress are different in every individual (Selye, 1973). Stress may elicit positive effects, negative effects, or no effect on biological systems. Different types and severities of stress have diverse biological effects on every system of the body. 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 the systems in the body and adapt to a constantly changing environment (Selye, 1973).

### ***Hypothalamic-Pituitary-Adrenal Axis***

It has been established that stressors cause activation of the hypothalamic-pituitary-adrenal axis (HPA axis) to mediate a stress response resulting in elevated circulating concentrations of cortisol in the blood (Butcher and Lord, 2004). In response to stress, the neurosecretory neurons of the hypothalamic paraventricular nucleus (PVN) synthesize corticotrophin-releasing hormone (CRH) and arginine vasopressin (VP) (Brown, 1994). Corticotrophin-releasing hormone is a 41 amino acid peptide that regulates anterior pituitary gland secretion (Vale et al., 1981). The secretion of CRH is increased in response to stress (Rivier and Vale, 1983; Gibbs, 1985; Plotsky et al., 1985). The hypothalamus is the primary, but not the sole origin of CRH, as it is also secreted by other tissues such as the placenta; therefore, affecting maternal and fetal

circulation (Challis et al., 1995). Corticotrophin-releasing hormone peptide neurohormone travels through the hypophyseal portal vessels to act on corticotrophes in the anterior pituitary gland. Corticotrophes in the anterior pituitary gland produce a precursor protein known as proopiomelanocortin (POMC), which can be cleaved into the peptides adrenocorticotrophic hormone (ACTH) and  $\beta$ -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 GR, the GR 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, and to allocate resources to produce increased blood glucose for energy expenditure, as well as having catabolic, lipogenic, antireproductive and immunosuppressive effects, in which prolonged exposure can be detrimental to the body (Sapolsky et al., 2000; Gerrard, 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 in 1939 as a “fight or flight response” and was found to be associated with acute changes in adrenal gland secretion. Selye described this response as an “alarm reaction”(Selye, 1973). 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). 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, 2003; Verbrugghe et al., 2012).

## **Fetal Programming**

### ***Prenatal Stress***

Fetal 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). Alterations in fetal environment due to prenatal stress are involved in programming the fetal HPA axis to select for enhanced vigilance to better prepare the neonate to survive in a stressful postnatal environment (Matthews, 2002). Prenatal stress has been shown to affect fetal development and alter health later in life (Barker, 2004). Prenatal and perinatal stress is known to cause increased HPA axis activity in many species such as mice, pigs, sheep, and calves (Lay et al., 1996; Lay et al., 1997a; Lay et al., 1997b; Meaney et al., 2000; Roussel et al., 2004; Williams, 2007). Hypothalamic-pituitary-adrenal axis function has been shown to be affected by an animal’s temperament (Curley et al., 2006; Curley et al., 2008). Circulating concentrations of cortisol and basal adrenal production of cortisol have been shown to be greater in temperamental Brahman calves relative to calmer herd mates (Curley et al., 2006). Temperament is defined as the reactivity of cattle to humans and novel environments (Fordyce et al., 1988). Temperamental calves are more stress responsive than calmer calves (Curley et al., 2006; Curley et al., 2008). Our laboratory recently examined the relationship of prenatal transportation stress with calf temperament, finding that prenatal

transportation stress resulted in Brahman calves being more temperamental through weaning (Littlejohn et al., 2013). Prenatal stress has also been shown to alter fetal testosterone secretion in rats (Ward and Weisz, 1980) and reduce fertility in sexually mature mice (Politch and Herrenkohl, 1984; Herrenkohl, 1986; Crump and Chevins, 1989). Elevated temperament or increased excitability in calves is not desirable from a managerial standpoint, because temperamental cattle have been reported to be harder to manage, have compromised immune function, reduced growth rate, and inferior carcass characteristics (Voisinet et al., 1997; Fell, 1999; King et al., 2006; Cafe et al., 2010). Specifically, temperamental cattle have been reported to produce meat that is more likely to be borderline dark cutting and tougher (Voisinet et al., 1997). Prenatal stress results in fetal alterations that favors enhanced vigilance in attempts to better prepare the neonate to survive in a stressful postnatal environment. In addition, the traits associated with temperamental livestock are typically negative in terms of producing quality meat products. While all living animals constantly undergo some form of stress as stated by Selye in 1973, it is imperative to understand the biological effects and control managerial related stress incurred by gestating livestock.

### ***Mechanisms of Prenatal Stress***

The HPA axis is susceptible to programming during the fetal and perinatal period of life (Matthews, 2000; Meaney, 2001; Seaman-Bridges et al., 2003). Potential mechanisms by which alterations in fetal HPA axis function occur 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  $11\beta$ -hydroxy steroid dehydrogenase type II ( $11\beta$ -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  $11\beta$ -HSD2. Holmes et al. (2006) found that blocking  $11\beta$ -HSD2 with carbenoxolone, a  $11\beta$ -HSD2 inhibitor, in pregnant mice caused an upregulation in glucocorticoid receptors in basolateral, central and medial nuclei in the amygdala portion of the brain and greater basal concentrations of corticosterone in the blood of offspring as adults. The amygdala is located in the midtemporal lobe of the brain and is involved in emotions, social behavior, and memory (Aggleton, 1993). 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). Kraszpulski et al. (2006) reported that prenatal stress (i.e., random handling in a new environment and intramuscular saline injection once a day) in rats was associated with decreased volume, number of neurons, and number of glial cells in the basolateral, central, and lateral nuclei of the amygdala at 25 days of age. However, at 45 days of age no differences in volume, number of neurons, or number of glial cells were observed in prenatally stressed compared to control rats. This suggests that prenatal stress had short-term effects on the physiology of the amygdala in rats. This study also showed that males had a bigger medial amygdala relative to females (Kraszpulski et al., 2006). Contrasting results were reported by Salm et al. (2004) 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. Prenatal stress has been shown to affect the hippocampal portion of the brain in Rhesus monkeys and rats. The hippocampus is located in the medial temporal lobe of the brain and is involved in memory storage and spontaneous activity (Green, 1964). The hippocampus 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 shown to be associated with decreased neurogenesis in the dentate gyrus portion of the hippocampus (Coe et al., 2003; Lemaire et al., 2000 and 2006; Zuena et al., 2008; Kawamura et al., 2006), 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. The sexually dimorphic nucleus in the preoptic area (SDN-POA) of the hypothalamus is involved in sexual behavior (Gorski et al., 1980). Under normal conditions the area comprising SDN-POA exhibits greater neuronal density, larger cells, and larger neurons in males relative to females (Gorski et al., 1980). Anderson et al. (1985) reported that prenatally stressed (i.e., heat from bright lights and restraint) rats exhibited a cross-sectional SDN-POA area that was double the size of the SDN-POA in control males at birth. However, there was a 50% decrease in the area of the cross-sectional SDN-POA in prenatally stressed males compared to control male rats at 20 and 60 days of age. This was similar to the size of the cross-sectional area of the SDN-POA of control females in the study, suggesting feminization likely as a result of lack of exposure to gonadal hormones in the SDN-POA in males (Anderson et al., 1985; Gorski et al., 1980). The previously described mechanisms reveal that prenatal stress is in fact associated with various physiological alterations in the brain of the affected offspring, and that it may produce sexually dimorphic effects.

### ***Epigenetics***

Prenatal stress induced fetal programming results from epigenetic alterations in the genome of the fetus. However, it is important to note that epigenetic signals alter the function of the genome without altering the DNA sequence itself (Waddington, 1957). Epigenetic signaling involves chemical modifications to the DNA or the regions surrounding the DNA, identified 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 DNA histone combination, 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, causing a space between DNA and histones and resulting in transcription factors gaining access to regulatory sites on DNA, such as DNA methylations, or histones (Zhang and Meaney, 2010). Methylation of DNA can result in gene silencing and occurs when enzymes cause the addition of a methyl group to a cytosine within the DNA. This generally takes place during early fetal development, affecting cellular differentiation, and is considered to be irreversible (Zhang and Meaney, 2010). Therefore, environmental effects on cellular differentiation during fetal development can affect genotypic function in addition to phenotype in a mature individual. For example, Lillycrop et al. (2005) reported that the stressful occurrence of protein restriction during fetal development is associated with decreased methylation of a glucocorticoid receptor promoter region resulting in increased expression of glucocorticoid receptor and

decreased 11 $\beta$ -HSD2 expression in the liver, lung, kidney, and brain of rats (Lillicrop et al., 2005).

### ***Factors Affecting Prenatal Stress***

Fetal programming elicits effects differently in different individuals, as Selye (1973) pointed out that different individuals respond differently to stress. Prenatally stressed individuals may be affected by factors such as the type of prenatal stress, the duration of prenatal stress, and the stage of gestation at which the prenatal stress was experienced. It is also imperative to note that many studies have revealed differences in effects of prenatal stress between males and females, specifically on neurological development (Kapoor et al., 2006). Postnatally, female lambs whose dams were 50% nutrient restricted during gestation exhibited greater circulating concentrations of cortisol and ACTH in response to a challenge with exogenous CRH relative to their male counterparts (Chadio et al., 2007). Female but not male rats born to dams that were subjected to noise and light stress at random times throughout pregnancy had decreased GR binding sites in the hippocampus compared to controls (Weinstock et al., 1992). Thus in times of high circulating concentrations of glucocorticoids, when the hippocampus would typically send negative feedback signals to suppress CRH production from the hypothalamus leading to decreased glucocorticoid production, there might be less negative feedback from the hippocampus, less suppression of the production of glucocorticoids, and a prolonged stress response. Montano et al. (1993) reported that there is a greater transfer of glucocorticoids across the placenta in the

female fetal compared to male fetal mice. Prenatal stress, specifically sex-specific responses to prenatal stress, may additionally be affected by gestational timing of the stressor. Females rats whose dams were restrained daily during the last week of gestation exhibited greater basal corticosterone concentrations, lower density of hippocampal corticosteroid receptors (Szuran et al., 2000) , and greater circulating concentrations of corticosterone and ACTH in response to restraint (McCormick et al., 1995), than controls and no difference was observed in prenatally stressed males. Contrasting results have been reported by Mueller and Bale (2008), stating that males whose dams underwent exposure to a different stressor (36 h of constant light, 15 min of fox odor exposure, novel object exposure overnight, 5 min of restraint stress in a 50 ml conical tube, novel noise overnight, multiple cage changes throughout the light cycle, and saturated bedding overnight) on each of the first seven days of gestation exhibited maladaptive behavior in response to stress, increased HPA axis activity, long term alterations in GR expression, and alterations in CRH and GR gene methylation correlated with altered gene expression. This study also reported that early gestational stress resulted in increased expression of PPAR $\alpha$  (peroxisome proliferator-activated receptor  $\alpha$ ), IGFBP-1 (insulin-like growth factor binding protein 1), HIF3 $\alpha$  (hypoxia-inducible factor 3a), and GLUT4 (glucose transporter 4) in placentas of dams of males but not females. Mueller and Bale (2008) suggest that placental alterations in dams of prenatally stressed males are the key to understanding the epigenetic effects of prenatal stress, as the brain is not present in rats during the stage of gestation that these rat's mothers were exposed to stressors (days 1-7 of gestation). Rakers et al. (2012) examined

effects of prenatal stress in the ovine between two separate models, stress during early gestation (30-100 days of gestation) compared to stress during late gestation (100-120 days of gestation). Dams were held in an isolation box (fully isolated from auditory, tactile, or visual contact with flock mates) twice each week for 3 hours, is an exceptionally stressful event for sheep, given they are gregarious animals. Rakers et al. (2012) reported that progeny exposed to prenatal stress early in gestation had higher cortisol concentrations relative to offspring exposed to prenatal stress later in gestation. Therefore, early prenatal stress had the greatest effect on alteration of the fetal HPA axis in the ovine. Contrasting and varying results reveal that prenatal stress is not monotone, but instead complex and multifaceted resulting in diverse outcomes and effects.

## **Male Endocrinology**

### ***The Hypothalamic-Pituitary-Testicular Axis***

The relationship between hypothalamic, pituitary, and testicular function has been well established (Thompson, 1951; Schally and Kastin, 1970; Schanbacher, 1982). Gonadotropin releasing hormone (GnRH) is secreted from the hypothalamus to cause the release of gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), from the anterior pituitary. Luteinizing hormone acts on receptors on Leydig cells in the testes to cause the secretion of androgens such as testosterone (Thompson, 1951; Schanbacher, 1982). Normal patterns of LH pulsatility vary from animal to animal, and can be affected by factors such as breed, age, reproductive status, and environment (Mongkonpunya et al., 1975; Lacroix and Pelletier, 1979; Godfrey et al., 1990; Chase et

al., 1997). Katongole and associates (1971) reported that mature bulls exhibited 5-10 pulses in a 24-h period. Circadian rhythms have been reported to have no effect on LH production in the bull (Katongole et al., 1971; Smith et al., 1973). Testosterone is the major regulatory feedback hormone for production of LH from the anterior pituitary in males (Boyd and Johnson, 1968; Schally and Kastin, 1970; Schanbacher, 1980).

Katongole and associates (1971) reported a time lag relationship between LH and testosterone in mature bulls. This study demonstrated that a peak in LH concentration resulted in a peak in testosterone concentration approximately 30 min following the peak in LH concentration. On average, an endogenous peak in LH is near 2.4 ng/ml in dairy bulls (Smith et al., 1973). The same study showed that stimuli, such as the sight of a cow, could induce LH release from the anterior pituitary to cause increased testosterone production. However, if testosterone concentrations were already at maximum levels, there was no further increase in testosterone concentration as a result of the stimuli-induced increase in LH concentration (Katongole et al., 1971).

Administration of exogenous GnRH has been shown to cause an increase in circulating concentrations of testosterone in mature dairy bulls (Zolman and Convey, 1973). Tannen and Convey (1977) reported that the magnitude of an LH peak is directly related to the magnitude of the following peak in testosterone in intact dairy bulls in response to GnRH. Rutter and associates (1991) reported that Brahman bulls had similar GnRH-induced LH responses compared to pubertal dairy bulls in a study by Tannen and Convey (1977). Mongkonpunya and associates (1975) reported that circulating concentrations of testosterone were increased threefold in dairy bulls at 6 months of age

in response to GnRH-induced LH secretion. In this study, researchers administered one of three doses of exogenous GnRH on different days, including 200, 400, and 800 µg given in the muscle. Of the three doses, researchers found that the 800 µg dose resulted in a prolonged return to baseline for LH concentrations. Prior to GnRH administration, basal LH concentrations were 1 ng/ml on average regardless of dose or age.

Approximately 45 min following GnRH administration, peak LH concentration occurred at 24 ng/ml (Mongkonpunya et al., 1975). Schanbacher and Echterkamp (1978) reported that intravenous administration of a 500 µg dose of GnRH in mature Herford bulls induced a 30-fold increase in LH concentration in peripheral blood. Testosterone concentration was increased 7-fold in response to the GnRH-induced LH release.

Testosterone concentration did not return to basal concentrations until 8 h after GnRH administration (Schanbacher and Echterkamp, 1978). A dose of 200 µg GnRH has been used effectively in Brahman bulls to elicit a response in LH and testosterone (Godfrey et al., 1989; Godfrey et al., 1990; Rutter et al., 1991). After administering 200 µg GnRH, Rutter and associates (1991) collected blood samples every 15 min for 6 h.

Concentration of LH returned to baseline within the 6 h sampling period, but the testosterone response concentration may not have reached basal values within the 6 h sampling period. Therefore, it may have been useful to extend the sampling period.

Although 200 µg of GnRH was effective in inducing an LH and testosterone response, the common dose for every animal did not account for differences in body weight.

Rocha et al. (1995) controlled for potential differences due to BW by administering an



effective dose of 10 ng/kg of BW into an indwelling jugular catheter to examine response to GnRH in Brahman bulls (Rocha et al., 1995).

### **Relationship of the Hypothalamic-Pituitary-Testicular Axis with Adrenal Secretion**

It has been established that a close relationship exists between adrenal and testicular function in bulls (Welsh et al., 1979a; Welsh et al., 1979b; Welsh Jr, 1981; Welsh and Johnson, 1981; Welsh et al., 1981; Johnson et al., 1982). Hardin and Randel (1983) reported that catecholamines epinephrine and norepinephrine have a negative relationship with LH secretion in prepubertal heifers. Welsh and collaborators (1979a,b) demonstrated that circulating concentrations of cortisol are negatively associated with circulating concentrations of LH and testosterone in bulls. In this study, 93% of LH peaks took place at the time of basal or declining concentrations of cortisol. Welsh and Johnson (1981) reported that increased production of corticosteroids in response to a stressor results in suppression of LH and testosterone in bulls. This study demonstrated that peak concentrations of serum cortisol occurred 15 min after encountering a stressor, which in this case was electroejaculation. Concentrations of serum LH were significantly lower from 45 min to 4 h following electroejaculation. Concentrations of serum testosterone decreased after 1 h, and this decrease was believed to be delayed due to a testosterone peak immediately preceding electroejaculation. In the proposed study, bulls will be given a minimum acclimation period of 15 min between placement in stanchions and the first sampling period. However, if animals remain in an excited state following the acclimation period, cortisol concentrations are expected to be elevated in the blood,

resulting in suppression of LH and testosterone as previously discussed. Evaluating endocrine profiles of bulls in the summer raises additional concerns which must be controlled to accurately analyze LH and testosterone production. Concentrations of endogenous cortisol in peripheral blood have been shown to be greater in June compared to January (Welsh et al., 1981). The well-established relationship between LH and testosterone was less consistent in June compared to January (Welsh et al., 1981). Season has been shown to affect fertility in Brahman bulls (Fields et al., 1982; Godfrey et al., 1990). Godfrey and associates (1990) reported that young ( $20 \pm 0.8$  mo at the start of the study) sexually mature Brahman bulls have decreased semen quality and testosterone production in the winter. Therefore, summer is an appropriate time to examine endocrine profiles in tropically adapted *Bos indicus* bulls in order to avoid effects of seasonality and cooler weather.

Because one's health begins *in utero*, this study sought to investigate how a prenatal stressor, such as transportation, may affect the progeny of dams undergoing chronic instances of stress during gestation. Specifically, this project was designed to determine if transportation of pregnant Brahman cows (prenatal stress, PNS) affects their bull calves' secretion of reproduction related hormones LH and testosterone, and the stress hormone cortisol, as sexually mature bulls. Specifically, the goals of this study were to:

1. Characterize endogenous pulses and profiles of LH and testosterone in prenatally stressed compared to control bulls;

2. Characterize LH and testosterone profiles in response to exogenous GnRH in prenatally stressed compared to control bulls;
3. Examine the relationship between LH and testosterone in PNS relative to control bulls; and,
4. Examine effects of cortisol on endogenous LH and testosterone secretion prior to GnRH administration in prenatally stressed relative to control bulls.

CHAPTER II  
INFLUENCE OF PRENATAL TRANSPORTATION STRESS ON LH,  
TESTOSTERONE, AND CORTISOL SECRETION IN SEXUALLY MATURE  
BRAHMAN BULLS

**Introduction**

It is a relatively common occurrence in most production situations for cows to encounter stressors during gestation. Stressors can be derived from natural or managerial origins, including circumstances such as predation, weaning, castration, handling, and transportation. When an individual encounters a stressor, the hypothalamic-pituitary-adrenal (HPA) axis is activated, resulting in the release of cortisol from the adrenal cortex and catecholamines from the adrenal medulla (Sapolsky et al., 2000; Chrousos, 2009). Cortisol and catecholamines are known to have catabolic, lipogenic, lypolytic, antireproductive and immunosuppressive effects, in which prolonged exposure can be detrimental to the body (Sapolsky et al., 2000; Gerrard, 2003; Charmandari et al., 2005; Chrousos, 2009). Transportation of the pregnant bovine is a common practice with realistic production concerns. As previously reported by our laboratory, transportation during gestation is a stressor for Brahman cows (Lay et al., 1996; Price et al., 2012). Specifically, the transportation process increased body temperature as well as circulating concentrations of cortisol and glucose in pregnant Brahman cows (Price et al., 2012). Elevated maternal cortisol may alter the fetal environment, therefore affecting fetal development. The concept that environmental insults experienced by a fetus during

gestation can have lasting effects on an individual's health through adulthood was explored by Barker (1990, 2002). Prenatal stress can affect development of the fetus and lead to adverse health issues later in life, such as coronary heart disease, and type 2 diabetes (Barker et al., 2002). Temperament is defined as the reactivity of cattle to humans and novel environments (Fordyce et al., 1988). Temperament is known to influence stress responsiveness, and temperamental animals have been found to exhibit greater cortisol and catecholamine concentrations after encountering a stressor when compared to calm animals (Stahringer et al., 1990; Curley et al., 2006; Curley et al., 2008; Burdick et al., 2010). Increased circulating concentrations of cortisol in peripheral blood are known to have suppressive effects on LH and testosterone secretion in bulls (Welsh et al., 1979a; Welsh et al., 1979b; Welsh, 1981; Welsh and Johnson, 1981; Welsh et al., 1981; Welsh et al., 1982). Additionally, Hardin and Randel (1983) reported that catecholamines have suppressive effects on LH secretion. The current study examined the secretion of LH, testosterone, and cortisol before and after GnRH stimulation in 12 control and 11 prenatally stressed (PNS) sexually mature Brahman bulls. Control bulls were derived from non-transported pregnant cows, and PNS bulls were derived from cows transported for a 2-h period at 60, 80, 100, 120, and 140 ± 5 d of gestation. The bulls in this study were found to have elevated temperament scores through weaning compared to control bulls (Littlejohn et al., 2013). This study sought to investigate how a prenatal stressor, such as transportation, may affect the production of LH, testosterone, and cortisol in progeny of dams undergoing chronic instances of stress during gestation.

## **Materials and Methods**

All proposed experimental procedures comply 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 prior to experimentation (AUP # 2013-008A).

### ***Animal Procedures***

Ninety-six mature pregnant Brahman cows (*Bos indicus*) were assigned to one of two treatment groups based on age, parity, and temperament. Forty-eight cows were maintained as controls throughout the study and 48 cows underwent transportation events during gestation. The stressor consisted of 5 transportation events of 2 hours each at 60, 80, 100, 120, and  $140 \pm 5$  days of gestation. Control cows were maintained in the same pastures as the stressed cows (at the Texas A & M AgriLife Research Center in Overton) and fed the same diet. The prenatally stressed calves utilized in this study were born in the Spring of 2012 and reached sexual maturity during the Summer of 2013. The control group consisted of 25 males and the prenatally stressed treatment group consisted of 21 males. Temperament classification data were recorded at weaning, at which time calves were restrained in a working chute, and a whole blood sample ( $2 \times 10$  mL) was collected via jugular venipuncture in an uncoated tube (BD, Franklin Lakes, NJ) and serum isolated for determination of cortisol concentrations. From this calf crop, all bulls were electroejaculated every 2 wk beginning when each bull reached a

minimum scrotal circumference of 24 cm and concluding when each bull reached sexual maturity (i.e., 500,000,000 sperm/ejaculate). The level of sexual maturity of the bulls to be challenged was a concern when designing the project, because if there are less than adequate gonadotropin receptor sites in the testes, there will not be an ample response to GnRH (Schally and Kastin, 1972; Roth et al., 1973). Of these bulls, the initial 11-12 bulls from each treatment group to reach sexual maturity were utilized to compare pituitary and testicular function between prenatally stressed and control animals. The challenges were carried out between 1 and 3 wk after reaching sexual maturity. During each challenge day, the bulls that had reached sexual maturity were utilized for a period of 14 h (6 h before GnRH injection and 8 h after GnRH injection) while being confined within a stanchion. Sampling was initiated at a similar time of day for each bull (0730 h  $\pm$  45 min). A 6 h sampling period prior to GnRH administration was chosen in an attempt to observe a minimum of one endogenous LH pulse in each animal. An 8 h sampling period following GnRH administration was chosen to allow ample time for LH and testosterone concentrations to return to basal concentrations. Each animal was fitted with an indwelling jugular cannula immediately prior to the blood sampling period. At the time of cannulation, one 10 mL blood sample was collected from each animal to test the patency of the cannula. Following cannulation, each animal was moved from the chute into a stanchion, where each bull was allowed to acclimate for a minimum of 15 min prior to the first sampling period. Each bull remained in its stanchion for the remainder of the sampling period. Blood sampling commenced following the cannulation of the final animal to be sampled that day and continued at 15-min intervals

for a period of 6 h (360 min) to determine the pattern of LH release. Blood samples were collected in 10-mL vacutainers, centrifuged to obtain serum, and stored at -20°C. After the 360-min blood sample, GnRH was administered in the form of Cystorelin (50 µg/mL), gonadorelin diacetate tetrahydrate, to each animal at a dose of 10 ng /kg of BW through the jugular cannula. Following the GnRH treatment, collections were resumed and continued at 15-min intervals for 8 h to determine response of LH and testosterone to GnRH injection. A total of 57 blood samples were taken from each animal. After each collection, 10 mL of sterile saline were injected for fluid replacement. Heparinized-saline was used to flush the extension tubing to prevent clotting and sustain patency of the cannula. After the final blood sample was collected, the cannulas were removed and the animals returned to the herd.

### ***Measures for Temperament Classification***

To quantify temperament, three measures of temperament were assessed at weaning, including pen score, exit velocity, and an overall temperament score. Pen score is a subjective measurement, in which a single experienced evaluator individually scores calves in groups of 3-5 animals within a confined pen. Reactions to that evaluator are assessed on a scale of 1-5 (Hammond et al., 1996) . A pen score of 1 is defined as nonaggressive or docile, and 5 is defined as easily excitable, very aggressive, or dangerous (Table 1). For scoring consistency, the same individual was responsible for assigning all pen scores for the animals in the study. Exit velocity is an objective measurement that is defined as the rate, measured in m/s, at which an animal traverses



1.83 m upon exiting a squeeze chute (Burrow, 1988; Curley et al., 2006). Time is initiated when the animal crosses the first infrared beam sensor and stopped upon crossing the second infrared beam sensor. Temperament score is defined as the numerical average of pen score and exit velocity [TS = (PS + EV)/2] (Curley et al., 2006; King et al., 2006). Calves 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 1.** Observations associated with the individual categories of pen scores (Hammond et al., 1996)

Pen Score	Description
1	Walks slowly, can be approached slowly, not excited by humans
2	Runs along fences, stands in corner if humans stay away
3	Runs along fences, head up and will run if humans come closer, stops before hitting gates and fences, avoids humans
4	Runs, stays in back of group, head high and very aware of humans, may run into fences and gates
5	Excited, runs into fences, runs over anything in its path

### ***Cortisol Assay***

Serum concentrations of cortisol were determined using a single antibody radioimmunoassay (Coat-A-Count Cortisol Kit # TKC05, Siemens Medical Solutions Diagnostics, USA) that utilized antiserum coated tubes according to the manufacturer's directions (Burdick et al., 2009; Hulbert et al., 2012) (Appendix C). The minimum detectable cortisol concentrations for this assay are known to be 3 ng/mL and the intra- and inter-assay coefficients of variation were 5.20% and 15.71%, respectively. Serum cortisol concentrations were determined by comparison of unknown samples with a standard curve generated from known concentrations of cortisol. Data from this assay are presented as concentrations in ng/mL.

### ***Luteinizing Hormone Assay***

Concentrations of LH in serum were assayed with a double antibody radioimmunoassay (RIA) using rabbit anti-ovine LH (AFP192279; National Hormone and Peptide Programme, Torrance, CA, USA). A highly purified ovine LH (AFP8614B; National Hormone and Peptide Programme) was used for the iodinated tracer and reference standard preparation using the method of Rivera et al. (2011) (Appendix A). The intra-assay coefficient of variation was 10.85% and the inter-assay coefficient of variation was 10.24%. The Pulse XP algorithm program was utilized in the period preceding GnRH administration to assess endogenous LH pulse incidence and area under the concentration data. An endogenous peak in LH should result in a peak in

testosterone. Therefore, occurrence of an elevation in testosterone in response to LH was also used as criteria to classify LH peak occurrence. Data from this assay are presented as concentrations in ng/mL.

### ***Testosterone Assay***

Concentrations of testosterone in serum were assayed using the method of Vera-Avila et al. (1997) (Appendix B). The antibody (II-BSA#250), obtained from G. D. Niswender (Colorado State University, Fort Collins, CO). The inter-assay coefficient of variation was 12.98%, and the intra-assay coefficient of variation was 7.14%. The Pulse XP algorithm program was utilized to assess area under the curve. Data from this assay are presented as concentrations in ng/mL.

### ***Statistical Analysis***

Data were analyzed (SAS, 2011) using a fixed effect model, with treatment included in the model. Endocrine profiles from time -240 to time 360, -240 to 0, and 0 to 360 relative to GnRH administration were analyzed for LH and testosterone using repeated measures ANOVA. Time -360 to time 0 relative to GnRH administration were analyzed for cortisol using repeated measures ANOVA. The incidence of LH pulses during the pre-GnRH period as well as the occurrence of a resulting endogenous testosterone response was compared between treatment groups by chi-square analysis (JMP\_Pro, 2012).

## **Results**

### ***Temperament***

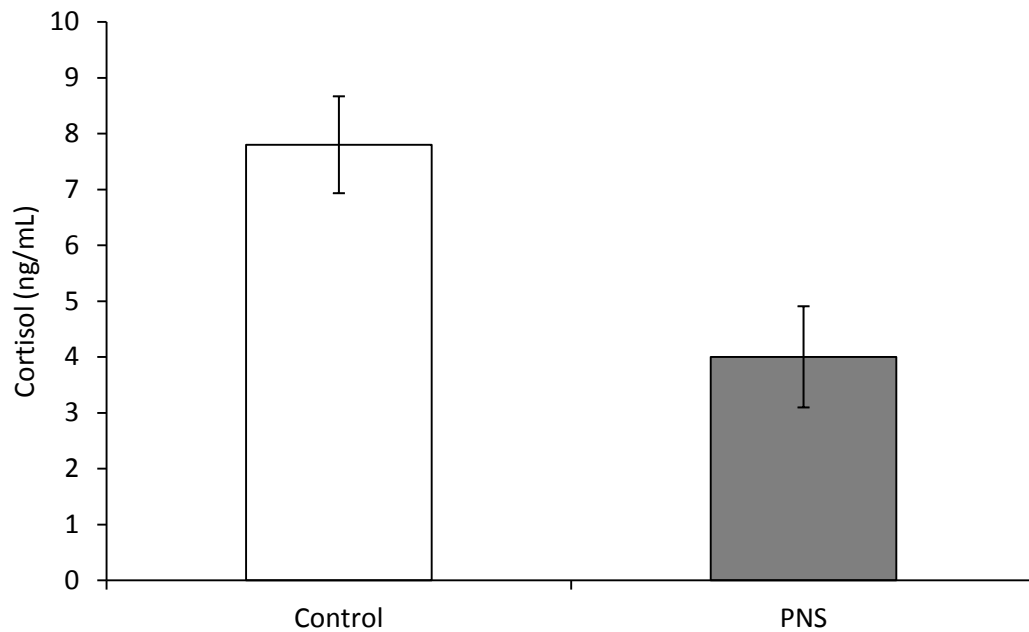
Temperament data were not analyzed in this study due to uneven temperament groups. Because the first bulls to reach sexual maturity were utilized in this study, there was no means to balance the number of test subjects within temperament groups. As a result, the sample group consisted of 13 calm (8 control and 5 PNS), 7 intermediate (2 control and 5 PNS), and 3 temperamental (2 control and 1 PNS) bulls. Season and photoperiod have been shown to affect LH production in Brahman bulls (Godfrey et al., 1990). Godfrey et al. (1990) reported that young ( $20 \pm 0.8$  mo at the start of the study) sexually mature Brahman bulls have decreased semen quality and testosterone production in the winter. Because bulls were sampled between July 6 and September 13, continuing to sample bulls later in the year would have resulted in negative effects of season on LH and testosterone secretion.

### ***Cortisol***

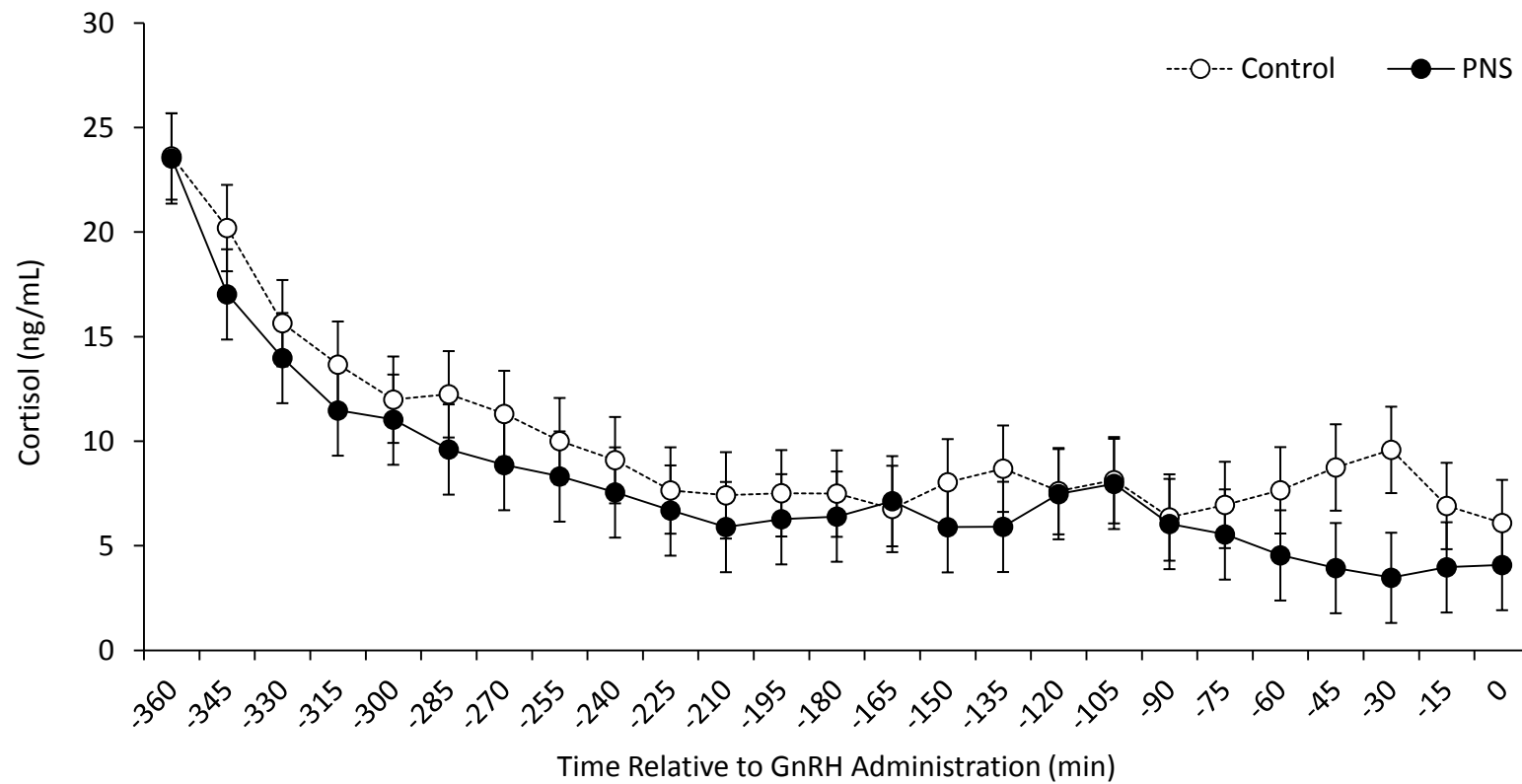
Cortisol was measured from 360 min prior to GnRH treatment (time -360) until

the time of GnRH treatment (time 0). Measurements taken to assess endogenous cortisol included area under the curve (AUC) during the first two h of sampling (time -360 to -255), AUC during the following 4 h (time -240 to 0), average cortisol concentration in the h prior to GnRH administration, as well as cortisol concentration at time -360, -240, and time 0. Cortisol concentration profiles were also assessed and compared between control and PNS bulls.

Although the overall cortisol concentration profile was not statistically different in control compared to PNS bulls, average cortisol concentration in the h prior to GnRH administration was greater ( $P = 0.0064$ ) in control bulls ( $7.8 \pm 0.8673$  ng/ml) relative to PNS bulls ( $4.0 \pm 0.9059$  ng/ml) (Fig. 1). There was no statistical difference in the overall cortisol concentration profiles between control and PNS bulls (Fig. 2). However, the cortisol concentration profile reveals that there was a time effect ( $P = <0.0001$ ) on cortisol concentrations, with the greatest cortisol concentrations at the beginning of the sampling period and then decreasing over time.



**Figure 1.** Average cortisol concentration in the h prior to GnRH administration in prenatally stressed (PNS; grey) compared to control (white) bulls. The numerical average was taken of cortisol concentrations for the 4 samples in the h preceding GnRH administration. PNS ( $4.0 \pm 0.9059$ ) bulls had lower ( $P = 0.0064$ ) cortisol concentration in the h preceding GnRH administration relative to control ( $7.8 \pm 0.8673$ ) bulls.



**Figure 2.** Cortisol concentration profiles prior to GnRH administration in prenatally stressed (PNS; black circles with solid line) compared to control (white circles with dashed line) bulls. Overall, there was no difference ( $P = 0.3306$ ) in cortisol concentration from time -360 to time 0 relative to GnRH administration between PNS bulls and control bulls.

### *Luteinizing Hormone*

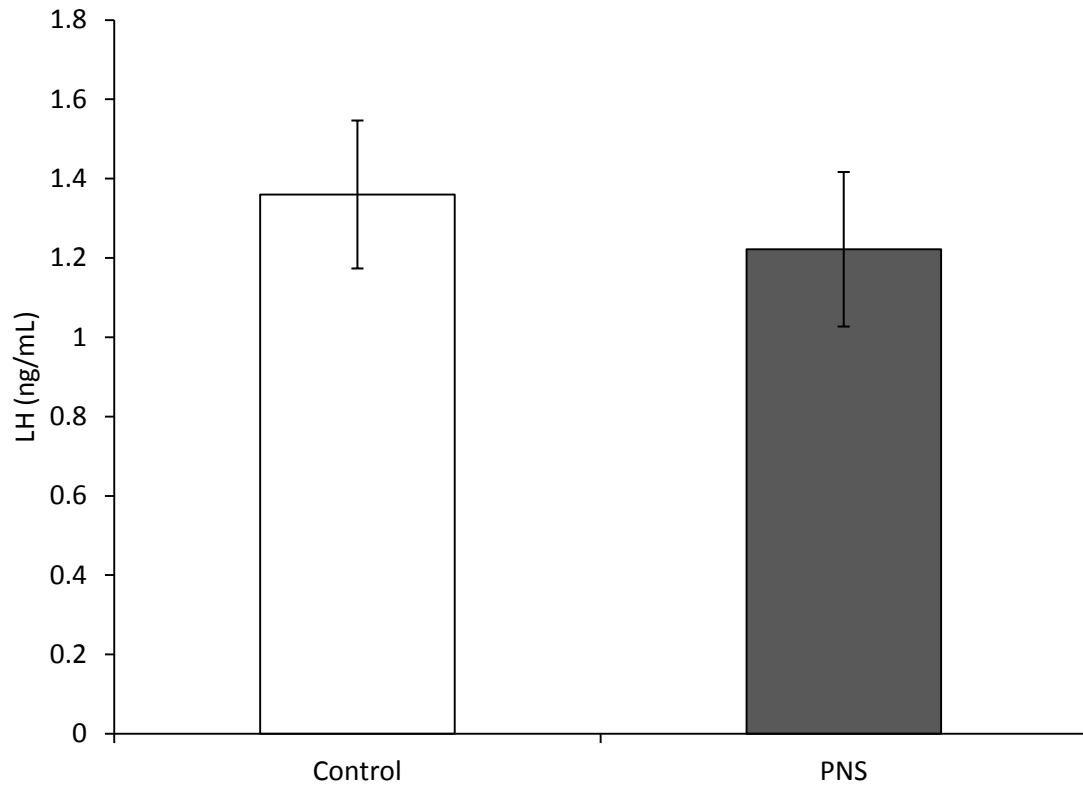
Measurements taken to assess endogenous LH included maximum concentration and amplitude of a detectable LH pulse, baseline concentration of LH, area under the LH curve (AUC), LH concentration at time -240, average LH concentration in the h preceding GnRH administration, and profile of LH prior to GnRH administration. These variables were calculated for the 4-h period immediately preceding GnRH administration. Measurements taken to assess exogenous GnRH-induced LH response included maximum concentration and amplitude (maximum concentration – baseline) of the GnRH-induced LH release, AUC post-GnRH administration, the duration of the GnRH-induced LH release, LH concentration at time 0 and time 360, as well as the profile of LH following GnRH administration. More PNS bulls exhibited an LH pulse in the period prior to GnRH administration, which consisted of a sampling period from 240 min prior to GnRH administration to the time of GnRH administration, (9 of 11;  $P < 0.01$ ) relative to control bulls (3 of 12). These results as well as simple means for the measurable LH pulses are shown in Table 2. The PNS bulls exhibited greater means for maximum concentration of and pulse amplitude of detectable pulses relative to control bulls. Endogenous pulses were classified not detectable if they failed to reach the maximum concentration of the pulse prior to time 0 (i.e., time of exogenous GnRH administration), because exogenous GnRH affected LH concentrations after that point. There was no difference ( $P = 0.6140$ ) in average LH concentration in the h prior to GnRH administration between PNS bulls ( $1.2 \pm 0.2$  ng/mL) relative to control bulls ( $1.4 \pm 0.2$  ng/mL) (Fig. 3). There was no difference ( $P = 0.67$ ) in the profile of LH



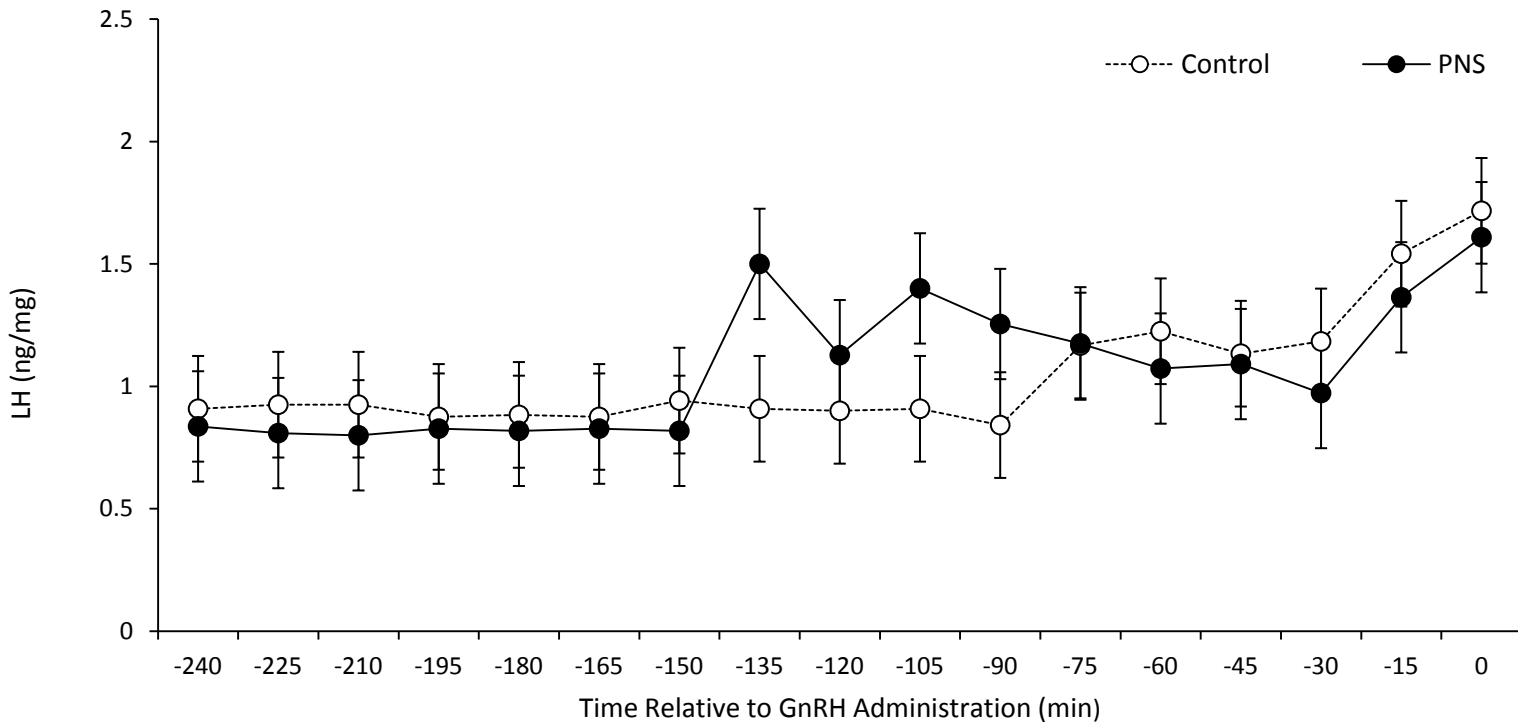
concentration prior to GnRH administration between PNS and Control bulls (Fig. 4). No other characteristic associated with the endogenous release of LH prior to GnRH treatment evaluated in this study differed between treatment groups ( $P > 0.1$ ). All bulls responded similarly to exogenous GnRH, with the exception of the duration of the GnRH-induced LH response. Duration of GnRH-induced LH response was calculated as the duration of time taken to return to basal concentrations of LH. Basal concentrations were defined as baseline, which was calculated as the average of every time point in the 4-h period immediately preceding GnRH administration that was not a component of an endogenous LH pulse. Prenatally stressed bulls had a longer ( $P = 0.02$ ) duration of GnRH-induced LH response ( $268 \pm 18$  min) relative to control bulls ( $207 \pm 16$  min) (Fig. 5). There was no difference ( $P = 1.0$ ) in the profile of LH concentration prior to GnRH administration between PNS and control bulls (Fig. 6). No other characteristic associated with the release of LH following GnRH administration evaluated in this study differed between treatment groups ( $P > 0.1$ ). Luteinizing hormone concentration profiles from time -240 to time 360 relative to GnRH administration were not different ( $P = 1.0$ ) between PNS and control groups (Fig. 7). Variables that differed between PNS and control bulls included pattern of LH secretion before GnRH and duration of GnRH-induced LH release.

**Table 2.** Luteinizing hormone pulse incidence, mean maximum concentration of a pulse, and mean pulse amplitude in prenatally stressed (PNS) and control bulls. Luteinizing hormone pulse incidence was analyzed using chi-square analysis. More ( $P < 0.01$ ) Prenatally stressed bulls exhibited an LH pulse in the 240-min period prior to GnRH injection compared to control bulls. PNS bulls exhibited greater means for maximum concentration of a pulse and pulse amplitude of detectable pulses relative to control bulls.

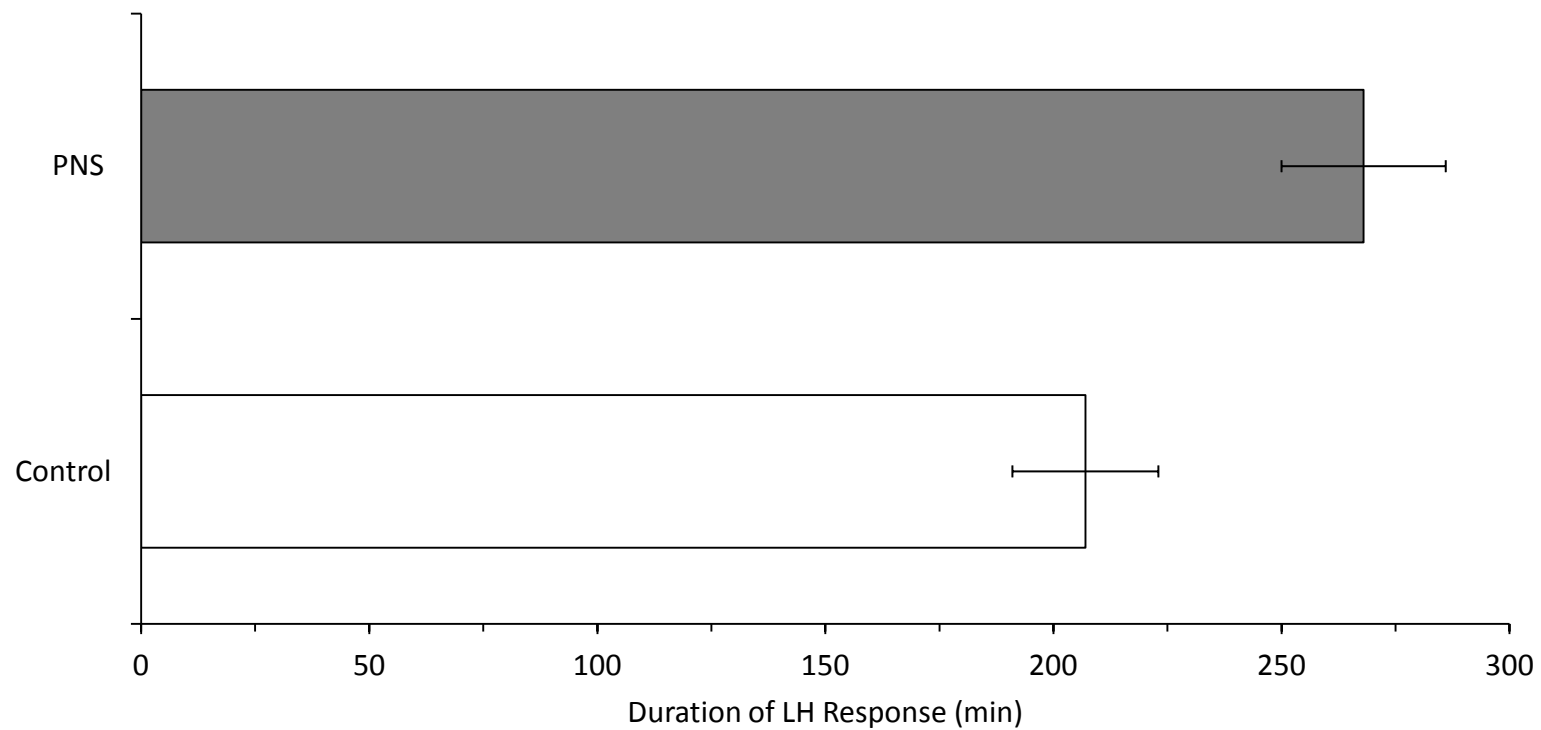
<b>Treatment</b>	<b>Pulse Incidence</b>	<b>Total n</b>	<b>Detectable Pulse n</b>	<b>Mean Maximum Concentration of Pulse</b>	<b>Mean Pulse Amplitude</b>
Control	Pulse	3	3	2.3 ng/mL	1.4 ng/mL
Control	No Pulse	9	—	—	—
PNS	Pulse	9	7	3.3 ng/mL	2.5 ng/mL
PNS	No Pulse	2	—	—	—



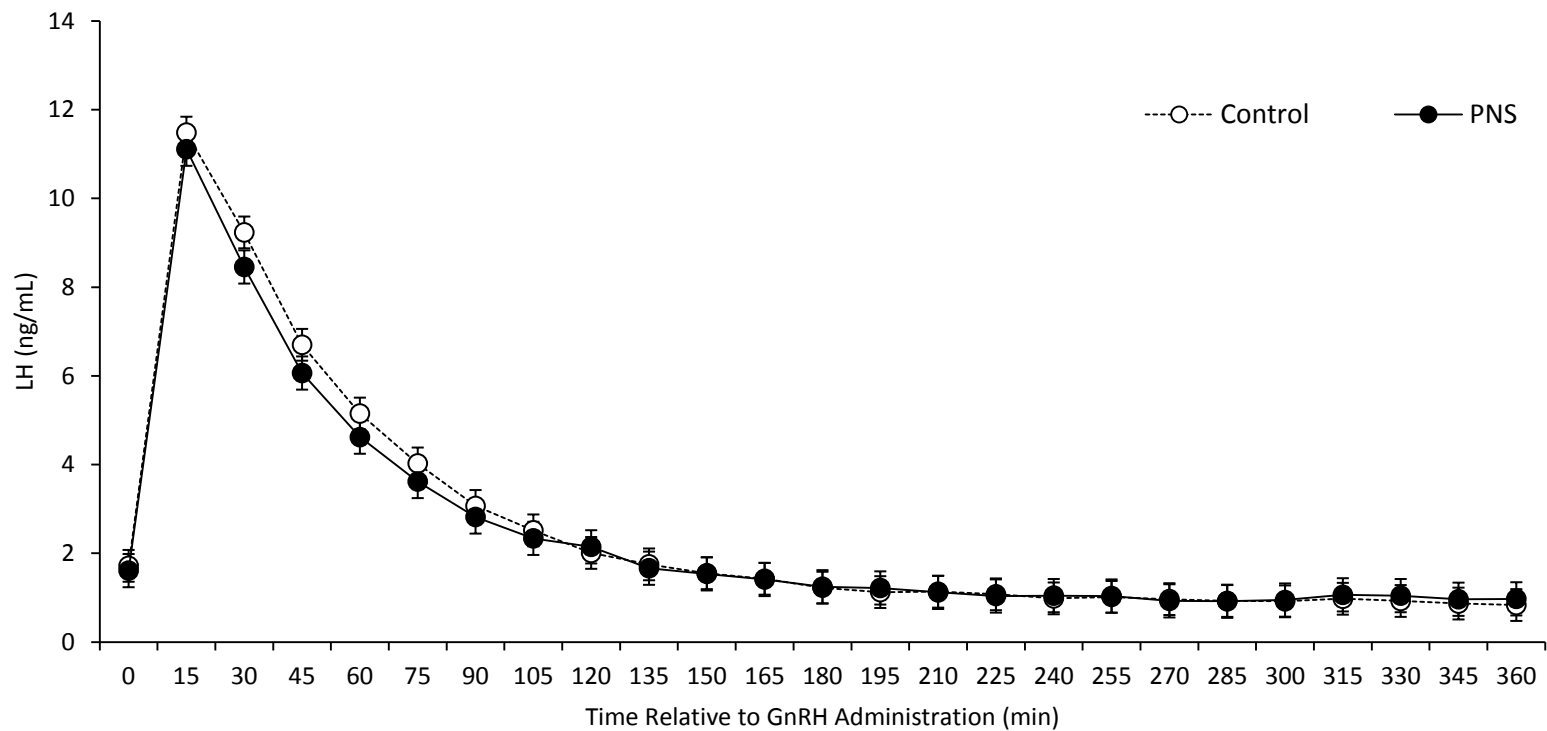
**Figure 3.** Average LH concentration in the h prior to GnRH administration between prenatally stressed (PNS; grey) and control (white) bulls. There was no difference ( $P = 0.614$ ) in average LH concentration in the h prior to GnRH administration between PNS and control bulls.



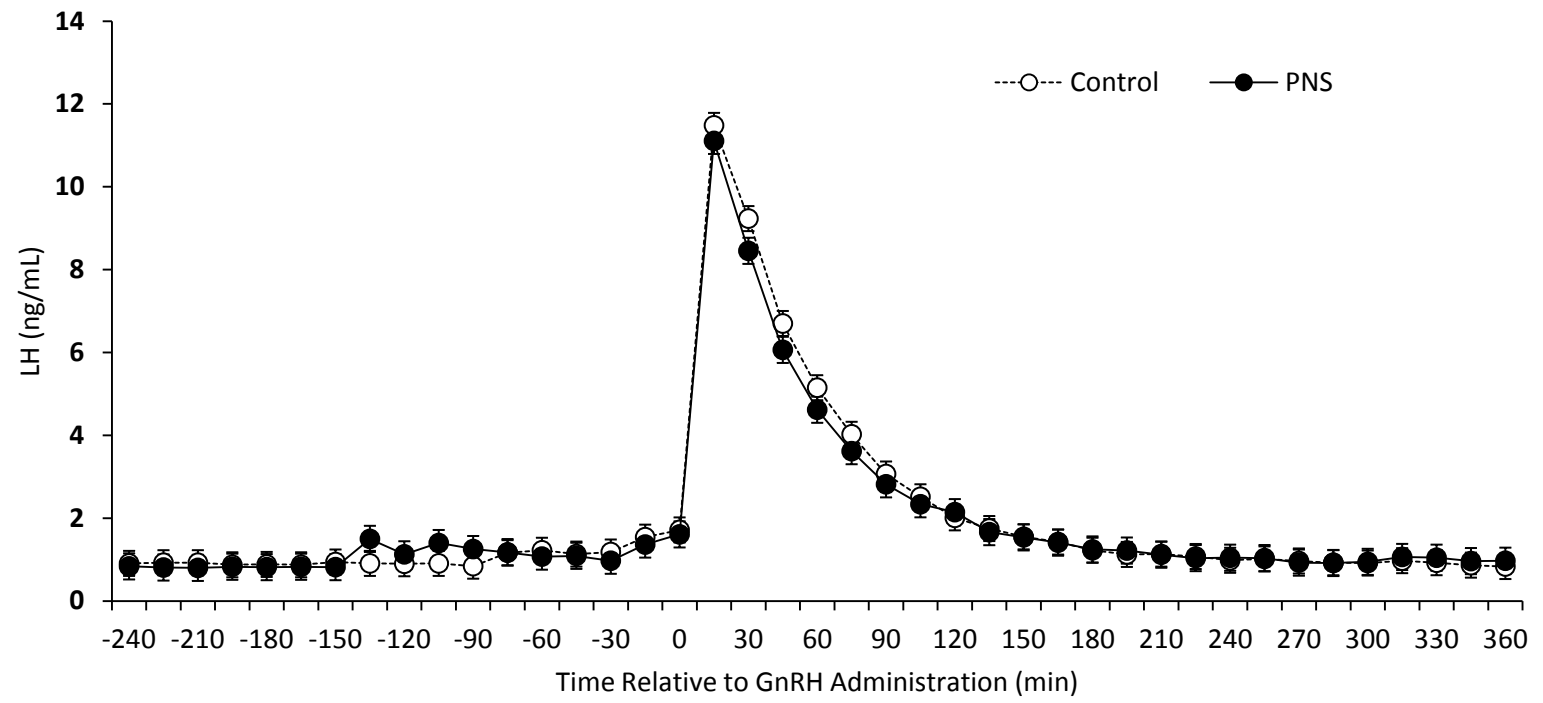
**Figure 4.** Luteinizing hormone concentrations prior to GnRH administration between prenatally stressed (PNS; black circles with solid line) and control (white circles with dashed line) bulls. There was no difference ( $P = 0.67$ ) in the profiles of LH prior to GnRH administration between PNS bulls and control bulls.



**Figure 5.** Duration of GnRH-induced LH release in prenatally stressed bulls (PNS; grey) compared to control bulls (white). Duration of GnRH-induced LH release was greater ( $P = 0.02$ ) in PNS bulls relative to control bulls.



**Figure 6.** Luteinizing hormone concentration profile following GnRH administration in prenatally stressed (PNS; black circles with solid line) and control (white circles with dashed line) bulls. There was no difference ( $P = 1.0$ ) in the profile of LH following GnRH administration between PNS bulls and control bulls.



**Figure 7.** Luteinizing hormone concentration profiles from time -240 to time 360 relative to GnRH administration between prenatally stressed (PNS; black circles with solid line) and control (white circles with dashed line) bulls. LH concentration profiles from time -240 to time 360 relative to GnRH administration were not different ( $P = 1.0$ ) between prenatally stressed and control bulls.

### ***Testosterone***

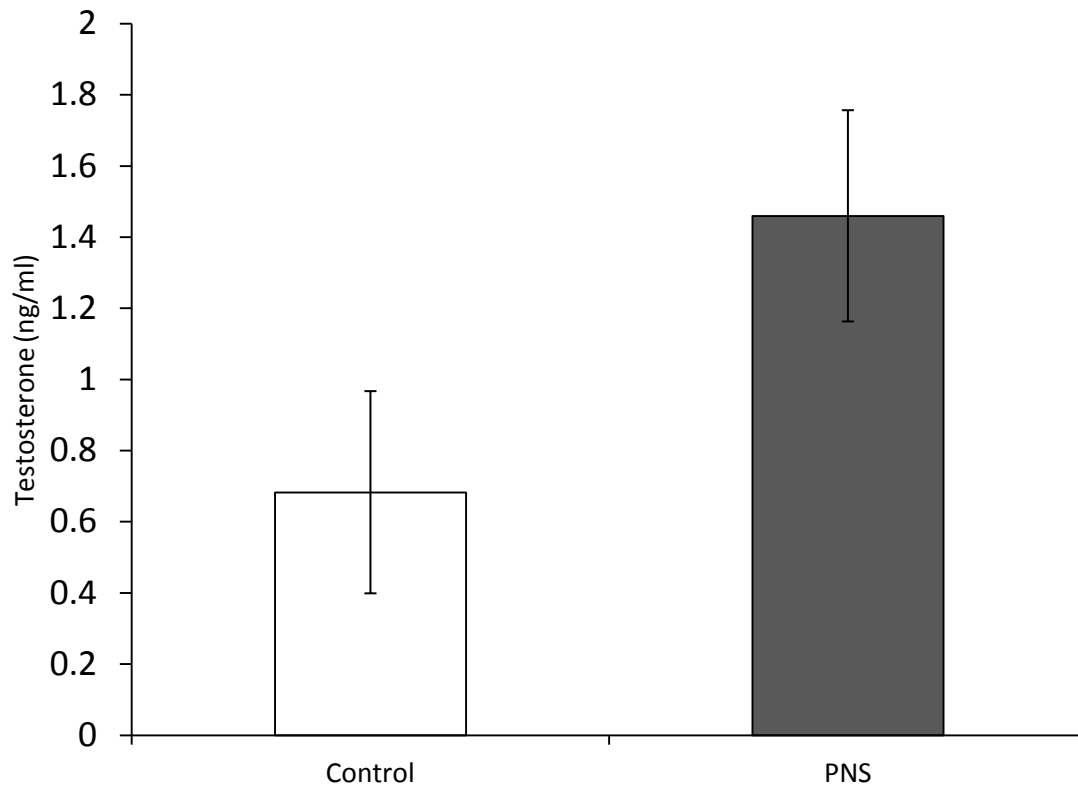
Measurements taken to assess endogenous testosterone included testosterone response incidence, baseline concentration of testosterone (i.e., 5 time points preceding time 0 that were not a component of an endogenous testosterone response), area under the testosterone curve (AUC), testosterone concentration at time -240, maximum concentration and amplitude of a detectable response, and profile of testosterone concentration prior to GnRH administration. These variables were calculated for the 4-h period immediately preceding GnRH administration. Measurements taken to assess exogenous GnRH induced testosterone response included maximum testosterone concentration and amplitude (Maximum concentration – baseline) following GnRH administration, testosterone AUC post-GnRH administration, time at maximum testosterone response to GnRH, testosterone concentration at time 0 and time 360, and profile of testosterone concentration following GnRH administration. More PNS bulls exhibited an endogenous testosterone response (9 of 11;  $P = 0.02$ ) relative to control bulls (4 of 12). These results, as well as simple means for the detectable testosterone pulses are shown in Table 3. PNS bulls exhibited greater means for maximum concentration and pulse amplitude of detectable pulses relative to control bulls. Endogenous pulses were classified as not detectable if they failed to reach the maximum concentration of the response prior to time 0 (time of exogenous GnRH administration), because exogenous GnRH effected LH concentration after that point. One bull in the control group exhibited a testosterone response not preceded by a detectible elevation in LH, speculatively due to inadequate sampling interval for that particular test subject.



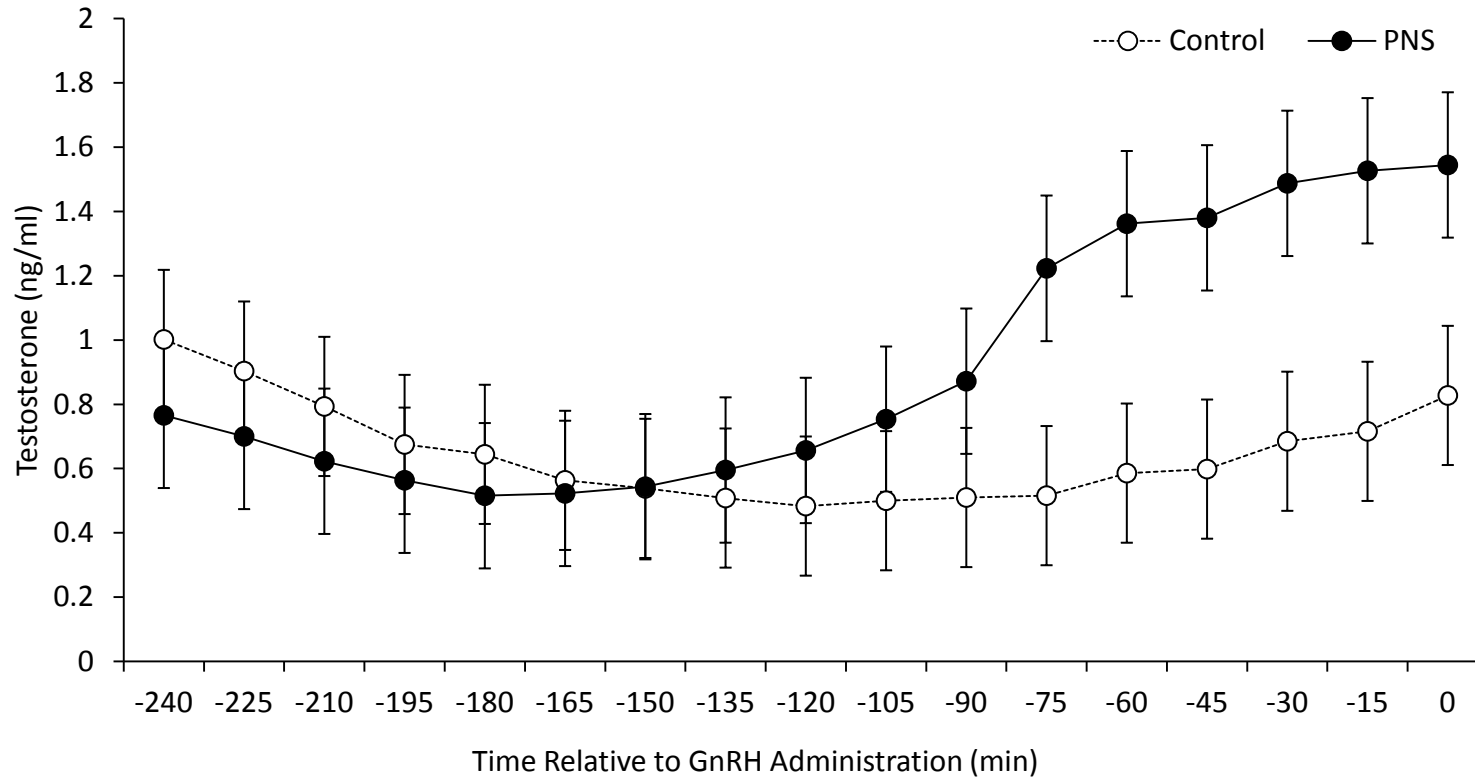
Average testosterone concentration in the h prior to GnRH administration tended to be greater ( $P = 0.0726$ ) in PNS bulls ( $1.5 + 0.3$  ng/mL) relative to control bulls ( $0.7 + 0.3$  ng/mL) (Fig. 8). Profiles of testosterone in the period prior to GnRH administration tended to be greater ( $P=0.0756$ ) in PNS bulls after time -90 relative to GnRH administration compared to control bulls (Fig. 9). No other characteristic associated with the endogenous release of testosterone prior to GnRH treatment evaluated in this study differed between treatment groups ( $P > 0.1$ ). Profiles of testosterone in the period following GnRH administration tended ( $P = 0.09$ ) to be different between treatment groups (Fig.10). No other characteristic associated with testosterone secretion in the period following the administration of exogenous GnRH evaluated in this study differed between treatment groups ( $P > 0.1$ ). The testosterone concentration profile from time -240 to time 360 relative to GnRH administration differed between treatment groups (Fig. 11).

**Table 3.** Testosterone response incidence, mean maximum concentration of a response, and mean response amplitude in prenatally stressed (PNS) and control bulls. Testosterone response incidence was analyzed using chi-square analysis. More ( $P = 0.02$ ) PNS bulls exhibited a testosterone response in the 240-min period prior to GnRH injection relative to control bulls. Prenatally stressed bulls exhibited greater means for maximum concentration of a pulse and pulse amplitude of measurable pulses relative to control bulls.

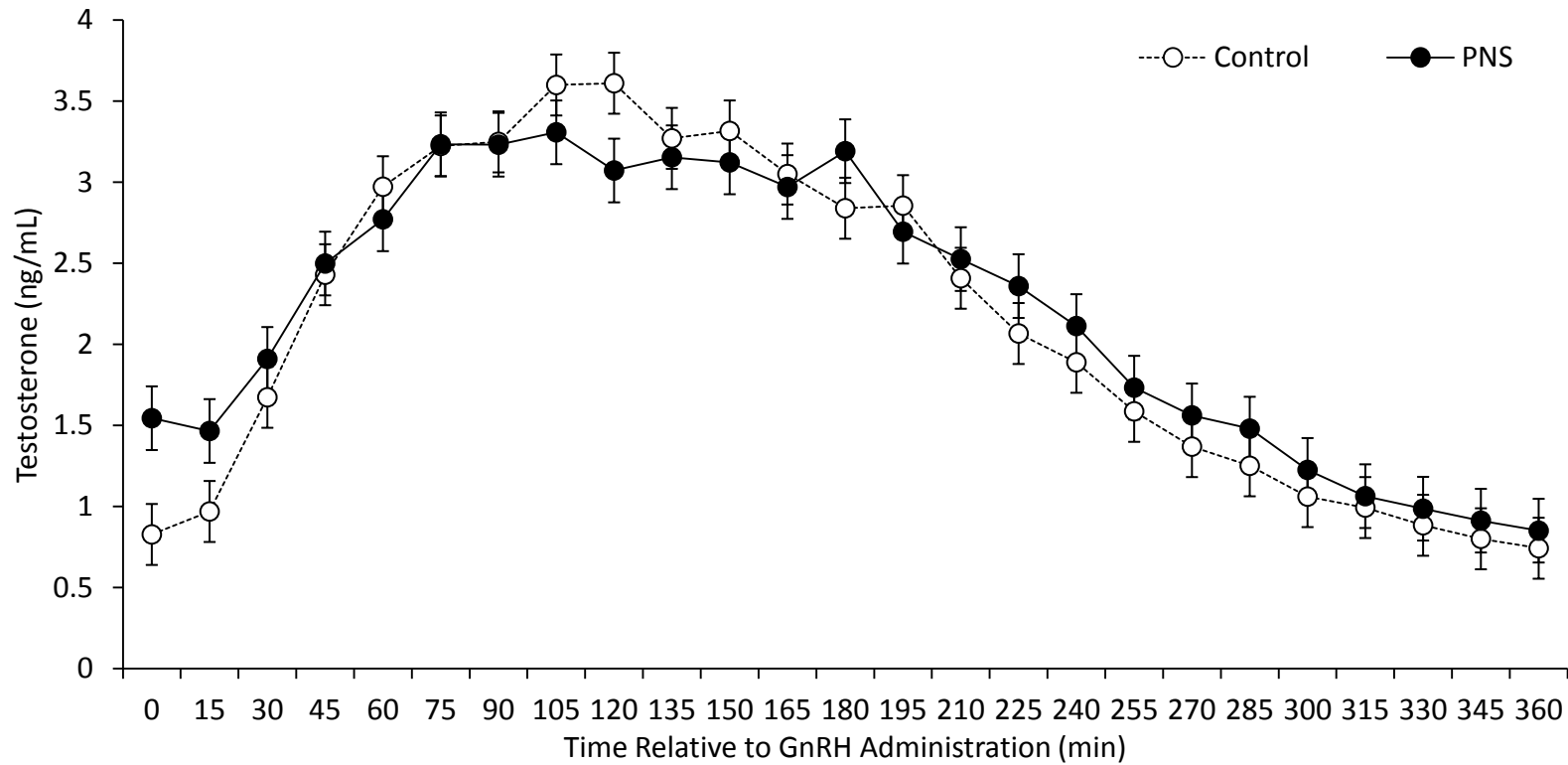
<b>Treatment</b>	<b>Response Incidence</b>	<b>Total n</b>	<b>Measurable Response N</b>	<b>Mean Maximum Concentration of Pulse</b>	<b>Mean Pulse Amplitude</b>
Control	Response	4	2	2.9 ng/mL	2.4 ng/mL
Control	No Response	8	—	—	—
PNS	Response	9	5	3.0 ng/mL	2.5 ng/mL
PNS	No Response	2	—	—	—



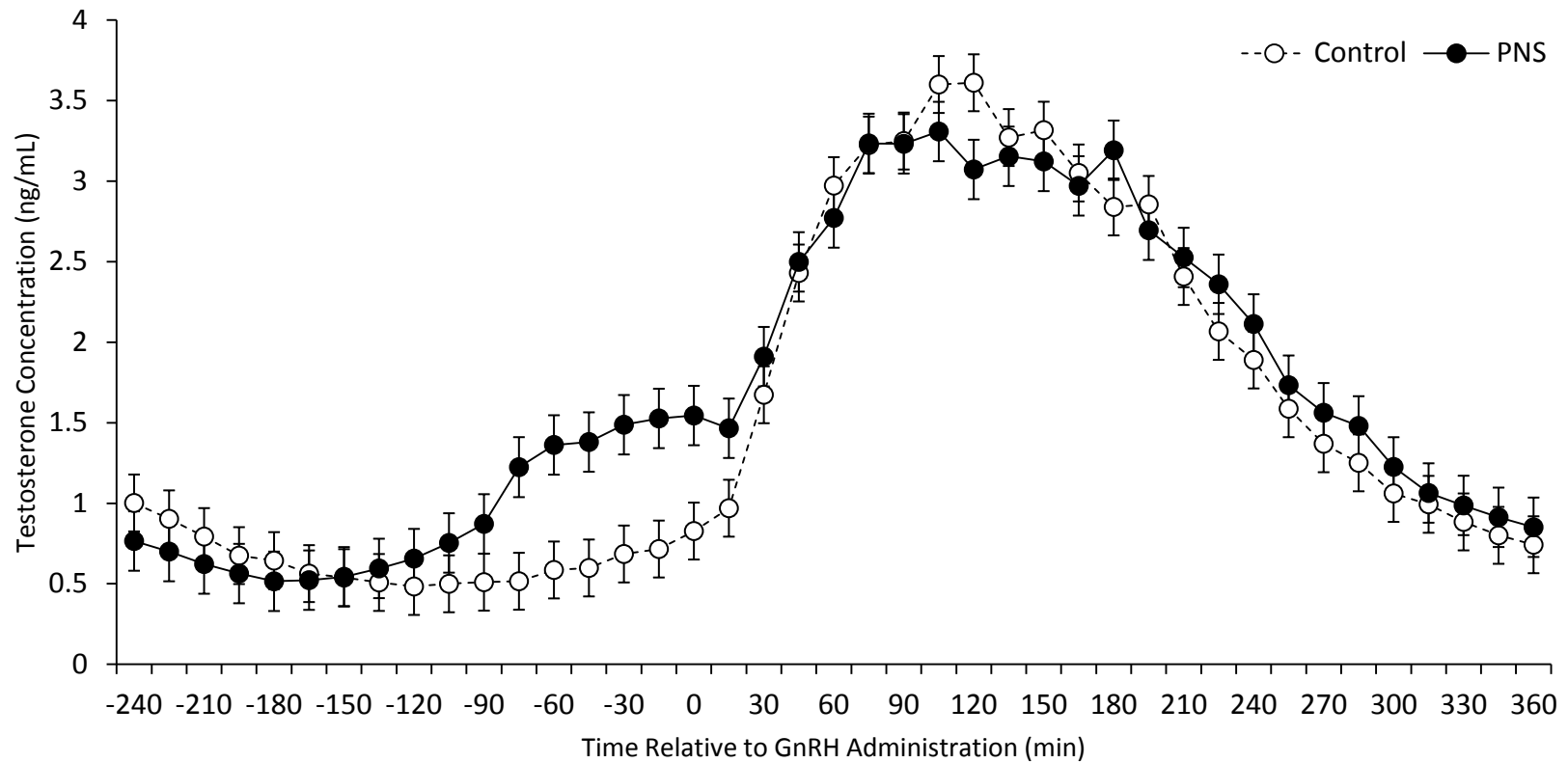
**Figure 8.** Average testosterone concentration in the h prior to GnRH administration in prenatally stressed (PNS; grey) compared to control (white) bulls. Average testosterone concentration in the h prior to GnRH administration tended ( $P = 0.0726$ ) to be greater in PNS bulls relative to control bulls.



**Figure 9.** Testosterone concentration profiles prior to GnRH administration in prenatally stressed (PNS; black circles with solid line) compared to control (white circles with dashed line) bulls from time -240 to time 0 relative to GnRH administration. Profiles of testosterone in the period prior to GnRH administration tended to be greater ( $P=0.0756$ ) in PNS bulls compared to control bulls after time -90 relative to GnRH administration.



**Figure 10.** Testosterone concentration profiles following GnRH administration between prenatally stressed (PNS; black circles with solid line) and control (white circles with dashed line) bulls. Testosterone concentration profiles tended to be different ( $P = 0.09$ ) with PNS bulls having a greater testosterone concentration relative to control bulls from time 0 to time 15 and PNS bulls having a lower testosterone concentration at time 120 compared to control bulls.



**Figure 11.** Testosterone concentration profiles from time -240 to time 360 relative to GnRH administration in Prenatally stressed (PNS; black circles with solid line) compared to control (white circles with dashed line) bulls. PNS bulls had greater ( $P = 0.03$ ) concentrations of testosterone from time -75 to time +15 relative to GnRH administration compared to control bulls.

## ***Relationship of Cortisol, LH, and Testosterone***

### **Regression Analysis**

Cortisol AUC from time -360 to -255 and cortisol AUC from time -240 to 0 can be utilized to predict LH and testosterone concentrations prior to GnRH administration (Tables 4 and 5). Luteinizing hormone baseline and LH pre-GnRH AUC as well as testosterone baseline and testosterone pre-GnRH AUC each have a negative relationship with cortisol AUC from time -360 to -255 (Table 4) and cortisol AUC from time -240 to 0 (Table 5). Luteinizing hormone concentrations prior to GnRH administration can be utilized to predict testosterone concentrations prior to GnRH administration (Table 6). Luteinizing hormone AUC in the pre-GnRH period has a positive relationship with testosterone AUC in the pre-GnRH period, while LH baseline has a positive relationship with testosterone baseline (Table 6). Luteinizing hormone concentration in the period following GnRH administration can be predicted by cortisol and testosterone concentration (Table 7). Cortisol from time -240 to 0 is negatively related to the time taken for LH to return to basal concentrations following GnRH in control bulls and positively related to the time taken for LH to return to basal concentrations following GnRH in PNS bulls (Table 7). There is a positive overall relationship of post-GnRH

**Table 4.** Regression analysis: LH and testosterone concentrations as predicted by cortisol AUC from time -360 to -255.

Independent Variable	Dependent Variable	Treatment	Regression Equation	R-Square	P value
Cortisol AUC -360 to -255	LH Pre-GnRH AUC	Overall	LH Pre-GnRH AUC = 290.7768 - 0.0252 x Cortisol AUC -360 to -255	0.044	0.3366
		Control	LH Pre-GnRH AUC = 292.7073 - 0.0272 x Cortisol AUC -360 to -255	0.0524	0.4742
		PNS	LH Pre-GnRH AUC = 288.2932 - 0.0222 x Cortisol AUC -360 to -255	0.0322	0.5976
	LH Baseline	Overall	LH Baseline = 0.9394 - < 0.0001 x Cortisol -360 to -255	0.0151	0.5766
		Control	LH Baseline = 1.0657 - < 0.0001 x Cortisol -360 to -255	0.1401	0.2306
		PNS	LH Baseline = 0.8256 - < 0.0001 x Cortisol -360 to -255	0.0042	0.85
	Testosterone Pre-GnRH AUC	Overall	Testosterone Pre-GnRH AUC = 19.2786 - 0.0052 x Cortisol AUC -360 to -255	0.2698	0.0111
		Control	Testosterone Pre-GnRH AUC = 15.9401 - 0.0041 x Cortisol AUC -360 to -255	0.5141	0.0087
		PNS	Testosterone Pre-GnRH AUC = 21.8746 - 0.0039 x Cortisol AUC -360 to -255	0.2014	0.1662
	Testosterone Baseline	Overall	Testosterone Baseline = 0.5818 - < 0.0001 x Cortisol AUC -360 to -255	0.284	0.0088
		Control	Testosterone Baseline = 0.5798 - < 0.0001 x Cortisol AUC -360 to -255	0.4111	0.0246
		PNS	Testosterone Baseline = 0.5796 - < 0.0001 x Cortisol AUC -360 to -255	0.1741	0.2017



**Table 5.** Regression analysis: LH and testosterone concentrations as predicted by cortisol AUC from time -240 to 0.

Independent Variable	Dependent Variable	Treatment	Regression Equation	R-Square	P value
Cortisol AUC -240 to 0	LH Pre-GnRH AUC	Overall	LH Pre-GnRH AUC = 309.5587 - 0.0313 x Cortisol AUC -240 to 0	0.083	0.1825
		Control	LH Pre-GnRH AUC = 305.122 - 0.02654 x Cortisol AUC -240 to 0	0.069	0.4095
		PNS	LH Pre-GnRH AUC = 328.1448 - 0.0485 x Cortisol AUC -240 to 0	0.119	0.2989
	LH Baseline	Overall	LH Baseline = 0.9337 - <0.0001 x Cortisol AUC -240 to 0	0.0009	0.6626
		Control	LH Baseline = 0.9943 - <0.0001 x Cortisol AUC -240 to 0	0.0288	0.5983
		PNS	LH Baseline = 0.9324 - <0.0001 x Cortisol AUC -240 to 0	0.018	0.6938
	Testosterone Pre-GnRH AUC	Overall	Testosterone Pre-GnRH AUC = 21.3883 - 0.0054 x Cortisol AUC -240 to 0	0.3534	0.0028
		Control	Testosterone Pre-GnRH AUC = 15.5933 - 0.0029 x Cortisol AUC -240 to 0	0.3442	0.045
		PNS	Testosterone Pre-GnRH AUC = 28.2925 - 0.0098 x Cortisol AUC -240 to 0	0.4406	0.0259
	Testosterone Baseline	Overall	Testosterone Baseline = 0.5595 - <0.0001 x Cortisol AUC -240 to 0	0.1403	0.0782
		Control	Testosterone Baseline = 0.5316 - <0.0001 x Cortisol AUC -240 to 0	0.1211	0.2677
		PNS	Testosterone Baseline = 0.5944 - <0.0001 x Cortisol AUC -240 to 0	0.1403	0.2563

**Table 6.** Regression analysis: Testosterone concentrations as predicted by LH concentration

Independent Variable	Dependent Variable	Treatment	Regression Equation	R-Square	P value
LH Pre-GnRH AUC	Testosterone Pre-GnRH AUC	Overall	Testosterone Pre-GnRH AUC = 2.4553 + 0.0385 x LH Pre-GnRH AUC	0.2092	0.0282
		Control	Testosterone Pre-GnRH AUC = 6.1692 + 0.0154 x LH Pre-GnRH AUC	0.1011	0.3139
		PNS	Testosterone Pre-GnRH AUC = -1.4415 + 0.0623 x LH Pre-GnRH AUC	0.354	0.0535
LH Baseline	Testosterone Baseline	Overall	Testosterone Baseline = 0.4313 + 0.0432 x LH Baseline	0.0077	0.6914
		Control	Testosterone Baseline = 0.3419 + 0.1198 x LH Baseline	0.057	0.455
		PNS	Testosterone Baseline = 0.4822 + 0.0081 x LH Baseline	0.0003	0.9611

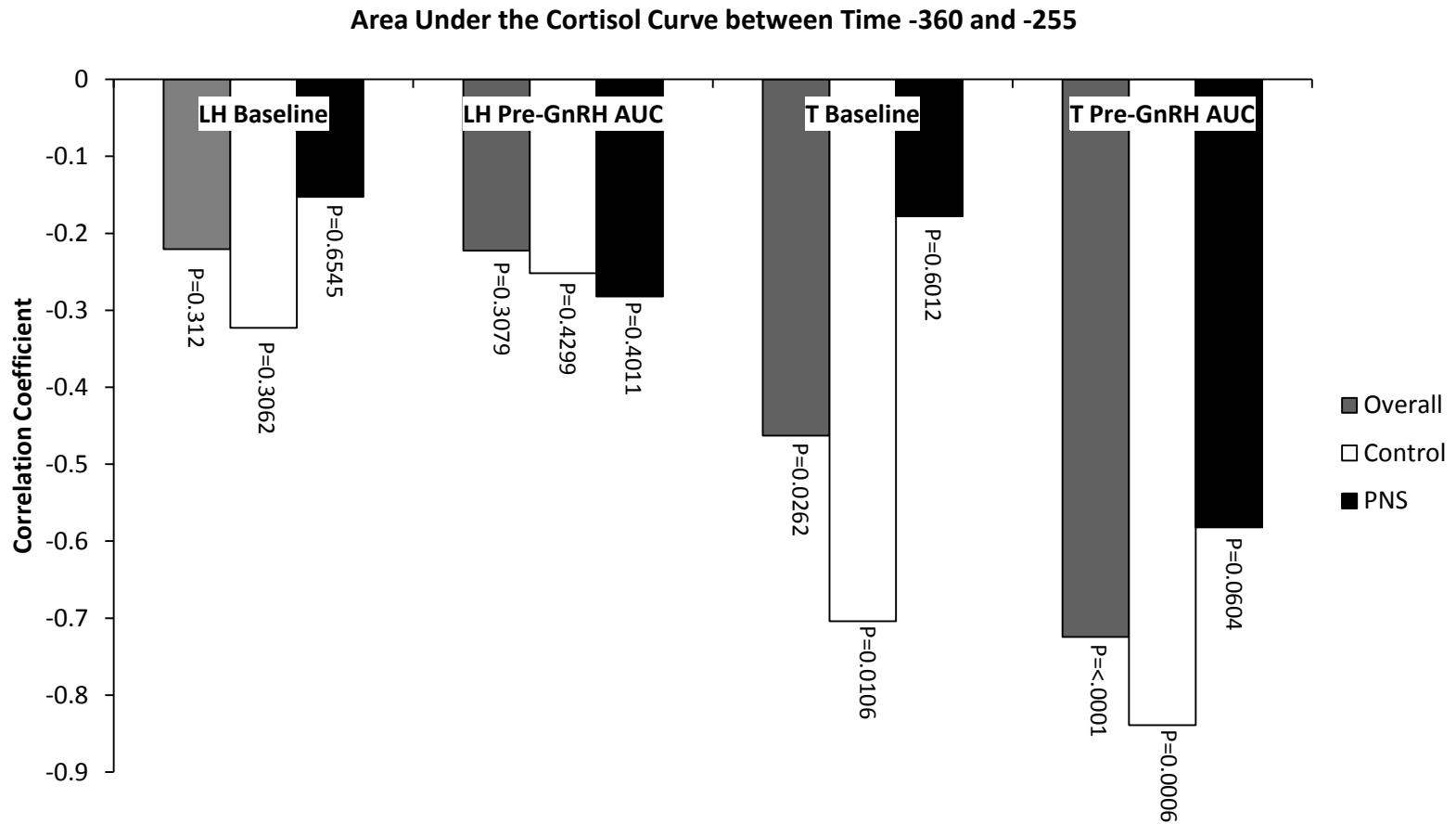
**Table 7.** Regression analysis: LH concentration following GnRH administration as predicted by cortisol and testosterone concentrations

Independent Variable	Dependent Variable	Treatment	Regression Equation	R-Square	P value
Cortisol AUC -240 to 0	LH Return to Baseline	Overall	LH Return to Baseline = 212.5167 - 0.0075 x Cortisol AUC -240 to 0	0.0165	0.5592
		Control	LH Return to Baseline = 216.2655 - 0.0026 x Cortisol AUC -240 to 0	0.0036	0.8534
		PNS	LH Return to Baseline = 167.3492 + 0.05293 x Cortisol AUC -240 to 0	0.3968	0.0378
LH Post-GnRH AUC	Testosterone Post-GnRH AUC	Overall	LH Post-GnRH AUC = 703.2333 + 0.1125 x Testosterone Post-GnRH AUC	0.0394	0.3639
		Control	LH Post-GnRH AUC = 812.2089 - 0.0388 x Testosterone Post-GnRH AUC	0.0072	0.7931
		PNS	LH Post-GnRH AUC = 493.7074 + 0.3978 x Testosterone Post-GnRH AUC	0.3011	0.0805
Testosterone Pre-GnRH AUC	LH Post-GnRH AUC	Overall	LH Post-GnRH AUC = 648.3338 + 10.7627 x Testosterone Pre-GnRH AUC	0.1136	0.1158
		Control	LH Post-GnRH AUC = 719.5049 + 6.8414 x Testosterone Pre-GnRH AUC	0.0123	0.7312
		PNS	LH Post-GnRH AUC = 572.1919 + 13.559 x Testosterone Pre-GnRH AUC	0.3757	0.0449

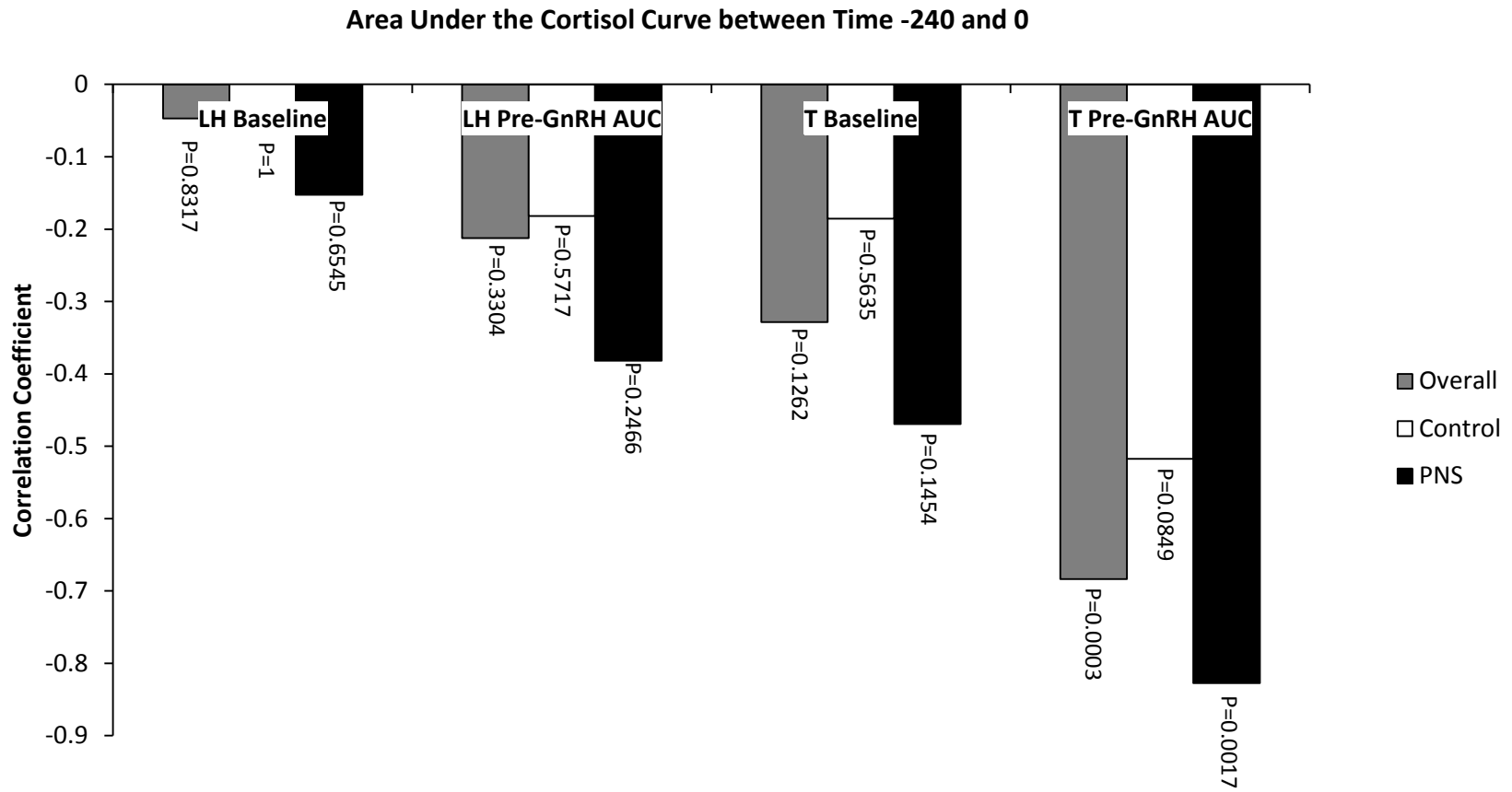
AUC for LH and post-GnRH AUC for testosterone (Table 7). Testosterone AUC prior to GnRH administration is positively correlated with LH AUC in the period following GnRH administration, indicating the lack of a negative effect of pre-GnRH testosterone concentrations on post-GnRH LH concentrations.

### Correlations

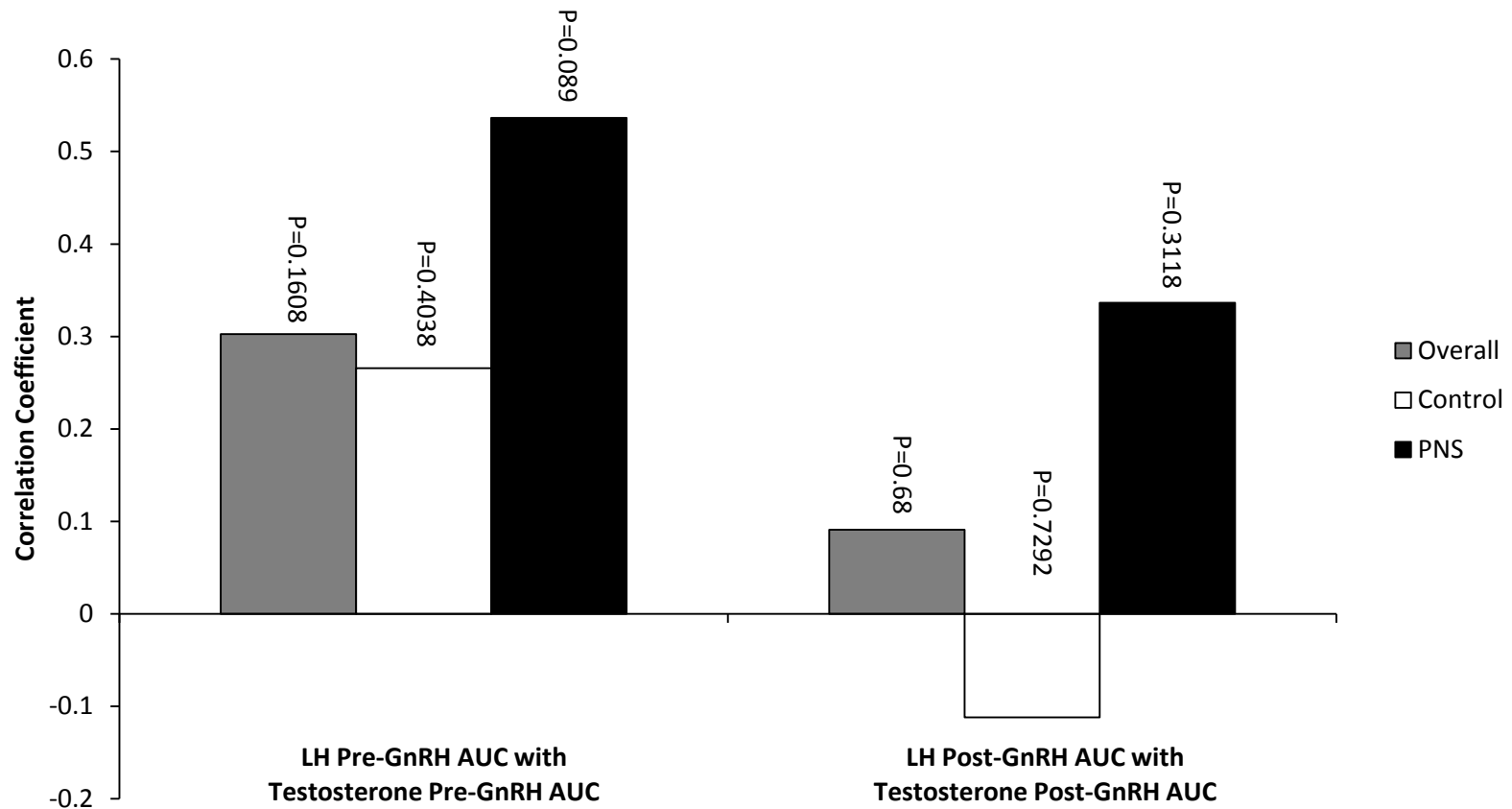
Luteinizing hormone baseline and LH pre-GnRH AUC as well as testosterone baseline and testosterone pre-GnRH AUC each were negatively correlated with cortisol AUC from time -360 to -255 (Fig. 12) and cortisol AUC from time -240 to 0 (Fig.13). Luteinizing hormone AUC in the period prior to GnRH administration was positively correlated with testosterone AUC in the period prior to GnRH administration (Fig. 14). Luteinizing hormone AUC in the period following GnRH administration was positively correlated with testosterone AUC in the period following GnRH administration (Fig. 14).



**Figure 12.** Correlations of area under the curve (AUC) for cortisol between time -360 and -255 with LH baseline, LH pre-GnRH AUC, testosterone baseline, and testosterone pre-GnRH AUC overall (grey) and between prenatally stressed (PNS; black) and control (white) groups.



**Figure 13.** Correlations of area under the curve (AUC) for cortisol between time -240 and 0 with LH baseline, LH pre-GnRH AUC, testosterone baseline, and testosterone pre-GnRH AUC overall (grey) and between prenatally stressed (PNS; black) and control (white) groups.



**Figure 14.** Correlations of LH pre-GnRH area under the curve (AUC) with testosterone pre-GnRH AUC and LH post GnRH AUC with testosterone post-GnRH AUC, overall (grey) and between prenatally stressed (PNS; black) and control (white) groups.

## **Discussion**

Stress is known to activate the HPA axis and cause the release of cortisol from the adrenal cortex (Butcher and Lord, 2004). Cortisol is known to have suppressive effects on LH and testosterone secretion in bulls (Welsh et al., 1979a; Welsh et al., 1979b; Welsh, 1981; Welsh and Johnson, 1981; Welsh et al., 1981; Welsh et al., 1982). This negative relationship was consistent with the results of this study and was maintained throughout the sampling period. The first two h of samples in this study were not assayed for LH or testosterone concentrations to allow a longer rest period to ensure that animals were not experiencing a stress response to cannulation and handling. This would result in increased cortisol secretion and suppressed secretion of LH and testosterone. Of the two corticosteroid receptors in the brain, GR is highly expressed throughout the structures of the limbic system (Sapolsky et al., 1983), but has a 5-10 fold less affinity for glucocorticoids compared to mineralocorticoid receptors (MR). Therefore, MR is extensively bound during basal glucocorticoid secretion, and GR is only extensively bound during times of greater circulating concentrations of glucocorticoids in the peripheral blood supply (Reul and de Kloet, 1985; Herman et al., 2005). Therefore, GR play a greater role in the management of the stress response. Glucocorticoid receptors have been shown to be present in human fetuses as early as the first trimester of pregnancy (Costa et al., 1996). Adrenalectomy has been shown to result in increased GR densities in the hippocampus and prefrontal cortex (Lowy, 1991). Prenatal stress is known to cause alterations and damage to the limbic system and brain (Anderson et al., 1985; Coe et al., 2002; Coe et al., 2003), and cause reduced GR



expression in the hippocampus and cortex of the limbic system (Reul et al., 1994; Maccari et al., 1995). Studies in mice have shown that reduced GR in the limbic system 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). Therefore, this lab expected to observe greater circulating concentrations of cortisol in the peripheral blood supply, resulting in suppression of LH and testosterone in prenatally stressed mature Brahman bulls relative to bulls that did not undergo prenatal stress. Rakers et al. (2012) examined the effects of maternal isolation stress on fetal HPA axis of offspring during early gestation (30 - 100 d), and late gestation (100 - 120 d). Prenatal stress resulted in greater cortisol concentrations relative to controls, with early prenatal stress resulting in greater fetal cortisol concentrations relative to late prenatal stress. Lay et al. (1997b) reported that prenatal transportation stress resulted in elevated plasma cortisol concentrations for a longer period relative to controls in suckling calves. Contrastingly, PNS bulls in this study did not have greater cortisol, and in fact had lower concentrations of cortisol in the h prior to GnRH administration. Additionally, PNS bulls in this study exhibited greater LH and testosterone pulsatility relative to control bulls. Major differences between the Lay et al. study and the current study are as follows. The transported dams in the Lay et al. study were not balanced by temperament. The number of test subjects used was less than half of the number of test subjects used in the current study (42 pregnant cows). It is important to note that this is a novel study to examine the effects of prenatal stress on LH, testosterone, and cortisol production in sexually mature bulls. Therefore, it is critical to acknowledge species differences when examining the

effects of perinatal stress, as perinatal development is vastly diverse among species, specifically between lower and higher order species (Dobbing and Sands, 1979). The vast majority of existing published literature pertaining to perinatal stress and the HPA and HPG axis has been reported in mice and rats, which are known to give birth to immaturely developed young (Sapolsky and Meaney, 1986). At term, the limbic system in the rat fetus is developmentally equivalent to that of the human fetus at less than 25 percent of its gestation length (Darlington et al., 1999). In the fetal rat, on and following day 13 GR mRNA can be found in the anterior hypothalamus, hippocampus, and pituitary gland (Diaz et al., 1998). In contrast to results from this study, prenatal stress in the form of dexamethasone administration in fetal male rats after d 14 of gestation has been reported to be associated with decreased testosterone secretion (Page et al., 2001) and demasculinization of aromatase activity in the preoptic area (Reznikov et al., 2004). Prenatal exposure of the synthetic glucocorticoid betamethasone to male rat pups has been reported to cause a decreased testosterone surge, which is required for fetal masculinization on d 18 and d 19 of gestation (Reznikov et al., 2004; Manojlović-Stojanoski et al., 2012). Okret et al. (1986) reported a down regulation GR mRNA in rat hepatoma culture cells treated with the synthetic glucocorticoid dexamethasone. Pedrana et al. (2008) reported contrasting results in prenatally stressed Merino sheep. Pregnant sheep were intramuscularly injected with either saline or the synthetic glucocorticoid betamethasone at d 109, d 116, or both d 109 and d 116 of gestation. Fetal testes were obtained 5 d after the treatment was incurred. Immunohistochemistry showed greater GR immunoexpression in Leydig cells of sheep whose dams were injected with

betamethasone at d109 relative to control sheep. Lay et al. (1997b) reported that transportation of pregnant Brahman cows for 2 h intervals at 60, 80, 100, 120, 140 ± 5 d of gestation, resulted in reduced cortisol clearance rates in calves. Although the transportation stress times and intervals reported by Lay et al. (1997b) were identical to the methods in this study, temperament was not recorded or balanced in cows or calves and cortisol was only analyzed in immature calves. Temperament is known to affect circulating concentrations of cortisol and basal adrenal production of cortisol in temperamental Brahman calves relative to calmer herd mates (Curley et al., 2006). Littlejohn et al. (2013) previously reported PNS bulls in this study to have elevated temperament scores relative to control bulls through weaning. Age, maturity, time relative to encountering a stressor, and habituation may also play a critical roles in the endocrine profiles of prenatally stressed bulls in this study. The majority of previous literature has examined HPA axis and HPG axis epigenetic alterations in fetal and prepubertal prenatally stressed animals instead of sexually mature prenatally stressed animals. Therefore, future research should examine plasticity of epigenetic modifications induced by prenatal stress over time and as an animal ages. Mizoguchi et al. (2003) reported that chronic stress induced repeated elevated endogenous concentrations of cortisol in rats may cause decreased cytosolic GR in the hippocampus, followed by an increase in cytosolic GR when no longer exposed to the chronic stressor. Additionally, habituation may play a role in the stress response of the prenatally stressed bulls in this study. Habituation can be defined as repeated exposure to identical stressors, in which glucocorticoid responses to the stressor progressively decrease over time (Kant et al.,

1985; Herman et al., 2005). Chronic stress in the form of being confined to a wire cage and immersed in a water bath has been reported to cause the HPA axis to be less sensitive to dexamethasone in rats (Mizoguchi et al., 2003). Furthermore, it is known that cattle have the ability to adapt to a stressful environment over time, therefore causing decreased HPA axis activation and cortisol release (Lay et al., 1996). Therefore, habituation to an activated HPA axis could contribute to prenatally stressed bulls having a diminished stress response and consequently having enhanced HPT axis activity.

Stress during prenatal development affected secretion of cortisol, LH, and testosterone in sexually mature Brahman bulls. Alterations in hormone profiles due to prenatal stress may be associated with alterations in gene expression. Prenatal programming of epigenetic changes in gene expression are a result of methylations and/or histone acetylations of DNA and have been found to be heritable across generations (Drake and Walker, 2004; Zakharova, 2009). Drake et al., (2005) reported that prenatal exposure to glucocorticoids results in low birth weight, adult hyperinsulinemia, hyperglycemia, and increased phosphoenolpyruvate carboxykinase (PEPCK) activity in rats. The male offspring of females that were prenatally stressed also had low birth weights, glucose intolerance, and increased PEPCK activity. However, upon examining the third generation, no effects were observed to be associated with the first generation of prenatal stressed rats. Therefore, epigenetic effects of PNS may affect future but limited generations. Furthermore, any epigenetic modifications incurred by the bulls in this study could affect future generations. This suggests that not only could herd management practices affect progeny of gestationally

stressed cows and potentially affect their production related traits, but also affect future generations and potentially those calves' production related traits.

## CHAPTER III

### CONCLUSIONS

Because cortisol has been shown to be elevated in prenatally stressed animals (Lay et al., 1996; Lay et al., 1997a; Lay et al., 1997b; Meaney et al., 2000; Roussel et al., 2004; Williams, 2007), this lab expected to see greater cortisol in PNS bulls relative to control bulls, and anticipated suppressed LH and testosterone secretion as a result. Instead, PNS bulls had lower circulating concentrations of cortisol in the h prior to GnRH administration relative to control bulls, and there was no other difference in overall cortisol secretion in these data. Additionally, PNS bulls had greater LH and testosterone pulsatility compared to control bulls. While some studies have examined effects of prenatal stress on reproductive development, very limited research has examined effects of associated with prenatal stress on reproductive function in sexually mature animals. Method, duration, interval, and stage in gestation prenatal stress may elicit different effects. The species, age, maturity, time relative to encountering a stressor, and habituation of an animal to stress at the time of examination may each play a role in HPA axis and HPG axis function.

This model of a higher order species provides insight into the genetic and/or epigenetic effects of prenatal stress on postnatal health and performance of animals and humans. In contrast to other studies done in lower order species, these data suggest that sexually mature Brahman bulls exposed to prenatal transportation stress may be more acclimated to an activated HPA axis. This potential adaptation may have allowed PNS

bulls to recover more rapidly from the stress of cannulation and handling prior to sampling relative to control bulls. Future studies will examine potential epigenetic modifications in these prenatally stressed bulls, the existence of which modifications could impact future generations.

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## APPENDIX A

### OVINE LUTEINIZING HORMONE RADIOIMMUNOASSAY

#### Reference:

Rivera, F. A. et al. 2011. Reduced progesterone concentration during growth of the first follicular wave affects embryo quality but has no effect on embryo survival post transfer in lactating dairy cows. *Reproduction* 141: 333-342.

### **RADIOIODINATION OF OVINE LH**

#### **I. Preparation of Iodination Grade oLH (AFP-8614B)**

\* Iodination grade oLH was obtained from Dr. A.F. Parlow, NHPP, UCLA

Weigh  $\pm$  25  $\mu$ g oLH; solubilize amount weighed with 0.5 M NaPO<sub>4</sub> to yield 1  $\mu$ g/ $\mu$ l

Pipette 10  $\mu$ l (10  $\mu$ g) into each microcentrifuge tube; store aliquots at -20° C for no more than 6 months

#### **II. Prior to iodination**

Prepare Anion Exchange Resin column (BioRad AG 1-XB; 50-100 mesh chloride form; see protocol for preparation in Laboratory Procedures File)

Weigh out appropriate amounts of Chloramine-T and Na Metabisulfite; solubilize Chloramine-T and Sodium Metabisulfite just prior to iodination

#### **III. Iodination**

\* Use only pipettes or Hamilton syringes located in the Iodination hood (room 407 C, Kleberg Center)

\*\* When Na<sup>125</sup> I is received, confirm it is in appropriate concentration: 1 mCi/10  $\mu$ l/vial. Add 50  $\mu$ l 0.5 M NaPO<sub>4</sub> to vial prior to first use and mix by flicking. To retrieve 0.5 mCi for iodination, remove 30  $\mu$ l from total volume (60  $\mu$ l).

Add 25  $\mu\text{l}$  0.5 M  $\text{NaPO}_4$  to reaction vial (vial containing 10  $\mu\text{g}$  oLH)  
Transfer 0.5 mCi  $^{125}\text{I}$  to reaction vial containing 10 $\mu\text{g}$  of oLH; vortex briefly;  
make sure all of the liquid is on the bottom of the tube  
Add 25 $\mu\text{l}$  (80  $\mu\text{g}$ ) of Chloramine-T; vortex (or flick) vial gently  
Allow reaction to proceed for 2 min  
Add 25  $\mu\text{l}$  (120  $\mu\text{g}$ ) of Sodium Metabisulfite to reaction vial; vortex  
Add 50  $\mu\text{l}$  0.5 M  $\text{NaPO}_4$  to reaction vial; vortex briefly; transfer product to column  
(may use a disposable, 1 ml syringe with 21 ga needle)  
Rinse reaction vial with 100  $\mu\text{l}$  0.5 M  $\text{NaPO}_4$  and transfer to column  
Elute column with 3 ml 0.05 M  $\text{NaPO}_4$  into plastic bottle containing 3 ml 1%  
BSA-PBS  
After collecting iodination product, vortex bottle briefly and count two 10  $\mu\text{l}$   
aliquots to determine radioactivity  
Determine total radioactivity (cpm) yielded from iodination

## **PREPARATION OF ANTISERUM FOR OVINE LH**

### **I. Preparation of rabbit anti-ovine LH (AFP-192279)**

\* Rabbit anti-ovine LH was obtained from Dr. A. F. Parlow in lyophilized state (in 2% normal rabbit serum in phosphosaline buffer (PBS))

1. A 1:100 dilution (stock) is prepared by adding 1 ml of distilled water
2. The 1:100 stock anti-oLH is stored in 25  $\mu\text{l}$  aliquots at  $-20^\circ\text{C}$

## **PREPARATION OF OVINE LH STANDARDS (AFP-8614B)**

\*oLH to be used as reference standards was obtained from Dr. A.F. Parlow, NHPP, UCLA

### **I. oLH Stock 10 µg/ml**

1. Weigh approximately 10 µg oLH (AFP-8614B)
2. Add appropriate volume of 1% bovine serum albumin (BSA)-PBS to yield 10 µg/ml solution
3. Vortex well; store 150 µl aliquots at -20° C

### **II. oLH Stock 1000 ng/ml**

1. Add 1,350 µl 1%EW-PBS to the vial containing 150 µl of 10 µg/ml stock to yield 1000 ng/ml
2. Vortex well

### **IV. Prepare standards as follow:**

1. 10 ng/200 µl (50 ng/ml)
  - a. Add 28.5 ml 1%EW-PBS to a conical tube
  - b. Add 1500 µl oLH Stock 1000 ng/ml into 1%EW-PBS solution; vortex
2. 6 ng/200 µl (30 ng/ml)
  - a. Dilute 15 ml oLH 50 ng/ml into 10 ml 1%EW-PBS; vortex
3. 4 ng/200 µl (20 ng/ml)
  - a. Dilute 14 ml oLH 30 ng/ml into 7 ml 1%EW-PBS; vortex
4. 2 ng/200 µl (10 ng/ml)
  - a. Dilute 10 ml oLH 20 ng/ml into 10 ml 1%EW-PBS; vortex
5. 1 ng/200 µl (5 ng/ml)
  - a. Dilute 10 ml oLH 10 ng/ml into 10 ml 1%EW-PBS; vortex
6. 0.5 ng/200 µl (2.5 ng/ml)
  - a. Dilute 10 ml oLH 5 ng/ml into 10 ml 1%EW-PBS; vortex

7. 0.25 ng/200  $\mu$ l (1.25 ng/ml)
  - a. Dilute 10 ml oLH 2.5 ng/ml into 10 ml 1%EW-PBS; vortex
8. 0.1 ng/200  $\mu$ l (0.5 ng/ml)
  - a. Dilute 8 ml oLH 1.25 ng/ml into 12 ml 1%EW-PBS; vortex
9. 0.05 ng/200  $\mu$ l (0.25 ng/ml)
  - a. Dilute 10 ml oLH 0.5 ng/ml into 10 ml 1%EW-PBS; vortex
10. 0.025 ng/200  $\mu$ l (0.125 ng/ml)
  - a. Dilute 9 ml oLH 0.25 ng/ml into 9 ml 1%EW-PBS; vortex
11. 0.0125 ng/200  $\mu$ l (0.0625 ng/ml)
  - a. Dilute 6 ml oLH 0.125 ng/ml into 6 ml 1%EW-PBS; vortex

\* Store standard solutions in 1 ml aliquots at -20° C

### **I. Assay Setup**

Label assay sheets and tubes (12 x 75 polypropelene tubes) as follow:

	<u>Tube #</u>
1. 4 NSB tubes	1-4
2. 9 Total count tubes	5-9
3. 3 “0 Standard” tubes	10-12
4. “Standard” tubes in triplicate	13-45
5. 2 “LOW Reference” tubes	46-47
6. 2 “MID Reference” tubes	48-49
7. 2 “HIGH Reference” tubes	50-51
8. Unknown sample tubes in duplicate	52-...



## II. Day 1

Pipette the following into each assay tubes

1. NSB: 500  $\mu$ l 1%EW-PBS
2. 0 Standard: 500  $\mu$ l 1%EW-PBS
3. Standards: 300  $\mu$ l 1%EW-PBS + 200  $\mu$ l standard
4. Ref Preps: 300  $\mu$ l 1%EW-PBS + 200  $\mu$ l reference preparation
5. Unknowns: 300  $\mu$ l 1%EW-PBS + 200  $\mu$ l sample

Add primary Antibody (check the most recent dilution used for approx. 25% total binding)

6. Pipette 200  $\mu$ l of PBS-EDTA + 1:400 NRS without primary antibody into NSB tubes ONLY.
7. Pipette 200  $\mu$ l anti-oLH (diluted in PBS-EDTA + 1:400 NRS) into all tubes EXCEPT NSB and TC
8. Vortex tubes briefly (if tracer is not added on same day, place tubes at 4° C overnight)

Add Tracer

9. Pipette 100  $\mu$ l  $^{125}$ I-oLH (20,000 cpm/100  $\mu$ l diluted in 0.1%EW-PBS) to all tubes.
10. Vortex tubes briefly and place at 4°C for 20-24 hr

## III. Day 2

Add Secondary Antibody (check the most recent dilution used)

(use the walk-in cooler; keep tubes and reagents at 4° C during entire procedure)

1. Pipette 200  $\mu$ l of Goat-anti-rabbit gamma globulin (GAR; 2<sup>nd</sup> Ab) diluted in PBS-EDTA without NRS into all tubes EXCEPT TC tubes.
2. Vortex tubes briefly and incubate at 4°C for 48-72 hr

#### **IV. Day 4**

Antigen+Ab complex precipitation

(use the walk-in cooler; keep tubes and reagents at 4° C during entire procedure)

1. Pipette 3 ml cold PBS (0.01 M; pH 7.0) to all tubes EXCEPT TC tubes (per spin basis)
2. Centrifuge tubes at 3600 rpm for 1 hr at 4°C EXCEPT TC
3. Decant the supernatant into a waste receptacle (dispose in Liquid Radioactive Waste Carboy)
4. Blot tubes in absorbent paper
5. Load tubes into the Gamma Counter and count each tube for 1 min

#### **REAGENT PREPARATION**

##### **I. 0.5 M Phosphate Buffer (0.5 M PB; pH 7.5)**

Weigh 3.42g  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  (monobasic); place in a 50ml volumetric flask  
Add ddH<sub>2</sub>O to volume; store at 4° C

Weigh 7.1g anhydrous  $\text{Na}_2\text{HPO}_4$  (dibasic); place in a 100ml volumetric flask  
Add ddH<sub>2</sub>O to volume; store at 4° C

0.5 M buffer with pH 7.5 is prepared by ratio of 16monobasic:84dibasic (1:5.25)  
Adjust pH to 7.5

##### **II. 0.2 M Phosphate Buffer (0.2 M PB; pH 7.4)**

\* Prepared by ratio of 19:81 monobasic:dibasic

Weigh 26.22 g  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  (monobasic; MW 137.99); place in the 5 L flask  
Weigh 115 g  $\text{Na}_2\text{HPO}_4$  (dibasic; MW 141.96); place in the 5 L flask  
Add approximately 4.5 L of ddH<sub>2</sub>O; stir until completely dissolved

Adjust pH to 7.4

Add ddH<sub>2</sub>O to final volume of 5 L; store at 4° C

**III. 0.01 M Phosphate Buffered Saline (0.01 M PBS; pH 7.4)**

Dilute 0.2 M PB 1:20 with ddH<sub>2</sub>O

Add 0.9 % NaCl; stir; store at 4° C

**IV. 0.01 M PBS; 0.05 M EDTA (PBS-EDTA; pH 7.0)**

Weigh 18.61 g disodium EDTA; place in a 1000 ml volumetric

Add 800 ml 0.01 M PBS

Warm and stir until dissolved

Adjust pH to 7.0

Add 0.01 M PBS to final volume of 1 L; store at 4° C

**V. 1:400 Normal Rabbit Serum (PBS-EDTA + 1:400 NRS)**

Add 0.25 ml NRS to 99.75 ml PBS-EDTA; store at 4° C

**VI. 1% Egg White, 0.01 M PBS (1%EW-PBS)**

Weigh 10 g egg white; dilute with 1000 ml 0.01 M PBS

Warm and stir until dissolved (do not let it warm too much)

Filter through glass wool using funnel with gauze

Adjust pH to 7.0 and store at 4° C

**VII. 0.1 % Egg White, 0.01 M PBS (0.1% EW-PBS)**

Dilute 1%EW-PBS 1:10 with 0.01M PBS

Stir and store at 4° C

**VIII. Chloramine-T (3.2 µg/µl)**

Weigh 0.032 g Chloramine-T and place into small glass vial covered with aluminum foil

Add 10 ml 0.05 M NaPO<sub>4</sub>

Prepare immediately before use; discard excess after use

**IX. Sodium metabisulfite (4.8 µg/µl)**

Weigh 0.048 g sodium metabisulfite and place into small glass vial

Add 10 ml 0.05 M NaPO<sub>4</sub>

Prepare immediately before use; discard excess after use

## APPENDIX B

### <sup>3</sup>H-BASED TESTOSTERONE RADIOIMMUNOASSAY

#### Reference:

Vera-Avila, H. R., T. D. A. Forbes, J. G. Berardinelli and R. D. Randel. 1997. Effect of dietary phenolic amines on testicular function and luteinizing hormone secretion In male Angora goats J Anim. Sci. 75: 1612-1620. In Press.

#### REAGENT PREPARATION

<u>PBSG (0.1% Gelatin, pH 7.5)</u>	<u>1 Liter</u>	<u>2 Liters</u>
1. Monobasic Sodium Phosphate (Sigma, S-9638; FW 138.0)	0.070 g	0.140 g
2. Dibasic Sodium Phosphate (Sigma, S-0876; FW 142.0)	1.350 g	2.700 g
3. Sodium Chloride (Sigma, S-9888; FW 58.44)	8.812 g	17.624 g
4. Sodium Azide (Sigma, S-2002; FW 65.01)	1.000 g	2.000 g
5. Disodium EDTA: dihydrate (Sigma; ED2SS, FW 372.2)	0.372 g	0.744 g
6. Gelatin (J.T. Baker, 2124-01)	1.000 g	2.000 g
7. Double Distilled H <sub>2</sub> O	1.00 liter	2.00 liter

Into dd H<sub>2</sub>O, at about 90% of the final volume, weigh out and add all reagents except EDTA and gelatin. Mix and pH to 7.5 using 1.0 N HCl or NaOH. Bring to

final volume in calibrated 2 L beaker or volumetric flask. Add EDTA and gelatin with continuous stirring over lowest heat until dissolved; this should take approx. 1 h. Transfer to storage bottle and store at 4 C. Replace at 30 to 40 d intervals. Sodium Azide is highly toxic – take appropriate precautions.

Charcoal Suspension for RIA Prepare at least 1 d in advance of RIA and discard at 20 d intervals. Can be stored at 4 C in a sealed beaker and must be maintained at approx. 4 C during additions. Use an ice bath with continuous stirring if addition time exceeds 5 min.

<u>Reagent/100 mL PBSG</u>	<u>T RIA</u>
Activated Charcoal (Sigma C-5260)	0.40 g
Dextran (Sigma D-4271)	0.04 g
<u>Addition Volume (uL/tube)</u>	<u>750</u>

#### Charcoal-Stripped Serum or Plasma Stock

1. Bleed, separate and collect 300+ mL sera or plasma from, preferably, an intact prepubertal female. Another reasonable source would be a mature female at 4 to 12 d postpartum. In cattle, “free-flow” bleeding with a large needle (14G), used-cleaned vacutainer tubes, and using intravenous pressure (i.e. no vacuum) will greatly reduce subsequent fibrin clots in sera stocks, both during and after processing.
2. Using a standard beaker that is ~200% of the pooled volume, pool the raw sera or plasma, and add a large magnetic stir bar. **For each 100 mL sera or plasma, add: 9.375 g Sigma C-5260 activated charcoal and 0.938 g Sigma D-4751 dextran.**
3. Cover and stir for 1.5 to 2 h at room temperature on stir plate.
4. While stirring by hand, pour suspension into 50 mL polycarbonate high-speed centrifuge tubes.

5. Centrifuge for 2.0+ h at 10,000 rpm x 4 C. Carefully remove tubes from rotor head and decant sera or plasma into a clean flask. Transfer only clear sera or plasma into this pool (e.g. leave the final 3 to 6 mL of charcoal-contaminated stock as waste).
6. Repeat centrifugation (Step 5.) using fresh centrifuge tubes. Carefully decant and pool clear sera or plasma stock into fresh flask.
7. Filter stock using Sartorius vacuum-filtration setup and hand-cut filters (derived from Whatman nos. 43 or 41 ashless 15.0 cm filter papers). Ideally this step should be repeated until no charcoal residue is visible on filter after procedure (about 5X; use dissecting scope to examine filters). In practice, we generally repeat the procedure twice for a total of three filtrations.
8. Aliquot at 5 to 7 mL in peti-vials, cap, label and freeze at -20 C until use.
9. This material is to be used as a base for Extraction Recovery estimates.

Trace Dilution Store at 4 C. Working dilution effective for at least 3 - 4 wks.

Stocks: NET-370 [1,2,6,7-<sup>3</sup>H] Testosterone, DuPont-NEN Research (4 – 8; 3.5/RIA)

1. Using micropipet, or Hamilton syringe for PGF<sub>m</sub>, introduce (n\*) uL of <sup>3</sup>H-tracer stock into 25 mL PBSG; mix for 5 min on stir-plate and let stand for 10 min at 4 C.
2. Prepare a triplicate set of scintillation vials containing the standard volume of cocktail (4 - 5 mL). Add a 100 uL aliquot of tracer solution base to each tube; mix by inversion, let stand 2 min and count for 1 min on LSC.
3. Calculate appropriate dilution. Currently 13,000 cpm/100 uL trace (i.e. mean cpm x original volume / 13000 = final volume).
4. Add appropriate volume of PBSG for working dilution of trace. Mix well and let stand overnight at 4 C before use.

Antibody Dilution: Prepare working dilutions daily from aliquoted storage dilutions. Store at 4 C.

Stocks: #250 anti-testosterone-11-BSA serum; Dr. G.D. Niswender, CSU, Ft Collins  
(1:500/1:50,000 to 1:60,000 for extr.)

1. Reconstitute lyophilized T anti-sera with 1.0 mL dd H<sub>2</sub>O (1:1). Always label and snap-freeze remainders in liquid N<sub>2</sub>, parafilm vial caps and store at -20C.
2. In order to minimize detrimental effects of repeated freeze-thaw cycles, use an aliquot of the full-strength antisera to prepare a second series of concentrated storage aliquots. Aliquot volumes should be appropriate for the simple preparation of adequate antisera to be used in a single RIA throughput.

Recommended PBSG dilutions for concentrated anti-sera storage aliquots:

400 uL x 1:400 for Testosterone

3. Working dilutions are prepared independently for each RIA in PBSG to achieve 30 to 50% max binding (%Ref/TC). Pre-labeled urine specimen cups are generally ideal for this step.

Recommended dilutions for anti-sera working stocks in 298-tube RIA:

30.0 mL x 1:40,000 for extraction Testosterone (.375 mL stock + 29.250 mL PBSG)

RIA Standards Prepare, aliquot at 1.0 mL and snap freeze in liquid nitrogen. Store at -20 C until required. Discard after 12 mo. Degradation may occur more rapidly in lowest concentrations of prepared standards. Minimal labeling requires analyte, concentration and date of preparation.

Testosterone Series:

1. Using Testosterone Stock I (on hand @ 10 ug/mL MeOH), construct Stock II by adding 10 uL Stock I to 10 mL volumetric flask and Q.S. to 10 mL with PBSG. Let sit overnight at 4 C.



2. Using above Stock II @ 100 ng/mL, construct 5000 pg/mL STD A by adding 500 uL Stock II to 10 mL volumetric flask and Q.S. to 10 mL with PBSG.
3. Using above STD A @ 5000 pg/mL, construct 1:1 serial dilutions in PBSG. These dilutions should be based on mass, rather than volume, to eliminate variability in volume associated with working with solutions at differing temperatures. Range produced currently for Extraction Testosterone RIA:

A= 5000.0 pg/mL

B= 2500.0 pg/mL

C= 1250.0 pg/mL

D= 625.0 pg/mL

E= 313.0 pg/mL

F= 156.0 pg/mL

G= 78.0 pg/mL

H= 39.0 pg/mL

I= 19.0 pg/mL

#### Protocol:

Get the samples, standards and control stocks to room temperature and begin pipetting by 0900. Turn down centrifuge bowl temperature to 4 to 6 C. During the thaw, load mini-scintillation vials with Ecolite(+) if this was not done the previous day.

1. Begin with the careful setup of all standard curves needed for the RIA using the appropriate **series table** above. Components should be pipetted in this order: Standards, PBSG, Charcoal-Stripped Plasma or Sera. Re-freeze the standard and plasma/sera stocks before continuing; hold the PBSG at room temperature on the bench.
2. Pipette the samples. The cell sequence of the storage flats should be used as the reference for the reaction tube sequence (e.g. sample of cell #4 pipetted into reaction tube #4). Rack individual “spins” as you work and group each with their respective standard curves so that they may be handled independently during the

- remainder of the protocol. For example, you may have two batches of n=135 samples plus n=13 standards that will require centrifugation. (Centrifuge capacity equals 148 tubes; TC tubes are not centrifuged.) With practice, this should require 1.5 to 2.0 h to complete.
3. Using the Eppendorf repeating pipette and the appropriate Combi-tip, add the appropriate volume of PBSG to the sample tubes (listed in **series tables**), shake each rack to mix. Set aside at room temperature.
  
  4. Referring to the **series tables** above, begin the reaction of Spin 1 at exactly 1030 h, regardless of whether the sample pipetting operation is complete. Using the Eppendorf repeating pipette and the appropriate Combi-tips:  
pipette the appropriate volume of 3H-Tracer into **all** tubes,  
then pipette the appropriate volume of antisera into all tubes **except TC and NSB tubes.**  
Shake racks vigorously or vortex. Place racks in plastic bags or parafilm the tubes.
  5. Incubate all tubes within each batch for exactly 90 min at room temperature.
  6. Transfer all tubes within each batch to refrigerator and incubate at 4 C for exactly 75 min.
  7. Remove Dextran/Charcoal suspension from refrigerator and place on a stir plate, at setting 5, for approximately 1 min before use. Referring to the **series tables** above, and using the Eppendorf repeating pipette and the appropriate Combi-tip, add Dextran/Charcoal suspension to all tubes, except TC. *Precise timing on this step is absolutely essential.* Start timer for 30 min countdown, then shake racks vigorously and return to the refrigerator for incubation at 4 C.
  8. At 30 min, remove batch from refrigerator and load all tubes, except TC, into centrifuge carriers (starting with standard curve) and centrifuge at 4000 rpm X 20 min X 4 C.
  9. Re-rack tubes (behind TC; in the same sequence as Step 8) and carry to the isotope lab (#138) for decanting. The reaction tubes must be handled carefully from this

point. Protect them from mechanical or thermal shock that might otherwise disturb the charcoal pellet. If this happens to a sample tube, take note, it must now be considered rerun. If this happens to a Standard tube, see step 1..

10. Starting with the standard curve, rack the tubes (in sets of 10) into the decanting bar and carefully decant supernatant into the 7 mL scintillation vials. Allow 10 seconds for complete pour-off and touch the rims of the reaction tubes to the surface of the cocktail to remove the last droplets. This step should be done precisely the same way for each bar of standards or samples across both batches.
11. Place the flat of scintillation vials on a tray and carry them to main lab (139) for capping, labeling and mixing. Cap the entire set. Label the cap of each standard vial with its ID or concentration. Label the cap of every fifth sample vial with its sequence number within the RIA (e.g. flat one = standard curve #1 plus samples 1 through 135; flat two = standard curve #2 plus samples 136 through 270). Place entire flat between two trays and mix thoroughly by 15 to 20 inversions. Leave the covered trays overnight in lab #138.

## Day 2

12. Re-mix the flats by inversion and count for 1.0 min each on TR2100 beta counter. Be sure to use the appropriate protocol-definition clip on the first cassette.
13. Transfer the quantification data from the TR2100 to a desktop PC and match the sequence of the RIA to the sequence of the sample array.
14. Transfer the counted vials to radioactive waste storage. Vials and solids (reaction tubes, paper wastes, etc.) must be boxed separately.

## Recovery Determination

1. Prepare recovery solution in a 13 X 100 mm PP culture tube by introducing appropriate volume (0.001 - 0.005 mL) of tritiated testosterone into 3.0 mL distilled EtOH. Vortex 1 min and let stand >30 min.

2. Pipette 0.010 mL recovery solution into each of three empty scintillation vials (total count set) and into each of three or more 16 X 100 mm BSG extraction tubes. Dry down solvent under gentle stream of nitrogen gas at ambient temperature.
3. Pipette 0.500 mL charcoaled plasma into BSG extraction tubes. Cap and incubate tubes in 35 - 38 C water bath for 5 min; mix by gentle vortex and inversion. Let stand at 4 C until extraction procedure.
4. Add appropriate volume of cocktail to scintillation vials, mix well, let stand overnight, count.
5. Extract recovery tube samples serially in triplicate with unknowns.
6. Determine recovery percentage after extraction/dry down/reconstitution (see below) by adding the total reconstitutes to scintillation vials, adding appropriate cocktail, counting and comparing to total count data.

#### Extraction Procedure

1. Pipette in duplicate plasma unknowns or pools into 16 X 100 mm BSG extraction tubes. Sample volumes used will vary from 0.050 to 0.500 mL; refer to the appropriate Series Table listed above for specific volumes to be used for a given analyses.
2. Working within the fume hood, pour ~250 mL of fresh diethyl ether into a clean beaker, then, using pre-marked glass-barrel syringe, add 3.0 to 4.0 mL to each BSG extraction tube and cap. This step should be carried out in such a manner that no tube stands uncapped for more than 2 min due to the high rate of evaporative loss of the solvent phase.
3. Place racked batch of 72 to 80 extraction tubes, including recovery tubes, in automatic shaker and cycle shaker for 2 min.
4. Let batch stand/separate for >2 min. Carefully freeze aqueous residue in pool of liquid nitrogen using the small bench-top cooler. Pay close attention to timing and handling characteristics of these tubes (i.e. time until organic phase begins to gel). If organic phase gels, allow tube to thaw completely and repeat process with

shortened exposure to nitrogen (hint: listen for the “sizzle” associated with the appropriate exposure time of about 18 to 24 seconds; this sound is the most useful marker regarding exact timing).

5. Pour off organic phase into appropriate BSG culture tubes (12 X 75 or 13 x 100 mm). Discard off aqueous residue.
6. For organic phase dry down, load culture tubes symmetrically into preheated Speed-Vac rotor. Close lid and start rotor cycle; do not start vacuum pump until rotor has reached top speed (approximately 30 sec).
7. Dry down tubes for 20 to 30 min. Shut off filter and vacuum pump and very slowly bleed the system back to atmosphere at the downstream connection between the condenser reservoir and vacuum tubing. Use a twisting motion as you slowly separate the tubing from the glass fitting). If the vacuum relief is too sudden, an oily residue from the hose will rush back into the centrifuge chamber contaminating it and all the tubes it contains—be careful here. Remove tubes and, using pasteur pipette, completely rinse tube inner surfaces with ~1.0 mL ether, vortex briefly on lowest setting, and replace tubes into Speed-Vac. Repeat above dry-down process until complete dryness is achieved.
8. Reconstitute dried tubes with 0.100 mL PBSG, tightly parafilm racks and store at 4°C for at least 12 h before RIA. Samples may be maintained for a maximum of two days at 4°C.

## APPENDIX C

### <sup>125</sup>I-BASED CORTISOL RADIOIMMUNOASSAY

Siemens Coat-A-Count Cortisol Kit # TKC05

Protocol:

1. If choosing to create additional standards for concentrations between those supplied, make them prior to starting.

Additional Standards created:

6.125 µg/dL – Add 25 µL of the 49 µg/dL standard to 25 µL of distilled H<sub>2</sub>O.

Vortex briefly to make 24.5 µg/dL standard. Vortex briefly. Add 25 µL of the

24.5 µg/dL standard to 25 µL of distilled H<sub>2</sub>O to make 12.25 µg/dL standard.

Vortex briefly. Add 25 µL 12.25 µg/dL standard to 25 µL of distilled H<sub>2</sub>O.

Vortex briefly.

2.45 µg/dL – Add 25 µL of the 4.9 µg/dL standard to 25 µL of distilled H<sub>2</sub>O.

Vortex briefly.

1.225 µg/dL – Add 25 µL of the 2.45 µg/dL standard to 25 µL of distilled H<sub>2</sub>O.

Vortex briefly.

0.355 µg/dL – Add 25 µL of the 0.71 µg/dL standard to 25 µL of distilled H<sub>2</sub>O.

Vortex briefly.

2. Label uncoated tubes for NSB and TC (total count) tubes. Label coated tubes for the remaining standards. Label coated tubes in duplicate for each unknown sample.

3. Pipette 25 µL of the zero standard into both the NSB and zero tubes. Pipette 25 µL of

each remaining standard and the pool into their respective tubes. . Pipette 25  $\mu$ L of each unknown into their respective tubes.

4. Add 1.0 mL of  $^{125}$ I Cortisol to each tube. Do this under the fume hood. Vortex for 5 seconds. Do not allow more than 10 min to elapse before continuing to the next step.

5. Cover with foil and incubate for 45 minutes in a 37°C water bath.

6. Remove from water bath and decant thoroughly using foam decanting racks. Do not decant the TC tubes. Allow tubes to drain for 3 min before striking on absorbent paper to absorb the rest of the moisture. Pat hard on paper to rid the tubes of any remaining droplets.

7. Place the tubes in a gamma counter for 2 min to be read.

**Materials Supplied by Kit:**

Antibody Coated Tubes (stable at 2-8°C until expiration date on bag.)

$^{125}$ I Cortisol (Iodinated cortisol)

6 standards of cortisol : 0  $\mu$ g/dL (2.0mL)

0.71  $\mu$ g/dL (1.0 mL)

4.9  $\mu$ g/dL (1.0 mL)

9.9  $\mu$ g/dL (1.0 mL)

20  $\mu$ g/dL (1.0 mL)

49  $\mu$ g/dL (1.0 mL)

**Materials Required but not Supplied by Kit:**

Plain 12 X 75 polypropylene test tubes for NSB and TC tubes

Test tube racks

Micropipets: 25 $\mu$ L and 1.0 mL and disposable pipet tips.

Repeat pipetter

Waterbath, capable of maintaining 37°C.

Foam decanting rack

Serum Pool for use as a standard (either make one or buy it prior to experiment)

Gamma Counter