EFFECTS OF PRENATAL STRESS ON INSULIN SENSITIVITY, PHYSIOLOGICAL STRESS RESPONSES, GROWTH, AND TEMPERAMENT OF BRAHMAN CALVES

A Thesis

by

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ABSTRACT

Stress incurred due to standard management practices applied to pregnant cattle may affect the health and productivity of the offspring. Separate studies were conducted to determine: 1) the extent to which transportation of cattle between 60 and 140 days of gestation affects the subsequent calves' endocrine stress response and insulin sensitivity, and 2) whether a chronic stressor applied during late gestation alters the birth weight, growth, or temperament of the offspring of Brahman cattle.

The function of the hypothalamic-pituitary-adrenal axis was studied with the use of intravenous ACTH and intravenous CRH challenges in 12 Brahman yearling heifers born to dams that were transported for 2 hours at 60, 80, 100, 120, and 140 days of gestation (prenatally stressed) and 12 yearling heifers whose dams were not transported (control). Prenatally stressed heifers did not differ from controls in their cortisol response to ACTH over time ($P = 0.12$) or in the cortisol ($P = 0.12$) and ACTH ($P = 0.90$) responses to CRH. The same group of heifers was also evaluated for insulin sensitivity and ability to clear circulating glucose with the use of an intravenous glucose tolerance test. Prenatally stressed heifers showed a decreased insulin response over time to the glucose bolus ($P = 0.03$), but did not differ from controls in serum glucose concentration over time ($P = 0.61$). The prenatally stressed heifers also took longer to reach peak insulin concentrations following the glucose challenge ($P < 0.01$) and returned to basal serum glucose and insulin concentrations in a shorter amount of time ($P < 0.01$).

A group of 13 multiparous cows and 20 nulliparous heifers was subjected to the Callicrate band dehorning procedure between days 93 and 168 of gestation. A control group consisting of 27 parous cows and 5 nulliparous heifers was not dehorned. The offspring produced by these cattle were evaluated for birth weight, temperament, and growth. The prenatally stressed calves (from the banded group) in this study had decreased birth weight ($P = 0.04$) compared to the control calves. However, the groups of calves did not differ in either temperament or growth at any point from 14 d of age through weaning.

In summary, it appears that a repeated transportation stressor applied between 60 and 140 days of gestation alters the insulin sensitivity, but not pituitary or adrenal function, of Brahman heifer calves. Additionally, a stressor applied later in gestation does not appear to have lasting effects on the growth or temperament of calves.

DEDICATION

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NOMENCLATURE

ACTH	Adrenocorticotropic hormone
ADG	Average daily gain
ANOVA	Analysis of variance
AUC	Area under the curve
BCS	Body condition score
BW	Body weight
CPM	Counts per miute
CRF	Corticotropin-releasing factor
CRH	Corticotropin-releasing hormone
CV	Coefficient of variation
EP	Epinephrine
EV	Exit velocity
GR	Glucocorticoid receptor
HPA	Hypothalamic-pituitary-adrenal
IACUC	Institutional Animal Care and Use Committee
IGR	Insulin:glucose ratio
IIND	Insulinogenic index
IVGTT	Intravenous glucose tolerance test
NE	Norepinephrine
PS	Pen score

RFI	Residual feed intake
SD	Standard deviation
TS	Temperament score
VFA	Volatile fatty acids
VP	Vasopressin
WW	Weaning weight

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Introduction

Livestock of all species are subjected to stressful events during life due to management practices. Whether stressful events experienced during gestation affect the postnatal productivity of the offspring is largely unclear. The cow-calf industry relies on raising healthy calves that grow rapidly to turn a profit. If stressful events during pregnancy negatively affect the productivity of the resulting calves, then it may be beneficial to producers to manage their cow herds with care. Factors that impact calf growth can include temperament, circulating cortisol concentration, and metabolic characteristics such as insulin sensitivity (Hostettler-Allen et al., 1994; Burrow and Dillon, 1997; Burdick et al., 2009).

Calves born to dams that were transported at 60, 80, 100, 120, and 140 d of gestation have altered growth rates, stress responsiveness, and temperaments (Littlejohn et al., 2013a,b). There is also evidence that repeated stress during this period influences the morphological development of the HPA axis and the rate of cortisol clearance (Lay et al., 1997a,b). However, it is not known if these calves display altered function of the HPA axis related to pituitary or adrenal sensitivity, or changes in insulin sensitivity independent of temperament. Because glucocorticoids are known to increase circulating glucose concentrations and decrease insulin sensitivity (Munck et al., 1984), it is important to determine whether or not calves exposed to prenatal stress exhibit

differences in glucose metabolism. Differences in energy metabolism may ultimately manifest as differences in feed efficiency and growth, which are both critical factors in beef production profitability.

A stressor applied at a later stage of gestation may, or may not, have effects similar to those reported for temperament and growth of the calves described by Littlejohn et al. (2013a,b). The Callicrate band method of dehorning adult cattle is possibly a viable strategy to induce a low level of chronic stress in the cow during the second trimester of pregnancy (Neely, 2013). It can take between 20 and 50 d, depending on the size of the horns, to fully remove the horns. This period of horn tissue death is stressful to the animal, and therefore could cause a significant chronic stress and possibly decreased feed intake resulting in weight loss or reduced weight gain.

During the second trimester of gestation (between 90 and 180 d post-conception), the female fetus develops its first primordial follicles (Vigier et al., 1976), but most of its other organ systems have already developed. Lu et al. (1991) showed that CRH receptor activity first appeared in the anterior pituitary gland at the beginning of the third trimester in sheep (100 d of gestation). Chronic exposure to elevated concentrations of glucocorticoids during this period may result in altered growth or temperament of calves. This is also a time of rapid fetal growth, although most will occur in the third trimester. Therefore, if cattle are exposed to a chronic stressful stimuli, such as the aforementioned dehorning method, near the end of the second trimester, it is possible that the stressful effects will continue into a portion of the third trimester and affect the rapid fetal growth that occurs at this time. Prenatal stress during the third trimester has resulted in

alterations of adrenal enzyme activity in goats and an altered response to ACTH stimulus in swine (Kranendonk et al., 2005; Roussel et al., 2005). Exposing sows to stress during mid-gestation has been shown to increase basal salivary cortisol in the offspring of these sows (Kranendonk et al., 2005). Because HPA axis function is associated with temperament in cattle (Curley, 2008; Burdick, 2010), prenatal stress may also result in altered temperament. However, there are currently no studies in the literature in which temperament has been assessed in cattle exposed to a mid- or late-gestation stressor. Further research in the area of prenatal stress, particularly during multiple stages of gestation, is important in order to make recommendations for the proper management of gestating livestock.

Objectives

Thus, the objectives for the proposed experiments include the following:

Experiment 1

The objectives of Experiment 1 are 1) to test whether prenatal stress affects postnatal pituitary responsiveness to exogenous CRH and 2) to test whether prenatal stress affects postnatal adrenocortical responsiveness to exogenous ACTH in Brahman heifers between 8 and 10 mo of age.

Experiment 2

The objective of Experiment 2 is to test whether the relationship between insulin sensitivity and glucose clearance of Brahman heifers born to cows transported for 2-h periods at 60, 80, 100, 120, and 140 d of gestation is altered by prenatal stress.

Experiment 3

The objective of Experiment 3 is to test whether a chronic stress (dehorning with Callicrate bands between 93 and 168 d of gestation) applied during the second trimester of gestation to Brahman cows alters the birth weight, growth, or temperament of the offspring.

The HPA Axis

Stress and its Significance for the Cattle Industry

The concept of stress was first introduced to the literature by Hans Selye in 1936. He described a general syndrome that affected rats subjected to a variety of agents such as cold exposure, surgery, exercise, or drug injection. The syndrome was consistent across the different treatments and was characterized by enlarged adrenal glands, diminished spleen, thymus, and lymph nodes, and bleeding gastrointestinal ulcers. What later became known as both the general adaptation syndrome and the biological stress syndrome, was generally defined as the body's reaction to stimuli which required habituation. Stress, in its most general form, is unavoidable and necessary because the body must constantly adjust to its changing environment (Selye, 1973).

Routine management of cattle involves subjecting animals to a variety of stressors over their lifespan. Management-related stressors may include handling, castration, dehorning, weaning, mixing of social groups, and transportation (Stephens, 1980). Excessive stress caused by these and other management practices, can predispose animals to health problems such as bovine respiratory disease in feedlot cattle. Increased incidence of such diseases results in substantial economic losses for producers (Duff and Galyean, 2007). Therefore, it is economically important to understand and eliminate stress in domestic cattle.

The Structures and Hormones of the HPA Axis

The HPA axis is a network of endocrine glands that includes the hypothalamus, the pituitary gland, and the adrenal glands, all of which must be fully functional to properly regulate the primary steps in the hormonal regulation of the stress response in animals (Figure 1). The hypothalamus is a neuroendocrine gland in this axis that synthesizes CRH, catecholamines including EP and NE, and VP in response to a perceived stressful stimuli by the nervous system. Corticotropin-releasing hormone is a 41 amino acid peptide that was first isolated by Vale et al. in 1981. Its synthesis is increased in response to stress (Plotsky et al., 1985; Gibbs, 1985b, Rivier and Vale, 1983). Corticotropin-releasing hormone is secreted into the primary capillary plexus of the hypothalamo-hypophyseal portal system, which is a route of blood flow between the hypothalamus and anterior pituitary gland that is independent of systemic circulation. This allows small amounts of CRH to reach the anterior pituitary gland free of dilution

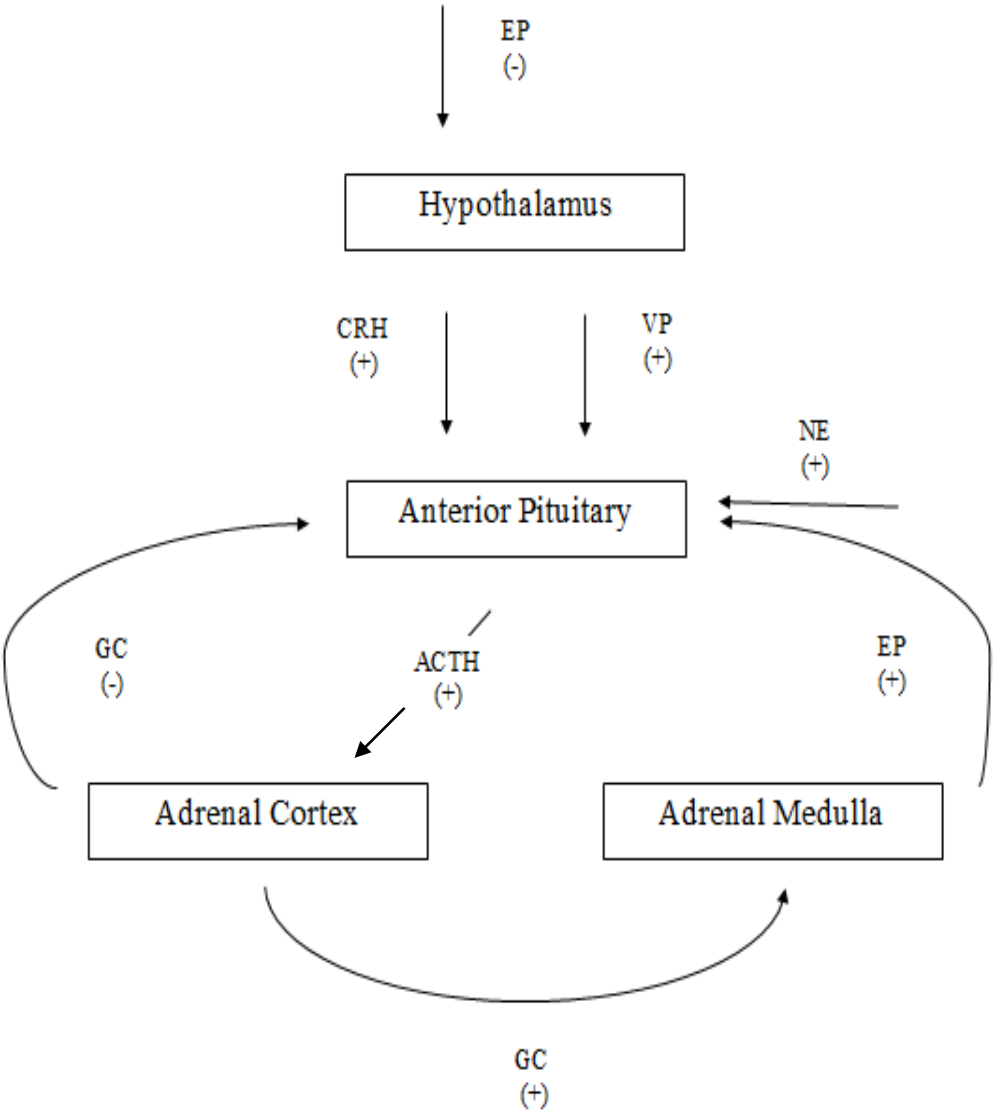

Figure 1. Regulation of glucocorticoid secretion (modified from Axelrod and Reisine, 1984). ACTH = Adrenocorticotropic hormone; CRH = corticotropin-releasing hormone; EP = Epinephrine; GC = Glucocorticoids; NE = norepinephrine; VP = vasopressin.

by systemic circulation (Plotsky, 1987). Although the hypothalamus is the primary source of CRH in the body, other tissues can also contribute to circulatory concentrations. For example, the placenta in pregnant females synthesizes and secretes CRH into systemic circulation (Challis et al., 1995). Stress also increases catecholamine synthesis in the hypothalamus. However, the adrenal medulla is the primary source of circulating catecholamines, as basal concentrations of systemic EP and NE originate almost exclusively from the adrenal gland (Gibbs, 1985a). Severe physical stress, such as hemorrhage, increases VP synthesis in the hypothalamus (Williams et al., 1985), which increases the activity of CRH (Yates et al., 1971; Gillies et al., 1982). While VP is synthesized in the hypothalamus, it is stored in and released from the posterior pituitary gland (Popenoe et al., 1952).

Corticotropin-releasing hormone and VP are both secretogogues of ACTH, but CRH is the primary modulator of the circulating concentration of ACTH (Whitnall, 1993). It has been shown that ACTH contains 39 amino acids in a single-chain polypeptide structure, and functions to stimulate the cortical cells of the adrenal glands to secrete glucocorticoids (Evans et al., 1966). This hormone is secreted by corticotrophic cells in the anterior pituitary gland and is down-regulated by glucocorticoid negative feedback (Herman and Cullinan, 1997).

Glucocorticoids are a group of hormones, the primary one being cortisol in humans and livestock, that have many physiological functions, both stimulatory and inhibitory, that allow an animal to cope with stress (Ingle, 1952; Munck and Naray-Fejes-Toth, 1992). This group of hormones was named as such when it was observed

that they are involved in glucose regulation. Specifically, cortisol increases circulating glucose concentrations in the body through increased gluconeogenesis, inhibited glucose uptake by muscle and adipose tissue, and increased mobilization of fatty acids in adipose tissue (Munck et al., 1984). In contrast to the initial release of the catecholamines, EP and NE, into circulation from the hypothalamus and adrenal medulla, the secretion of glucocorticoids is relatively slower and the actions are longer lasting (Brown and Fisher, 1986). These actions can either enhance or inhibit the initial stress response (Sapolsky et al., 2000; Munck and Naray-Fejes-Toth, 1994). Glucocorticoids also modulate the stress response by decreasing the secretion of CRH, ACTH, and VP, (Whitnall, 1993). Beginning about one h following the onset of acute stress, this negative feedback loop begins to take effect, along with other glucocorticoid actions (Sapolsky et al., 2000). In contrast to the negative feedback effects of glucocorticoids, these hormones also stimulate the synthesis of EP from NE by the chromaffin cells in the adrenal medulla (Wurtman, 2002).

HPA Axis Function

The function of the HPA axis is affected by the ability of each of its components to receive signals and secrete hormones. Furthermore, dysfunction of this system has been implicated in not only systemic disease (McEwen and Stellar, 1993), but also in mood disorders in humans (Kathol et al., 1989; Charney et al., 1993). The functional components of the HPA axis can be characterized through the use of CRH and ACTH intravenous challenges, in which a dose of either CRH or ACTH is administered and

blood samples are collected at intervals prior to and following the dose (Curley et al., 2008). A CRH challenge is useful in determining pituitary function and adrenal function, while an ACTH challenge can only characterize adrenal function. However, combining the results of these two tests provides a complete picture of HPA axis function. From this, abnormalities in cortisol concentrations can be traced to either a deficiency in pituitary or adrenal responsiveness.

Animal temperament is a factor that is associated with altered function of the HPA axis components. Curley et al. (2008) found that compared to calm heifers, temperamental heifers had a decreased ACTH and cortisol response to an intravenous dose of CRH as measured by area under the response curve. The cortisol response to an intravenous ACTH dose was also decreased in temperamental heifers compared to calm heifers. Basal cortisol concentrations were greater in the temperamental heifers and there was no significant difference in basal ACTH concentrations between the temperament groups. Ruis et al. (2000) found that gilt personality affected both cortisol response to handling and to an intramuscular ACTH dose. Low resistant gilts, or those with less aggressive personality types, had increased cortisol in response to both stimuli compared to high resistant gilts.

Sex and environmental conditions are also associated with altered HPA axis function. Female sheep exhibit a greater ACTH and cortisol response to exogenous CRH than males (Chadio et al., 2007). Furthermore, Hulbert et al. (2012) found that serum cortisol concentrations in heifers following a CRH challenge were greater than those seen in bulls. Wethers exposed to repeated handling and sham shearing showed a

progressive decrease in their cortisol response to an exogenous ACTH challenge, demonstrating the impact of acclimation on the stress response (Hargreaves and Hutson, 1990). For groups of bulls housed at the same stocking density, unfamiliar mixed groups have an increased cortisol response to an exogenous ACTH dose compared to familiar mixed groups. Within familiar mixed groups of bulls, those housed with an intermediate stocking density had a higher cortisol response to ACTH than low or high stocking density groups (Gupta et al., 2007).

Challenge Dose Response

When conducting an ACTH or CRH challenge, it is important to establish the appropriate dose needed to elicit potential biological differences in cortisol or ACTH responses between treatment groups. Curley et al. (2008) revealed significant differences between temperamental and calm Brahman heifers that underwent both an ACTH and CRH challenge. The dose for the ACTH challenge was 0.1 IU ACTH/kg BW. The dose for the CRH challenge was 0.1 μg bovine CRH/kg BW.

The optimal dose needed to elicit biological differences in the ACTH response between treatment groups during a CRH challenge has been studied in several species. Orth et al. (1983) evaluated the response of men to varying doses of synthetic ovine CRH between 0.001 and 30 μg/kg BW. They found that the threshold dose is between 0.01 and 0.03 μg/kg BW and the half maximal dose is between 0.3 and 1 μg/kg BW. When pre-injection losses were accounted for, the threshold dose was approximately 0.01 μg/kg BW. Veissier et al. (1999) administered a CRH challenge to 12-wk-old

Holstein bull calves at 4 different dose rates (0, 0.01, 0.03, and 0.1 μg/kg BW) and found that the lowest dose that produced a significant response in all calves was 0.1 μg/kg BW. This revealed that the bovine model needs a higher dose of CRH than human models to elicit a response.

Gupta et al. (2004) administered 4 increasing doses of CRH (0.1, 0.3, 1.0, and 1.5 μg/kg BW) to 14-mo-old Holstein-Friesian steers and compared ACTH and cortisol responses to a control group that received a saline infusion. Plasma ACTH concentration did not differ between the control group and the steers that received 0.1 μg/kg BW bCRH. However, in the other treatment groups there was an increase in the ACTH response compared to the control group.

Cooke et al. (2012) measured the acute phase response and cortisol concentrations in steers following CRH challenges at 2 different doses (0.1 and 0.5 μg/kg BW) and compared them to those of a control group that received a saline infusion. Both doses resulted in a significant increase in cortisol following the challenge. The authors did not report ACTH concentrations.

Regulation of Insulin and its Interaction with Glucose

The Discovery of Insulin and its Function

Von Mering and Minkowski (1890) were the first to develop the concept that secretions of the pancreas were involved in diabetes mellitus when they removed the pancreas from dogs and observed the subsequent development of the condition. After this experiment, there were differing ideas in the scientific community about the specific

mechanism by which the pancreas prevented diabetes. Some believed that the organ removed an agent from the blood that prevented the uptake of glucose, while others thought that the pancreas secreted a substance that promoted this action (Goldfine and Youngren, 1998). In 1915, Carlson and Ginsburg settled this disagreement by proving that blood from normal dogs contained a substance that would reduce diabetic symptoms when injected into depancreatized dogs. This pancreatic secretion was eventually isolated and named "insulin" (Banting et al., 1922a; Banting et al., 1922b). Later, Houssay and Biasotti (1931) discovered that the anterior pituitary gland, in addition to the pancreas, plays an important role in the regulation of blood glucose. Specifically, growth hormone increases glycogenolysis and decreases glucose uptake.

Regulation of serum glucose by insulin is an essential component of energy metabolism (Banting et al., 1922a,b,c). Glucose is a carbohydrate that is readily used by every cell in the body for energy. The brain, in particular, relies solely on glucose for energy and accounts for 10-15% of whole-glucose utilization in sheep (Hocquette et al., 1996). Insulin regulates serum glucose concentrations by stimulating its uptake by the body's liver, muscle, and adipose tissues and by inhibiting gluconeogenesis and glycogenolysis. Insulin also stimulates the secretion of pancreatic α-amylase into the small intestine, which facilitates the digestion and subsequent absorption of carbohydrates (Soling and Unger, 1972). Elevated blood glucose concentrations stimulate an insulin response (Bach and Holmes, 1937).

Insulin Structure and Synthesis

The chemical structure of insulin was first characterized by Sanger and Thompson (1953). They determined that the 51-amino acid peptide hormone is composed of two polypeptide chains (A and B) connected by disulfide bridges. Bovine and human insulin differ in the A polypeptide chain by three amino acid residues and display similar bioactivity. In human insulin, the specific chain sequence is Thr.Ser.Ile and in bovine insulin it is Ala.Ser.Val (Werner and Chantelau, 2011).

Insulin is stored as granules by β-cells in the pancreatic islets of Langerhans. It is synthesized from preproinsulin and proinsulin precursors, primarily in response to circulating glucose (Anderson and Long, 1947; Okamoto, 1981). The carbohydrates mannose and fructose also stimulate pancreatic secretion of insulin, but galactose, xylose, pyruvate, L-arabinose, and 2-deoxyglucose do not. This may occur because insulin secretion is triggered by a metabolite produced by the digestion of glucose, mannose, and fructose (Grodsky et al., 1963).

Glucoregulatory Mechanisms

Ruminant metabolism of glucose is unique from monogastrics due to their reliance on complex carbohydrates for nutrition. The rumen contains microorganisms that digest cellulose and hemicellulose present in fibrous plant material through fermentation (Hocquette and Abe, 2000). The fermentation process results in the production of VFA including acetate, propionate, and butyrate, which serve as the primary source of energy for the ruminant. These VFA provide approximately 70% of

the animal's metabolized energy. Glucose obtained directly from feedstuffs makes up a small proportion of the total glucose absorbed (Bergman, 1973). The remaining glucose is synthesized in the liver and kidneys (Bergman, 1973; Lindsay 1978). Propionate is the major precursor to the glucose synthesized by the liver and kidneys, accounting for up 76% of the total (Reynolds et al., 1994). Other compounds used by the liver in glucose synthesis include glycogenic amino acids, lactate, glycerol, i-butyrate, and n-valerate (Leng, 1970). In the fasted state, the ruminant animal relies on glycerol from adipose tissue and amino acids from muscle tissue to perform gluconeogenesis (Bergman, 1973).

Non-insulin-sensitive glucose transporters (GLUT 1, 3, 4, and 5) provide cells with the ability to take up and utilize glucose independent of insulin. GLUT 1 plays a large role in the glucose uptake of a wide variety of different tissues. Erythrocyte uptake of glucose is primarily promoted by GLUT 1, which is minimally expressed in the ruminant, compared to monogastric species. The uptake of glucose, galactose, and fructose into circulation by facilitating their diffusion in small intestine epithelial cells proximal to blood capillaries is catalyzed by GLUT 2. GLUT 3 is the primary glucose transporter for neuronal cells, but is also important in a variety of other tissues including the placenta and white blood cells. GLUT 5 catalyzes the uptake of both glucose and fructose in the small intestine epithelial cells, and also promotes glucose reabsorption in the kidney (Hocquette and Abe, 2000).

In contrast, GLUT 4 transporters, which are most commonly implicated in the pathology of human diabetes, are considered insulin-sensitive (Abe et al., 1996; Hocquette et al., 1996; Hocquette and Abe, 2000). Insulin is the primary regulator of

circulating glucose levels (Banting et al., 1923). When insulin binds to an insulin-sensitive cell, GLUT 4 is translocated from its basal state inside the cell to the plasma membrane. This allows for glucose to diffuse into the cell. In ruminants, GLUT 4 transporters primarily facilitate the glucose uptake by muscular and adipose tissues, and thus control the partitioning of energy between them (Hocquette and Abe, 2000). Insulin also regulates the circulating glucose concentration by stimulating liver and muscle tissue to synthesize glycogen and adipose tissue to increase lipogenesis from glucose (Hocquette et al., 1998). Due to the important role they play in determining body composition, glucoregulatory mechanisms involving insulin and GLUT 4 receptors should be of interest to livestock producers looking to increase meat yield.

Insulin Sensitivity

Ruminants generally have decreased insulin sensitivity compared to monogastric species (Sternbauer and Luthman, 2002). The maximally insulin-stimulated glucose utilization rate is approximately 10 mg•min-1•kg-1 BW for humans and 2-5 mg•min-1•kg-1 BW for ruminants (Hocquette et al., 1998). Increased insulin sensitivity in muscle tissue is associated with leanness in livestock due to increased glycogenolytic activity (Kelly et al., 2010). Insulin sensitivity has also been associated with decreased weight loss during periods of caloric restriction in obese humans (Hoffman et al., 1995).

Insulin sensitivity in ruminants varies with both the age of the animal and nutritional status (Hocquette and Abe, 2000). Glucose tolerance, and thus insulin sensitivity, has been shown to decrease with age in calves (Bauchart et al., 2000).

Similarly, insulin sensitivity and GLUT 2 expression has been shown to decrease in lambs with age (Gelardi et al., 1999). However, in contrast to monogastric species, ruminants do not appear to experience changes in GLUT 4 expression following weaning (Hocquette et al., 1997).

An IVGTT can be used to measure the response of insulin to glucose, and therefore an animal's insulin sensitivity. During the test, a bolus of glucose is administered intravenously and blood samples are collected at intervals of time to quantify changes in concentrations of glucose and insulin in serum over time. From these concentrations, it is possible to calculate markers of insulin sensitivity such as peak insulin concentration, time to peak insulin concentration, time to return to basal insulin and glucose concentrations, glucose half-life, area under the curve for both glucose and insulin, coefficient of glucose disappearance, the IGR, and the IIND. The IIND is calculated by dividing the change in insulin from basal insulin concentration by the change in glucose from basal glucose concentration (Abdelmannan et al., 2010; Cohn et al., 1999). The IIND can be calculated at any time point or as a maximal value in which the change in insulin to peak concentration is divided by the change in glucose to peak concentration. Basal insulin and glucose concentrations are determined by blood samples collected prior to the glucose infusion.

The Relationship between Stress, Insulin, and Glucose

Glucocorticoids are known to increase blood glucose concentrations and increase insulin resistance, which gives the body extra energy in times of stress (Thorn et al.,

1957; Frawley et al., 1959). Elevated blood glucose concentrations caused by a stress response will result in an elevation of insulin concentrations. During the stress response, catecholamines and glucagon inhibit the action of insulin and prevent uptake of glucose by certain tissue types (Porte and Robertson, 1973; Freeman and Manning, 1976). EP has been found to stimulate hyperglycemia through both glycogenolysis in the liver and the inhibition of the release of insulin (Anderson and Long, 1948; Coore and Randle, 1964). Most likely because of these actions, temperamental animals have an increased insulin response to glucose, and therefore lower insulin sensitivity in the body's tissues (Bradbury et al., 2011).

Following chronic stress, glycogen stores must be repleted. The rate of re-synthesis of glycogen may be positively associated with insulin sensitivity (Hocquette and Abe, 2000).

Prenatal Stress and Fetal Development

Fetal Programming

The concept of fetal programming is best defined by the Barker hypothesis in which it is proposed that conditions during fetal life may play a role in the risk of developing adult diseases and conditions. Barker et al. (2002) found that children born to mothers who were gestating during the Dutch Hunger Winter famine were more likely than their peers to develop coronary heart disease. They hypothesized that a poor uterine environment programs the fetus to exhibit a "thrifty phenotype" that gives them the ability to take up and utilize nutrients more efficiently. This then results in an increased

risk of obesity, type 2 diabetes, heart disease, and hypertension for the adult living in non-famine conditions.

Many different types of stressors, including both nutritional and psychological, at varying stages of gestation have been found to affect fetal development in diverse patterns (Barker et al., 2002; Entringer et al., 2012). The finding that gestating females respond to stress differently, depending on the stage of pregnancy they are in, plays a role in the wide range of outcomes that have been seen in prenatally stressed offspring. Entringer et al. (2010) reported that pregnant human females display both an attenuated physical and psychological response to psychosocial stress in late gestation (30 wk). Furthermore, the dependence of the fetus on maternal cortisol varies over the period of gestation (Challis et al., 2001), which may further contribute to the wide variation of prenatal stress effects that have been observed. Therefore, both the type and timing of a prenatal stressor must be considered in order to make assumptions about possible fetal effects.

Effects of Prenatal Stress on the HPA Axis

Maternal cortisol has been shown to increase expression of CRH mRNA in the placenta. This increase in fetal CRH results in a positive feedback loop that increases CRH, ACTH, and cortisol levels in both the maternal and fetal systems (Petraglia et al., 1996; King et al., 2001). Under normal circumstances, the exposure of the fetus to maternal glucocorticoids is low due to placental activity of 11β-HSD2, an enzyme which oxidizes cortisol to its inactive form (Seckl and Meaney, 2004). Only between 10 and

20% of maternal cortisol passes through the placenta un-oxidized. However, this reduced amount of cortisol exposure appears to be sufficient to alter fetal HPA axis function (Gitau et al., 2001; Weinstock, 2005).

Barbazanges et al. (1996) demonstrated that it is primarily exposure to maternal glucocorticoids that causes altered HPA axis function in offspring. Adrenalectomized pregnant rats were dosed with exogenous corticosterone to maintain normal basal levels and were submitted to stressful events. The offspring from these rats exhibited no signs of HPA axis alteration, which suggests that substances produced by the maternal adrenal glands, most likely glucocorticoids and catecholamines, are necessary for the changes in programming observed in other studies.

Prenatal stress appears to result in an alteration of the functioning of the HPA which can persist into pre-adolescence. Increased cortisol concentrations upon awakening were observed in the 10-year-old children of mothers who reported symptoms of anxiety and depression during pregnancy as part of a prospective longitudinal cohort study (O'Connor et al., 2005). However, children born to mothers living or working in close proximity to the World Trade Center bombings on September 11, 2001 while pregnant, and subsequently developed post-traumatic stress disorder, exhibit reduced cortisol concentrations (Yehuda et al., 2005). Similarly, Entringer et al. (2009) also found that young adults born to women who experienced severe psychosocial stress during pregnancy exhibited decreased basal cortisol concentrations. However, these prenatally stressed young adults displayed an increased adrenal response to both a psychosocial stressor and an ACTH challenge.

There is evidence that prenatal stress can cause alterations of HPA axis function that may inherited by subsequent generations. Radtke et al. (2011) found that women who experienced intimate partner violence during pregnancy did not exhibit altered expression of the GR gene, but their offspring showed an increase in methylation of the promoter region. Combined with the observation that violence experienced before and after pregnancy had no effect on the GR methylation status of the offspring, this suggests that the epigenetic regulation of this gene occurred during fetal development.

It has previously been established that transportation stress events at 60, 80, 100, 120, and 140 d of gestation create significant alterations in the HPA axis function and growth of Brahman calves (Lay et al., 1997a,b; Littlejohn et al., 2013a,b). Fetal calves at 266 d of gestation had significantly larger pituitary glands and BW than control calves, which could possibly be explained by a glucocorticoid-stimulated increase in somatotroph production of growth hormone. The basal concentrations of both ACTH and cortisol in these calves were not altered due to prenatal stress; however, the enlarged pituitary gland suggested a possible increased sensitivity to CRH (Lay et al., 1997a). This possible enhanced pituitary sensitivity to CRH could be the cause of increased basal glucocorticoid concentrations seen in prenatally stressed animals. Suckling calves that had been exposed to prenatal transportation stress exhibited a greater cortisol response to 3.5 h of restraint at 10 and 150 d of age than control calves and calves whose dams were given an ACTH dose of 1 IU/kg BW at 60, 80, 100, 120, and 140 d of gestation. However, there were no treatment differences in the cortisol response to branding, possibly due to the intensity or short duration of the stressor. Furthermore, the prenatal

transportation stress calves exhibited significantly slower clearance of exogenous cortisol than other groups, but did not have an altered response to exogenous ACTH (Lay et al., 1997b). In another study, prenatal transportation stress at the aforementioned d of gestation increased exit velocity, pen score, and temperament scores of calves (Littlejohn et al., 2013a,b).

Maternal nutrient restriction has also been proven to alter the postnatal HPA axis function of ruminants. Lambs that experienced nutrient restriction between d 0 and 30 of prenatal development have greater ACTH response to exogenous CRH at 2 mo of age than lambs that were nutrient restricted later in gestation or not at all, but the difference disappeared by 5.5 mo of age. The dams in the early nutrient restriction group showed elevated cortisol concentrations compared to the other groups from d 40 of gestation to term (Chadio et al., 2007).

Effects of Prenatal Stress on Metabolism

During gestation, glucose is the primary source of fuel for the fetus. Early in development, the fetus cannot perform gluconeogenesis, so it relies solely on maternally supplied glucose for energy (Faulkner, 1983). Expression of GLUT 4 increases in the perirenal and adipose tissue of the bovine fetus during the third trimester (Abe et al., 1999), but begins to decline in the adipose tissue near parturition (Hocquette et al., 1999). Pregnant sheep infused with glucose to induce fetal hyperglycemia gave birth to offspring with decreased myocardial and skeletal muscle GLUT 4 expression (Das et al., 1999). Decreased GLUT 4 concentrations in adult animals typically indicate an insulin

resistant state (Kahn et al., 1988; Garvey et al., 1989), but it is not known if fetal down-regulation of this transporter persists into adult life.

In rats, the administration of synthetic glucocorticoids to pregnant dams has been shown to impair glucose tolerance in the offspring (Muneoka et al., 1997; Nyirenda et al., 1998; Franko et al., 2010). However, natural glucocorticoids may have the opposite effect in some circumstances. Franko et al. (2010) found that rats born to dams repeatedly injected with saline between 15 and 20 d of gestation had improved glucose tolerance compared to an untreated group and a group treated with dexamethasone. In humans, a mother's psychosocial stress has also been associated with metabolic alterations in her child (Entringer et al., 2008).

Effects of Prenatal Stress on Behavior

Prenatal glucocorticoid exposure has been linked to morphological and behavioral changes associated with the central nervous system (Tamashiro and Moran, 2010). The extent of these changes appears to vary by the gender of the offspring and the timing of the stressor.

Several studies have found that prenatally stressed animals display altered behavioral patterns and an increased risk of mental illness. Vallée et al. (1997) found that adult rats, whose dams had experienced handling stress during the last week of gestation, displayed high anxiety behavior in a maze test. Children whose mothers experienced psychological stress during pregnancy are both more likely to develop ADHD and to display more severe symptoms than children whose mothers did not

experience moderate to severe stress during pregnancy (Clements, 1992; Grizenko et al., 2008).

The Dutch Hunger Winter that Barker described did not only increase the risk of cardiovascular disease in adults who were *in utero* at the time. Brown et al. (2000) found that the risk of those who were exposed during their second or third trimesters was significantly greater for being hospitalized due to the psychiatric condition major affective disorder. Furthermore, the individuals' risk of affective disorder and the severity of the famine they experienced was not related. Infant temperament has also been associated with prenatal stress. Salivary cortisol concentrations of mothers at 32 wk of gestation, along with an averaged measure of prenatal anxiety and depression between 19 and 32 wk, is positively correlated with infant negative reactivity, a measure which assesses the frequency of fearful behaviors (Davis et al., 2007).

Brain development has also been linked to the prenatal environment. In 2003, Coe et al. determined that prenatally stressed rhesus monkeys exhibited decreased neuronal growth of the hippocampus, which is essential for memory and spatial navigation. Similarly, Uno et al. (1990) found that exposing fetal rhesus monkeys to 2 d of dexamethasone 1 mo before parturition is sufficient to cause significant atrophy of hippocampal cells that does not resolve by 2 yr of age. According to Davis and Sandman (2010), exposure to elevated cortisol levels during early gestation is associated with a delayed rate of cognitive development during the first year of an infant's life. However, the same conditions late in pregnancy were associated with accelerated cognitive development.

Prenatal stress also appears to alter behavior in livestock species. Social mixing of pregnant sows during the second and third trimesters is associated with abnormal maternal behavior in the offspring. The prenatally stressed sows displayed significantly more restlessness following parturition and tended to bite their piglets more often than the control group of sows (Jarvis et al., 2006). Littlejohn et al. (2013a,b) found that prenatal stress alters the temperament of Brahman calves. The prenatally stressed calves were more temperamental, as determined by PS, EV, and TS, between the age of 14 d and the time of weaning.

Sex-Specific Effects of Prenatal Stress

Prenatal stress appears to affect the neurological development and HPA axis function of male and female offspring differently (Kapoor et al., 2006). Weinstock et al. (1992) found that only female offspring born to rats who were subjected to stress throughout pregnancy exhibited a decrease in hippocampal GR binding sites. This could result in decreased negative feedback to the hypothalamus, and thus excessive activation of the pituitary and adrenal glands in response to stress. Meaney et al. (2007) states that this results in increased basal cortisol concentrations, in addition to increased stress responsiveness. Szuran et al. (2000) also observed similar results in the hippocampal GR density and increased basal corticosterone concentrations in female rats born to dams who were restrained daily during their last week of gestation. Furthermore, it has also been reported that glucocorticoid transfer across the mouse placenta is greater in female fetuses than in male fetuses (Montano et al., 1993).

When pregnant rats are exposed to restraint stress during the final week of gestation, their offspring display differential programming of the HPA axis. McCormick et al. (1995) found that the prenatally stressed females exhibited increased ACTH and corticosterone responses to restraint stress compared to control rats, but the prenatally stressed male rats did not. Additionally, plasma corticosteroid-binding globulin and free corticosterone were elevated in the prenatally stressed females, but not the males. This further suggests that females may be more sensitive to stress *in utero* than males.

However, there is at least one study in the literature in which prenatally stressed male offspring were more severely altered than their female siblings. Mueller and Bale (2008) found that male mice exposed to prenatal stress in early gestation exhibited an increased HPA axis response to stress, demonstrated by elevated CRF and GR gene expression. The male mice also experienced methylation of the CRF and GR genes. The female offspring in this study did not differ from the control group.

It is clear that prenatal stress results in numerous postnatal effects across mammalian species. However, the interactions between the physiological stress response, metabolism, and behavior have yet to be completely explained.

CHAPTER II

EFFECTS OF PRENATAL STRESS ON PITUITARY AND ADRENAL FUNCTION

Introduction

The potential effects of stress *in utero* have been studied in both human and non-human species. The concept of fetal programming, in which the prenatal environment permanently "programs" the fetus to cope with adult life, was pioneered by David J. Barker in the early 1990s (Barker et al., 2002). Since his initial discovery of the relationship between birth weight and cardiovascular disease later in life, many other studies have found a relationship between fetal insult (either stress or under nutrition) and alterations in disease patterns, behavior, stress responsiveness, and metabolism across species (Barker et al., 2002; O'Connor et al., 2005; Yehuda et al., 2005; Kapoor et al., 2006; Muneoka et al., 2007; Franko et al., 2010).

If this phenomenon is manifested in livestock, then the welfare and nutrition of pregnant animals should be given careful consideration in order to minimize the risk of dampening the potential productivity of the offspring. In the beef industry, common management practices such as dehorning, transportation, and social mixing impose stress on cattle (Mench et al., 1990; Stafford and Mellor, 2005; Buckham Sporer et al., 2008). If it is found that prenatal stress negatively affects the productivity or efficiency of cattle, then this knowledge can be used to justify either avoiding submitting pregnant cows to stressful management practices altogether or during critical times during gestation.

The programming of the HPA axis is of special interest to beef cattle producers because of its association with animal health and productivity (Fell et al., 1999). Animals which are more responsive to stressful events, as evidenced by increased cortisol concentrations, are more vulnerable to immune challenges (Silberman et al., 2003; Compas et al., 2004; Zhao et al., 2008). Furthermore, Burdick et al. (2009) found that serum cortisol concentrations are negatively correlated with calf growth and serum immunoglobulin concentrations in young calves.

Lay et al. (1997a,b) found evidence that transporting pregnant cows at 5 different time points at 20-d intervals between 60 and 140 d of gestation was associated with an increased adrenal response to restraint stress, an enlarged pituitary gland, and decreased ability to clear exogenous cortisol from circulation. The objective of this study was to examine alterations in pituitary and adrenal function in yearling Brahman heifers that were exposed to prenatal stress at 60, 80, 100, 120, and 140 d of gestation.

Materials and Methods

All processes required to complete this project were approved by the Texas A&M University IACUC.

Animals and Experimental Design

Brahman heifers (n = 24) were utilized to compare pituitary and adrenal function between prenatally stressed (PNS) and control animals (C). Pituitary function was directly evaluated and adrenal function was indirectly evaluated using an intravenous

exogenous CRH challenge. Adrenal function, alone, was directly evaluated using an intravenous exogenous ACTH challenge. The dams of PS heifers were transported at 60, 80, 100, 120, and 140 d of gestation for 2-h periods, while the dams of the C heifers were not transported during gestation.

The group of tested heifers was equally divided into two subgroups (n = 12), with one subgroup being tested on a given day. The subgroups consisted of 6 PNS animals and 6 C animals that had been paired by temperament, which was determined by TS at weaning. Animals were selected from the population of PNS and C heifers by choosing the 6 most temperamental and 6 calmest heifers from each treatment group that had a similarly scored counterpart in the opposing group. The difference between matched animals ranged from 0 to 0.28 with a SD of 0.1. The mean TS of the sampled C and PNS heifers at weaning were 1.97 ± 0.94 and 2.01 ± 0.92 (Table 1), respectively. This experiment used a complete block design in which day of the challenge (1 or 2) was the block and the factors were temperament class (Calm or Temperamental) and treatment (C or PNS). The factor levels were PNS/Calm, PNS/Temperamental, C/Calm, and C/Temperamental. Each factor level was used and replicated the same number of times in each block.

The ACTH challenges were carried out on January 22nd and 24th, 2013, and the CRH challenges were carried out on February 20th and 22nd, 2013. During each test day, one subgroup was utilized for a period of 12.5 h while being confined within a stanchion.

Table 1. Characteristics of Brahman heifers utilized in an ACTH challenge and CRH challenge. The treatment groups did not differ in any of the analyzed characteristics ($P \geq 0.26$).

Variable	Treatment		P-value
	Control	Prenatal Stress	
ACTH Challenge Body Weight, kg	209.28 ± 24.18	207.65 ± 19.53	0.86
CRH Challenge Body Weight, kg	219.95 ± 24.72	219.28 ± 19.64	0.94
ACTH Challenge Age, d	283.75 ± 25.62	294.83 ± 20.88	0.26
CRH Challenge Age, d	312.75 ± 25.62	323.83 ± 20.88	0.26
Pen Score	2.42 ± 1.39	2.08 ± 1.08	0.53
Exit Velocity, m/s	1.45 ± 0.87	1.79 ± 0.79	0.32
Temperament Score	1.97 ± 0.94	2.01 ± 0.92	0.92

Temperament Evaluation

The Brahman heifers used in this experiment were evaluated for temperament at weaning by PS, EV, and TS. Pen score (Table 2) was assigned by a trained evaluator on a scale of 1 to 5 to describe the animal's willingness to be approached by a human (Hammond et al., 1996). A low PS indicates calmer or more docile temperament, while a higher PS indicates a reactive or aggressive animal. The same evaluator assigned a PS to each animal at weaning.

Exit velocity was measured as the velocity at which an animal travels 1.83 m immediately after exiting a squeeze chute (Burrow et al., 1988; Curley et al., 2006). It

was calculated from the time elapsed as the animal traversed 1.83 m after exiting the chute. Infrared sensors were used to start and stop a timer (FarmTek Inc., North Wylie, TX) that measured the time elapsed. A faster EV indicates a more temperamental animal, while a slower EV indicates a calmer animal.

A TS was calculated as the numerical average of EV and PS for each animal at weaning (Curley et al., 2006, 2008; King et al., 2006). A greater TS indicates a more temperamental animal while a lesser TS indicates a calmer animal. For the purpose of assigning animals to blocks using temperament, a TS ≥ 2 was considered "Temperamental" and TS ≤ 2 was considered "Calm" for this experiment.

Table 2. 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

Blood Collection Procedures

On the morning of each challenge day, heifers (n = 12) were fitted with jugular cannulas to allow for blood collection. A sterile 14-gauge thin-walled stainless steel biomedical needle (o.d. 2.11 mm) was inserted into the jugular vein and approximately

15 to 20 cm of PTFE (o.d. 1.66 mm; Cole-Palmer, Vernon Hills, IL) tubing was passed through the needle and into the vein. Approximately 15 cm of additional tubing was left outside of the animal. This tubing that extended from the vein was secured to the heifer's neck using stock glue (Santa Cruz Biotechnology, Santa Cruz, CA) and porous surgical tape. The cannula was then fitted with approximately 2 m of sterile plastic Tygon tubing (i.d. 1.59 mm, o.d. 3.18 mm; VWR Scientific, West Chester, PA) to allow for blood sampling in the stanchions. An 18-gauge needle with a 10-mL syringe was used to cap the end of the Tygon tubing. Before releasing the animal from the chute, the cannula was flushed with heparin solution (2 IU/mL) to ensure patency and the Tygon tubing was secured to the back of the animal using Vetrap Bandaging Tape (3M, St. Paul, MN) and surgical porous tape. Immediately following these procedures, the heifers were released from the chute and placed in a stanchion.

Once all heifers were placed in the stanchions, a period of at least 15 min was given as time for the animals to acclimate. Blood samples were collected into one 10-mL no additive Vacutainer tube (BD Biosciences, Franklin Lakes, NJ) at each time point during the ACTH challenge. Blood samples were collected into a 10-mL no additive Vacutainer tube (BD Biosciences, Franklin Lakes, NJ) and a 10-mL EDTA-coated Vacuatainer tube (BD Biosciences, Franklin Lakes, NJ) at each time point during the CRH challenge. Following each sample collection during the ACTH challenge, 10-mL of physiological saline solution (0.9%) was administered and followed with 7 mL of heparin solution (2 IU/mL). Similarly, each sample collection during the CRH challenge was followed with 20 mL of physiological saline solution (0.9%) and 7 mL of heparin

solution (2 IU/mL). These solutions were administered in order to replace fluid volume and prevent blood clotting in the cannulas.

Blood samples collected into the EDTA-coated tubes during the CRH challenge were immediately centrifuged at 4˚ C to separate the plasma. The plasma was then aliquoted into 2 polyethylene storage tubes and flash-frozen in liquid nitrogen. Plasma samples were stored at -80˚ C. The blood samples collected into the no additive tubes were immediately placed on ice and then stored at 4 ˚C for 12 h. Following this 12-h period, the samples were centrifuged at 4˚ C to separate the serum. The serum was then aliquoted into 2 polyethylene storage tubes and stored at -20˚ C.

ACTH Challenge

Blood samples (n = 31) were taken at the following time intervals in relationship to ACTH dose administration at 0 min: -360, -330, -300, -270, -240, -210, -180, -150, -120, -90, -60, -30, 0, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180, 210, 240, 270, 300, 330, and 360 min. Porcine ACTH (A6303 SIGMA, Sigma Chemical, St. Louis, MO) dissolved in physiological saline (0.9%) was administered at time 0 at a dose of 0.1 IU/kg BW via the cannulas. Porcine ACTH and bovine ACTH have identical biological activities in cattle due to the fact that they share the first 24 amino acid residues (Schwyzer, 1977).

CRH Challenge

Blood samples (n=35) were taken at the following time intervals in relationship to CRH dose administration at time 0 min: -360, -330, -300, -270, -240, -210, -180, -150, -120, -90, -60, -30, 0, 5, 10, 15, 20, 25, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180, 210, 240, 270, 300, 330, and 360 min. Bovine CRH (#4030530, Bachem Americas, Inc., King of Prussia, PA) was administered at a dose of 0.3 μg/kg BW at time 0, following the optimal dose recommendation of Gupta et al. (2004).

Cortisol RIA

Serum cortisol concentrations were determined from duplicate samples taken during both the ACTH and CRH challenges. Only the samples from the following time points were analyzed from the ACTH challenge: -120, -90, -60, -30, 0, 15, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180, 210, 240, 270, 300, 330, and 360 min. Similarly, only the samples from the following time points were analyzed from the CRH challenge: -120, -90, -60, -30, 0, 5, 10, 15, 20, 25, 30, 45, 60, 75, 90, 105, 120, 135, 150, 165, 180, 210, 240, 270, 300, 330, and 360 min. These time points were selected for analysis because the serum cortisol concentrations of many animals during the first 240 min of the acclimation period would show evidence of stress incurred during the cannulation process. By analyzing the samples beginning at -120 min, we were able to compare serum cortisol concentrations between animals without needing to account for exactly how much time had passed since they had been fitted with a cannula.

The samples were assayed using a single antibody RIA procedure (see Appendix C) as described by Curley et al. (2008). The procedure utilized rabbit anti-cortisol antiserum (Pantex, Div. of Bio-Analysis Inc., Santa Monica, CA) diluted 1:2,500, standards made by serial dilution (8,000 pg /100 µL to 3.9 pg/100 µL) of 4-pregnen-11β,17,21-triol-3,20-dione (Steraloids Inc., Newport, RI), and radio-labeled cortisol 3H-Hydrocortisone (1,2-3H, NEN, Boston, MA). Unknown cortisol concentrations were calculated using Assay Zap software (Biosoft, Cambridge, UK). The CPM were obtained from a liquid scintillation spectrophotometric beta-counter (Beckman Coulter LS 6500, Beckman Coulter, Inc., Brea, CA). The cortisol antiserum cross-reactivity was as follows: corticosterone, 60%; deoxycorticosterone, 48%; progesterone, 0.01%; and estradiol, 0.01%. For the ACTH challenge samples, the interassay and intraassay CV were 7.20% and 5.19%, respectively. For the CRH challenge samples, the interassay and intraassay CV were 13.25% and 12.74%, respectively.

ACTH RIA

Plasma ACTH concentrations were determined from duplicate samples taken during the CRH challenge. The samples were assayed using a double antibody RIA procedure (see Appendix D) as described by Curley et al. (2008). Only the samples from the following time points were analyzed: -30, 0, 10, 15, 20, 25, 30, and 45. This abbreviated schedule of time points was selected due to our need to include samples from all animals in a single assay. Based on data from Curley et al. (2008), we expected

all heifers to have reached their peak plasma ACTH concentrations by the 45 min time point.

The assay utilized a 1:2,000 dilution of IgG-ACTH-1 rabbit anti-(1-24)ACTH (IgG Corporation, Nashville, TN) as a primary antibody, goat anti-rabbit gamma-globulin (Calbiochem, La Jolla, CA) diluted 1:20 as the secondary antibody, standards made through serial dilutions (100 pg/100 μL to 0.05 pg/100 μL) of h,r(1-24)ACTH (Peninsula Laboratories, San Carlos, CA), and radio labeled ACTH 125I h(1-24)ACTH (ICN Biomedical, Carson, CA). The CPM were obtained from a Cobra II auto-gamma-counter (Perkin Elmer, Waltham, MA). Unknown ACTH concentrations were calculated using Assay Zap software (Biosoft, Cambridge, UK) and the intraassay CV was 9.62%.

Statistical Analysis

Once cortisol concentrations were obtained for each sample, the data were analyzed using the MIXED procedure in SAS 9.3 (SAS Institute, Inc., Cary, NC) to conduct a repeated measures ANOVA for each challenge. The fixed effects of time and treatment, with time being repeated, were analyzed for significance. A spatial power covariance structure was used in this model due to the uneven spacing of time points. Basal and peak concentrations of cortisol during each challenge were analyzed using the MIXED procedure with treatment as the fixed effect. Time to peak cortisol concentration and time to return to basal cortisol concentration were also analyzed similarly. The day of the challenge (1 or 2) and animal were random effects in all models.

The plasma ACTH concentrations were also analyzed using repeated measures ANOVA using the MIXED procedure of SAS 9.3 (SAS Institute, Inc., Cary, NC). A spatial power covariance structure was used in the model due to the uneven spacing of the time points. Basal ACTH concentration, peak ACTH concentration, and time to peak ACTH concentration were analyzed using the MIXED procedure with treatment as the fixed effect. The day of the challenge (1 or 2) and animal were random effects in all models.

Results and Discussion

ACTH Challenge

Over the sampling period, heifers in both treatment groups that were administered an ACTH dose at time 0 experienced an expected increase in serum cortisol concentration following the adrenal challenge. One PNS heifer was dropped from the study before the dose could be administered due to a failed cannula, and was therefore excluded from all statistical analysis. Therefore, there were 11 PNS heifers and 12 C heifers included in the analysis. Following dose administration, one PNS heifer and one C heifer were dropped from sampling following the 150 min time point due to cannula failure.

Over the entire sampling period (Figure 2) for which samples were analyzed (-120 to 360 min), neither treatment ($P = 0.12$), nor the interaction of treatment and time ($P = 0.16$), affected serum cortisol concentrations. Time; however, did affect serum cortisol concentrations ($P < 0.01$). This is not surprising because concentrations were

expected to increase immediately following the administration of ACTH and then gradually return to pre-challenge concentrations.

During the pre-challenge period (-120 to 0 min) (Figure 3), neither treatment ($P = 0.45$), time ($P = 0.11$), nor the interaction of treatment and time ($P = 0.65$) had significant effects on serum cortisol concentrations. Because time did not affect cortisol concentration, it can be assumed that the heifers were no longer adjusting hormonally to the handling stress and had reached, or come close to, basal concentrations. Given that treatment did not affect cortisol, it can be assumed that the two treatment groups entered the post-challenge period with similar levels of adrenal activity.

During the post-challenge period (0 to 360 min) (Figure 4), neither treatment ($P = 0.18$), nor the interaction of treatment and time ($P = 0.13$), had effects on serum cortisol concentrations. Time; however, did affect serum cortisol concentrations ($P < 0.01$), which was expected.

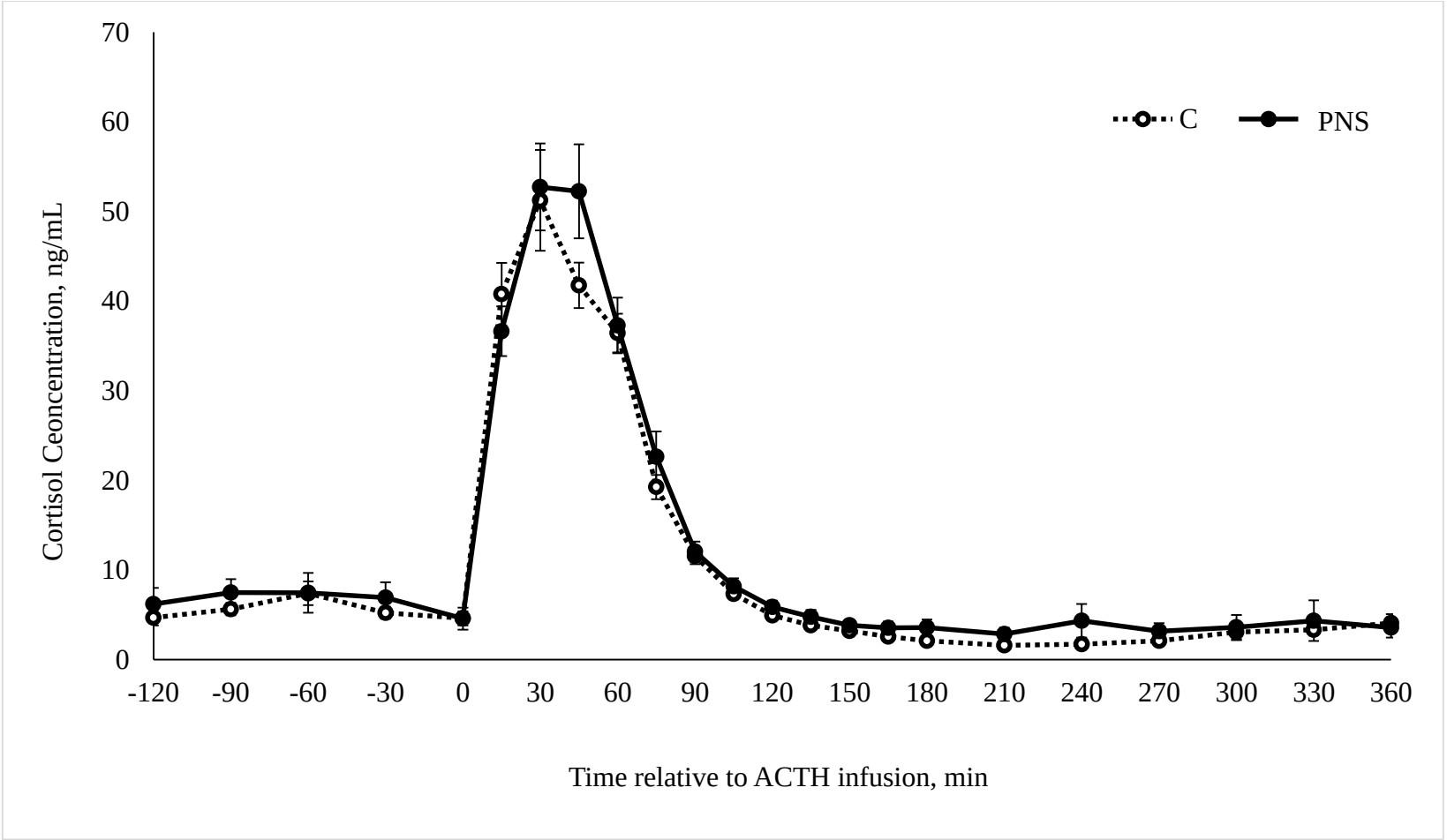

Figure 2. Mean serum cortisol concentrations before and after an intravenous ACTH challenge in both control (C; open circle) and prenatally stressed (PNS; closed circle) yearling Brahman heifers. The pACTH was administered at Time 0 at a dose of 0.1 IU/kg BW. Time influenced serum cortisol concentrations ($P < 0.01$), but neither treatment ($P = 0.12$), nor the interaction of time and treatment ($P = 0.16$), had an effect on serum cortisol.

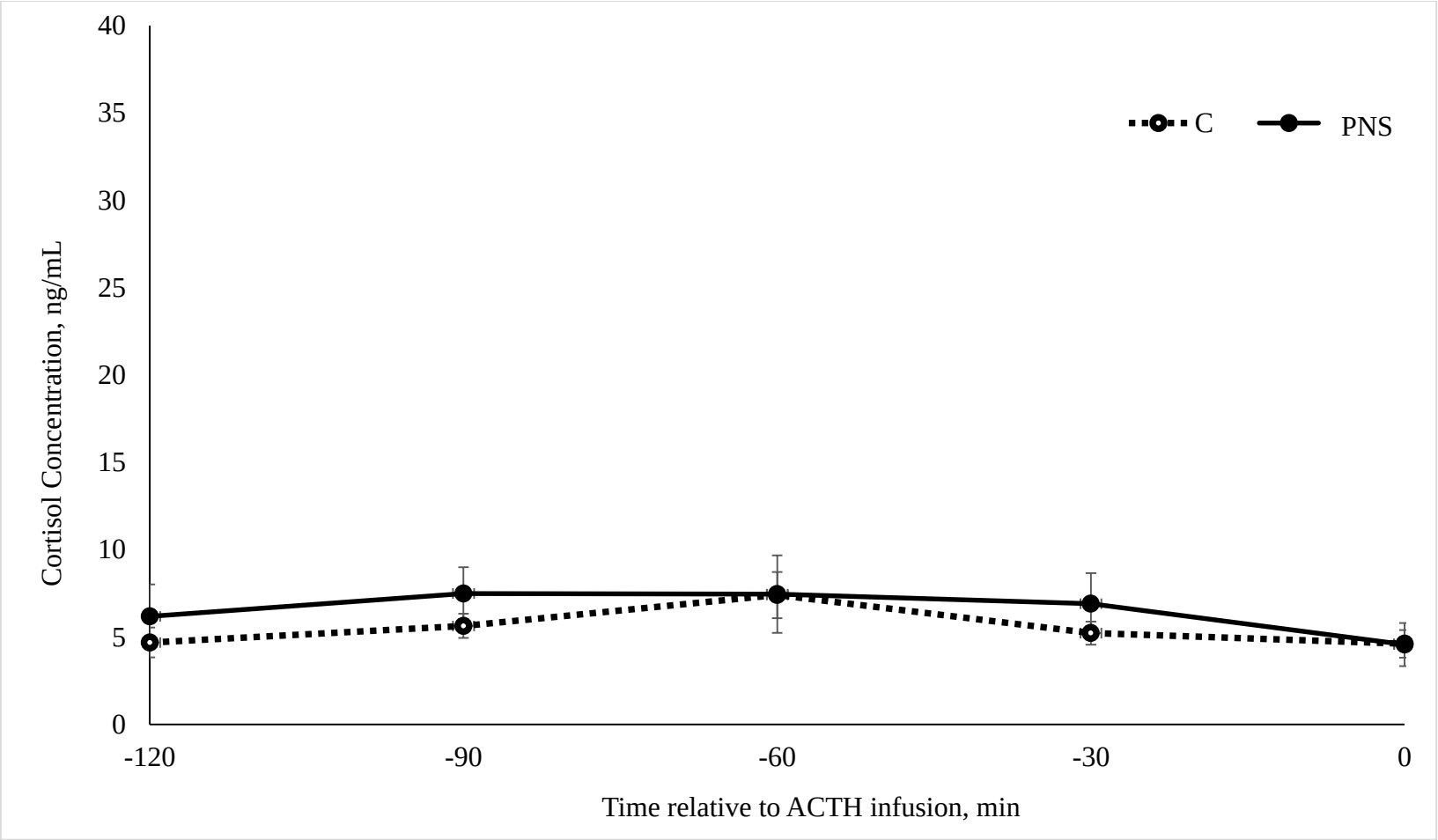

Figure 3. Mean serum cortisol concentrations before an intravenous ACTH challenge in both control (C; open circle) and prenatally stressed (PNS; closed circle) yearling Brahman heifers. The pACTH was administered at Time 0 at a dose of 0.1 IU/kg BW. Neither treatment (P = 0.45), time (P = 0.11), nor the interaction of time and treatment (P = 0.65), had an effect on serum cortisol prior to the ACTH infusion.

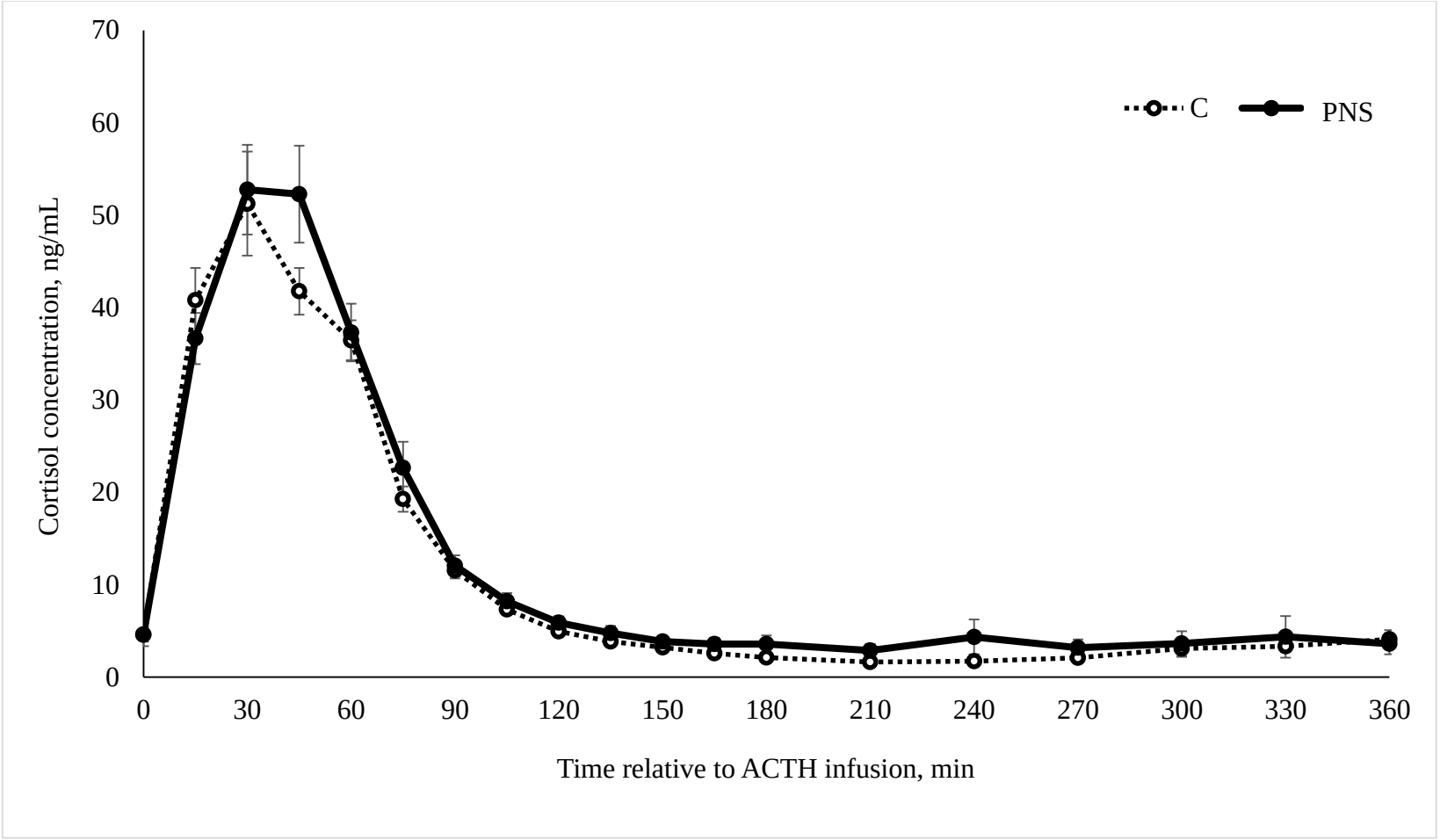

Figure 4. Mean serum cortisol concentrations following an intravenous ACTH challenge in both control (C; open circle) and prenatally stressed (PNS; closed circle) yearling Brahman heifers. The pACTH was administered at Time 0 at a dose of 0.1 IU/kg BW. Time influenced serum cortisol concentrations ($P < 0.01$), but neither treatment ($P = 0.18$), nor the interaction of time and treatment ($P = 0.13$), had an effect on serum cortisol concentrations following the infusion.

Treatment did not influence basal cortisol, which was calculated as the average serum cortisol concentration during the pre-challenge period for each heifer (P = 0.53). The C heifers had a mean basal serum cortisol concentration of 5.52 ± 1.10 ng/mL, while the PNS heifers had a mean concentration of 6.53 ± 1.15 ng/mL (Table 3). These values were similar to those that have been reported in Brahman heifers (4.30 ± 0.58 ng/mL), classified as calm based on EV, while confined in stanchions before an ACTH challenge (Curley et al., 2008). However, the basal cortisol values observed in this study were much lower than those observed in calm yearling Brahman bulls both pre- and post-transportation (Burdick et al., 2010), and slightly lower than those reported in calm yearling bulls reported by Curley et al. (2006). This was unexpected because the heifers in this study encompassed a wide range of temperaments. It is possible that the bulls in the other studies were agitated by handling, or that Brahman heifers tend to have lower basal serum cortisol concentrations than bulls of the same age. However, previous studies have shown that Angus heifers have decreased basal serum cortisol concentrations compared to Angus bulls (Henricks et al., 1984; Arthington et al., 2003). Furthermore, Hulbert et al. (2013) reported that Brahman heifers exhibited higher circulating cortisol concentrations than Brahman bulls 2 h prior to a CRH challenge, but that this difference had disappeared by the time the CRH dose was administered.

The peak serum cortisol concentration was also unaffected by treatment group (P = 0.57). The C heifers had a mean peak serum cortisol concentration of 54.04 ± 4.70 ng/mL, while the PNS heifers had a mean concentration of 57.84 ± 4.93 ng/mL (Table 3). These values are comparable to those reported by Curley et al. (2008) in calm heifers

Table 3. Cortisol response variables to an ACTH challenge in Control and Prenatal Stress yearling heifers. Treatment did not influence any of the cortisol response variables that were analyzed ($P \geq 0.48$).

Variable	Treatment Group		P-value
	Control	Prenatal Stress	
Basal cortisol, ng/mL	5.52 ± 1.10	6.53 ± 1.15	0.53
Time to basal cortisol, min	124.9 ± 10.1	130.3 ± 10.1	0.57
Peak cortisol, ng/mL	54.04 ± 4.70	57.84 ± 4.93	0.57
Time to peak cortisol, min	36.25 ± 3.20	39.55 ± 3.34	0.48

(55.14 ± 1.55 ng/mL) following an ACTH challenge with the same dose of ACTH (0.1 IU/kg BW). However, it is slightly higher than the concentrations reported in temperamental heifers (45.88 ± 7.65 ng/mL) in that study. The fact that the C and PNS heifers responded similarly to calm heifers is not too surprising because their basal cortisol concentrations were also similar. Animals with high basal cortisol concentrations sometimes display a muted response to an ACTH challenge (Curley et al., 2008).

Neither the time to reach peak cortisol concentration, nor the time to return to basal cortisol concentration, was affected by treatment ($P \geq 0.48$). The time to return to basal cortisol concentration (C = 124.88 ± 10.05 min; PNS = 130.33 ± 10.05 min) (Table 3) was markedly different in this group of cattle compared to those described by Curley et al. (2008), in which temperamental heifers took an average of 167.5 ± 17.5 min and calm heifers took an average of 305 ± 12.04 min. This was unexpected because the peak

and basal cortisol concentrations in this study were similar to the calm heifers and dissimilar to the temperamental heifers. When time to return to basal concentration is considered, this trend is reversed. One possible explanation for this may be that the present study lacked some environmental stressor that was present in the other study. Another possible reason for the discrepancy includes differences in the classification criteria used to assign animals to temperament groups. From these results, it appears that prenatal stress did not alter adrenal function in this group of heifers.

CRH Challenge

Adrenal Response to CRH

Over the sampling period, heifers in both treatment groups administered a CRH dose at time 0 experienced an expected increase in both plasma ACTH and serum cortisol concentration following the challenge. One PNS heifer did not experience an increase in ACTH or cortisol concentrations following the challenge, which indicated that it did not receive the proper CRH dose. Because of this, she was excluded from the study and all statistical analysis. Additionally, one C heifer experienced a failed cannula prior to dose administration and was also excluded from the study. Therefore, there were 11 PNS heifers and 11 C heifers included in the analysis. Following dose administration, one C heifer experienced cannula failure after 300 min and one PNS heifer experienced cannula failure after 150 min.

Over the full sampling period for which samples were analyzed (-120 to 360 min) (Figure 5), there was no difference between treatments in serum cortisol

concentration (P = 0.72). The interaction between treatment and time was also not different (P = 0.55). However, time did influence serum cortisol concentrations (P < 0.01). This was expected because the CRH dose at time 0 caused cortisol concentrations to rise and then gradually return to basal concentrations.

Over the pre-challenge period (-120 to 0 min) (Figure 6), there was no difference between treatments in serum cortisol concentration (P = 0.74). Furthermore, neither time (P = 0.37), nor the interaction between treatment and time (P = 0.12) were affected by treatment. Because time did not affect cortisol concentration between time -120 and time 0, it can be assumed that the heifers were no longer adjusting to handling stress at this point. Given that treatment did not affect cortisol, it can also be assumed that the two treatment groups entered the post-challenge period with similar basal adrenal activity.

Over the post-challenge period (0 to 360 min) (Figure7), neither treatment (P = 0.76), nor the interaction between treatment and time (P = 0.50) affected serum cortisol concentrations. However, as expected, time did affect cortisol concentrations (P < 0.01).

Basal serum cortisol concentration was not different between treatment groups (C = 4.11 ± 0.68 ng/mL, PNS = 4.44 ± 0.68 ng/mL; P = 0.71.). These cortisol concentrations were comparable to those observed prior to the ACTH challenge, which indicates that the environmental conditions during both challenges were similar. However, these basal concentrations of cortisol are lower than those reported for yearling Brahman bulls (Curley et al., 2006; Burdick et al., 2010). They are also slightly lower than those observed in Brahman heifers of similar age (Curley et al., 2008).

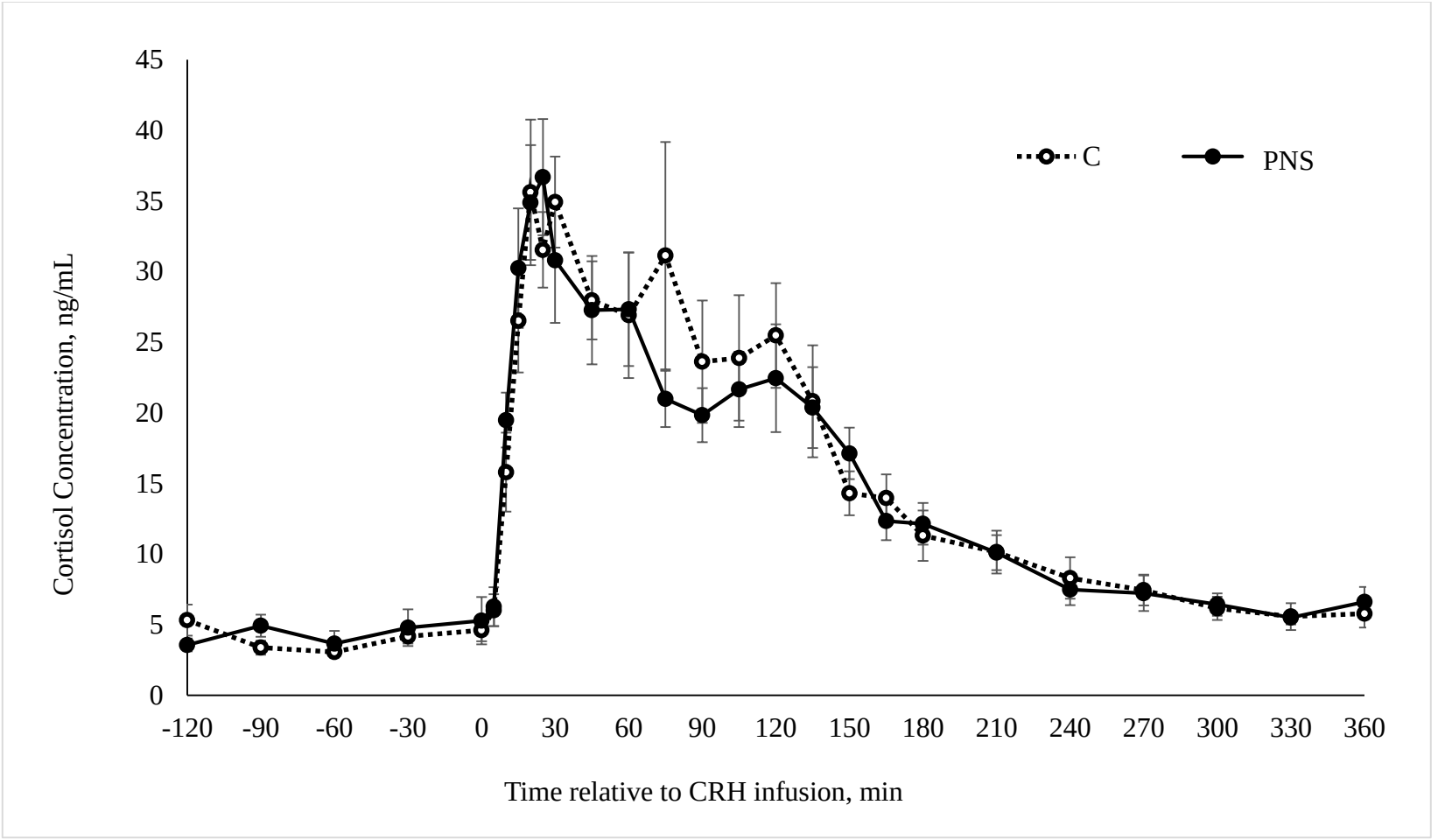

Figure 5. Mean serum cortisol concentrations before and after an intravenous corticotropin-releasing hormone (CRH) challenge in both control (C; open circle) and prenatally stressed (PNS; closed circle) yearling Brahman heifers. The CRH was administered at Time 0 at a dose of 0.3 μg/kg BW. Time influenced serum cortisol concentrations ($P < 0.01$), but neither treatment ($P = 0.72$), nor the interaction of time and treatment ($P = 0.55$), had an effect on serum cortisol.

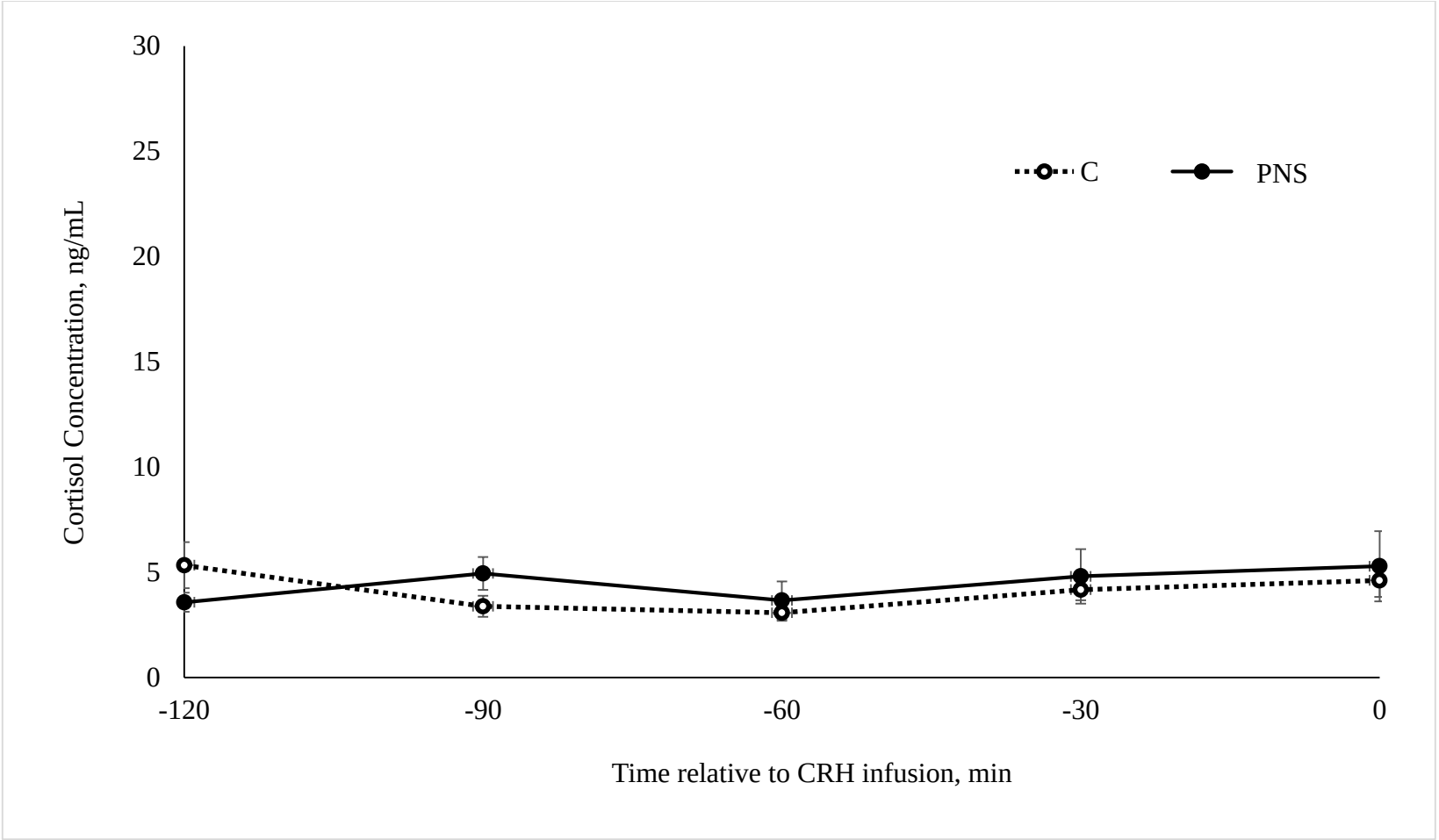

Figure 6. Mean serum cortisol concentrations before an intravenous corticotropin-releasing hormone (CRH) challenge in both control (C; open circle) and prenatally stressed (PNS; closed circle) yearling Brahman heifers. The CRH was administered at Time 0 at a dose of 0.3 μg/kg BW. Neither treatment (P = 0.74), time (P = 0.37), nor the interaction of treatment and time (P = 0.12), had an effect on serum cortisol concentrations prior to the infusion of CRH.

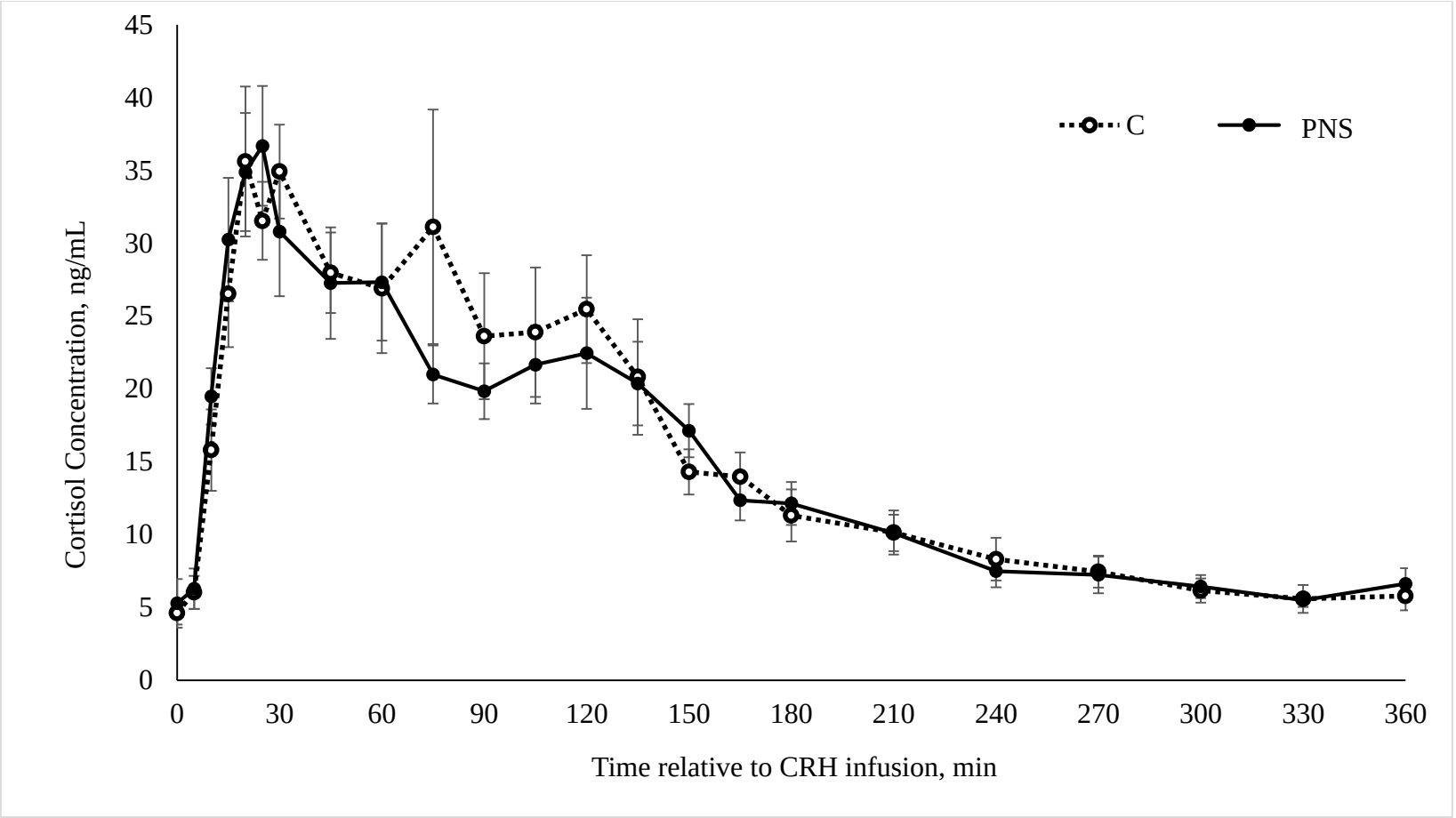

Figure 7. Mean serum cortisol concentrations following an intravenous corticotropin-releasing hormone (CRH) challenge in both control (C; open circle) and prenatally stressed (PNS; closed circle) yearling Brahman heifers. The bCRH was administered at Time 0 at a dose of 0.3 μg/kg BW. Time influenced serum cortisol concentrations ($P < 0.01$), but neither treatment ($P = 0.76$), nor the interaction of treatment and time ($P = 0.50$), had an effect on serum cortisol concentrations following the infusion of CRH.

Peak serum cortisol concentrations were not different between treatment groups (C = 44.77 ± 5.00 ng/mL, PNS = 48.88 ± 5.00 ng/mL; P = 0.57). These peak concentrations were slightly lower than those observed following the ACTH challenge. Interestingly, they were intermediate compared to the peak concentrations of calm and temperamental Brahman heifers given a dose of only 0.1 µg/ kg BW (Curley et al., 2008), but slightly elevated compared to those observed in Holstein steers given an identical dose of CRH (Gupta et al., 2004). These values were consistent with those observed in stressed cattle (Crookshank et al., 1979, Arthington et al., 2003; Cooke et al., 2011). Few other studies have sampled cattle prior to 30 min following a CRH dose, and thus it is difficult to compare peak concentrations of cortisol when several heifers in this study reached peak concentration prior to this time point.

The majority of animals did not return to basal serum cortisol concentrations following the challenge, and therefore the time to reach basal cortisol was not analyzed. Although this occurred on both test days, it is possible that the heifers were exposed to a mild stressor that caused cortisol to remain elevated throughout the challenge period. It should also be noted that the pre-challenge basal serum cortisol concentrations were obtained while the animals were experiencing daylight, while the final three hours of the challenge period took place after sunset. The heifers may have failed to return to their previous serum cortisol concentrations due to a natural diurnal variation.

The time to reach peak cortisol concentration was not influenced by treatment (C = 51.8182 ± 10.4298, PNS = 47.2727 ± 10.4298; P = 0.76). These values were larger than those observed in Brahman heifers given a dose of 0.1 µg/kg BW CRH (Curley et

al., 2008), but slightly smaller than those observed in Holstein steers given 0.3 µg/kg BW (60 min) (Gupta et al., 2004). This was expected because Veissier et al. (1999) observed that increasing the CRH dose from 0.1 µg/kg BW causes more substantial increases in the persistence of the cortisol response, rather than increasing peak cortisol concentrations. However, it should be noted that there was substantial variation in the data set with animals reaching peak cortisol concentration anywhere from 20 to 135 min post-CRH infusion. It is possible that an environmental stressor, such as increased human activity, disturbed some animals following the challenge, and thus caused an increase in cortisol concentrations.

Pituitary Response to CRH

Over the full sampling period for which samples were analyzed (-30 to 45 min) (Figure 8), there was no difference between treatments in plasma ACTH concentrations ($P = 0.90$). There was no interaction between treatment and time ($P = 0.86$). However, as expected, time did influence plasma ACTH concentrations ($P < 0.01$).

Treatment did not influence plasma ACTH concentrations over time during the pre- (-30 to 0 min) and post-challenge (0 to 45 min) periods ($P \geq 0.51$). Furthermore, the interaction between treatment and time did not influence ACTH concentrations during either of these periods ($P \geq 0.17$). Time affected ACTH concentration during the post-challenge period ($P < 0.01$), but not the pre-challenge period ($P = 0.24$).

Basal concentrations of plasma ACTH were similarly unaffected by prenatal treatment (C = 44.15 ± 5.14 pg/mL, PNS = 39.28 ± 5.14; $P = 0.51$), as was peak plasma

ACTH concentration (C = 166.66 ± 33.33 pg/mL, PNS = 185.76 ± 33.33 pg/mL; P = 0.57). The peak concentrations observed in this study are markedly lower than those reported in Holstein steers given the same dose of CRH (228 pg/mL), but the reported basal ACTH concentrations are similar (Gupta et al. 2004). Compared to Brahman heifers that received 0.1 µg/kg BW CRH, these peak concentrations are lower (Curley et al., 2008).

The time taken to reach peak plasma ACTH concentration (C = 21.82 ± 2.46, PNS = 23.18 ± 2.46) did not differ between treatment groups (P = 0.70) and was comparable to the 15-30 min range reported by Gupta et al. (2004).

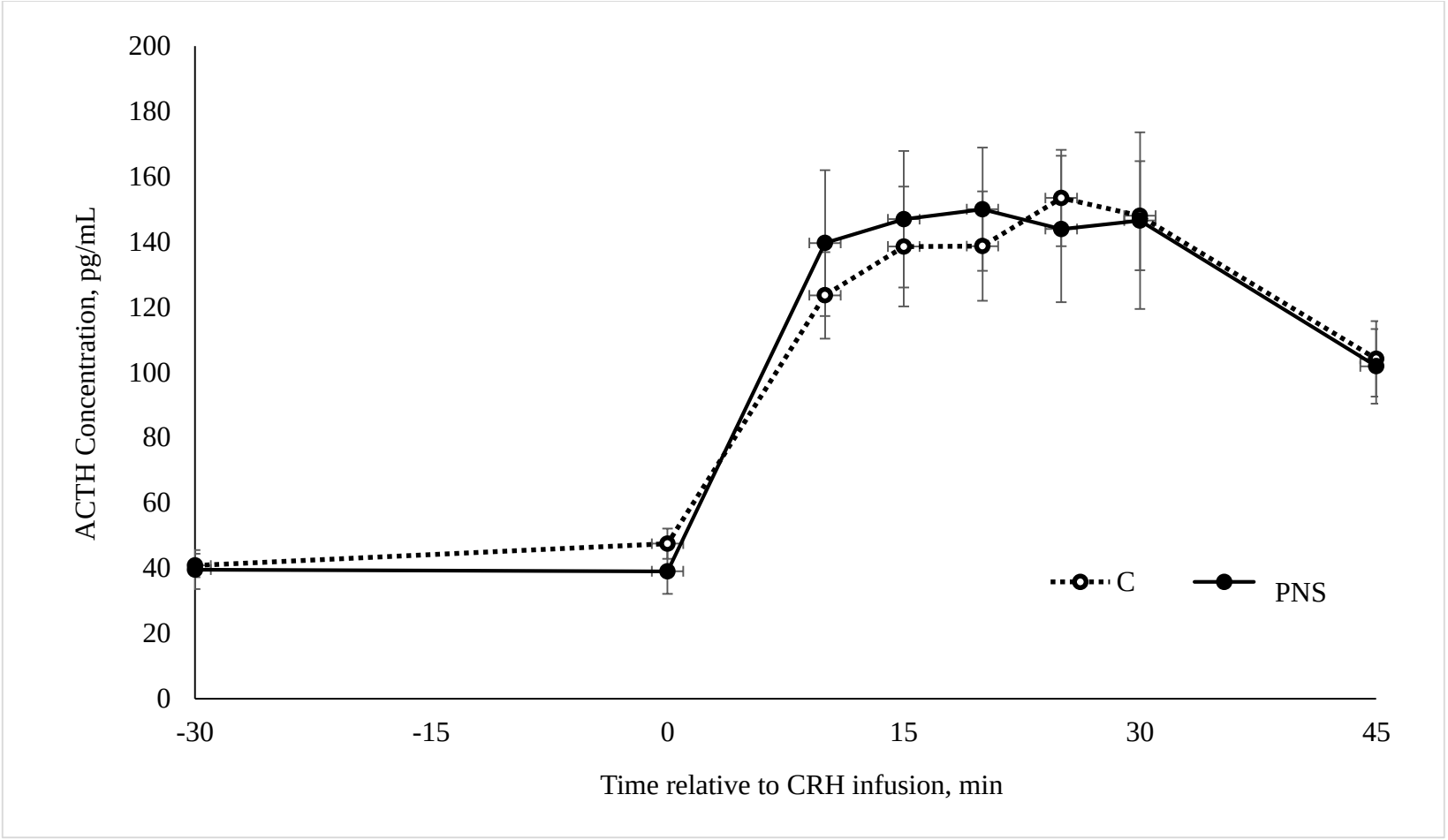

Figure 8. Mean plasma ACTH concentrations before and after an intravenous corticotropin-releasing hormone (CRH) challenge in both control (C; open circle) and prenatally stressed (PNS; closed circle) yearling Brahman heifers. The CRH was administered at Time 0 at a dose of 0.3 μg/kg BW. Time influenced plasma ACTH concentrations ($P < 0.01$), but neither treatment ($P = 0.90$), nor the interaction of time and treatment ($P = 0.86$), had an effect on plasma ACTH.

Conclusion

It appears that prenatal stress did not alter the pituitary or adrenal components of the HPA axis in these heifers. Although the findings reported by Lay et al. (1996a,b), particularly those regarding stress responsiveness and pituitary morphology, suggest that the described transportation schedule during gestation alters the endocrine stress response in calves, this was not found to be the case in the present study. However, it is important to consider that in the present study, temperament was excluded as a factor. Littlejohn et al. (2013a,b) reported that this particular population of PNS heifers is more temperamental than C heifers. Furthermore, Curley et al. (2008) found that heifers of differing temperaments differ in their HPA axis function. Therefore, if heifers were randomly selected from each treatment group instead of balancing for temperament, it is possible that treatment differences in HPA axis function would become apparent.

CHAPTER III

EFFECTS OF PRENATAL STRESS ON GLUCOSE TOLERANCE

Introduction

The potential effects of stress *in utero* have been studied in both human and non-human species. The concept of fetal programming, in which the prenatal environment permanently "programs" the fetus to cope with adult life, was pioneered by David J. Barker in the early 1990s (Barker et al., 2002). Since his initial findings regarding the relationship between birth weight and cardiovascular disease later in life, many other studies have found a relationship between fetal insult (either stress or undernutrition) and alterations in disease patterns, behavior, stress responsiveness, and metabolism across species (Barker et al., 2002; O'Connor et al., 2005; Yehuda et al., 2005; Kapoor et al., 2006; Muneoka et al., 2007; Franko et al., 2010).

If this phenomenon is present in livestock, then the welfare and nutrition of pregnant animals should be given careful consideration in order to maximize the productivity of their offspring. In the beef industry, common management practices such as dehorning, transportation, and social mixing impose stress on cattle (Mench et al., 1990; Stafford and Mellor, 2005; Buckham Sporer et al., 2008). If it is found that prenatal stress negatively affects the productivity or efficiency of cattle, then this knowledge can be used to justify either avoiding submitting pregnant cows to stressful management practices altogether or during critical times during gestation.

Lay et al. (1996a,b) found evidence that transporting pregnant cows at 5 different time points at 20-d intervals between 60 and 140 d of gestation is associated with an increased adrenal response to restraint stress, an enlarged pituitary gland, and decreased ability to clear exogenous cortisol from circulation. HPA axis function is associated with glucose tolerance and insulin resistance because glucocorticoids are known to increase blood glucose concentrations and increase insulin resistance (Thorn et al., 1957; Frawley et al., 1959). Because increased insulin sensitivity in muscle tissue is associated with leanness in livestock (Kelly et al., 2010) and decreased weight loss during caloric restriction (Hoffman et al., 1995), animals which have higher circulating glucocorticoids could be less feed-efficient and productive than animals with lower levels.

The objective of this study was to examine alterations in glucose tolerance and insulin resistance in Brahman heifers exposed to prenatal stress at 60, 80, 100, 120, and 140 d of gestation.

Materials and Methods

All processes required to complete this project were approved by the Texas A&M University IACUC.

Animals and Experimental Design

Brahman heifers (n = 24) were utilized to compare glucose tolerance and insulin sensitivity between prenatally stressed (PNS) and control animals (C). Glucose tolerance and sensitivity to insulin were evaluated using an IVGTT. The dams of PNS heifers were

transported at 60, 80, 100, 120, and 140 d of gestation for 2 h periods, while the dams of the C heifers were not transported during gestation.

The group was equally divided into two subgroups (n = 12), with one subgroup being tested on a given day. The subgroups consisted of 6 PNS animals and 6 C animals that had been paired by temperament, which was determined by TS at weaning. Animals were selected from the population of PNS and C heifers by choosing the 6 most temperamental and 6 calmest heifers from each treatment group that had a similarly scored counterpart in the opposing group. The difference between matched animals ranged from 0 to 0.28 with a SD of 0.1. The mean TS of the sampled C and PNS heifers at weaning were 1.97 ± 0.94 and 2.01 ± 0.92 (Table 4), respectively. This experiment used a complete block design in which day of the challenge (1 or 2) was the block and the factors were temperament class (Calm or Temperamental) and treatment (C or PNS). The factor levels were PNS/Calm, PNS/Temperamental, C/Calm, and C/Temperamental. Each factor level was used and replicated the same number of times in each block.

The IVGTT were carried out on January 31st and February 1st, 2013. During each test day, one subgroup was utilized for a period of 5 h while being confined within a stanchion.

Table 4. Characteristics of Brahman heifers utilized in an IVGTT. The treatment groups did not differ in any of the analyzed characteristics ($P \geq 0.26$).

Variable	Treatment		P-value
	Control	Prenatal Stress	
Body Weight, kg	212.20 ± 23.74	209.81 ± 19.88	0.79
Age, d	292.25 ± 25.50	303.33 ± 20.90	0.26
Pen Score	2.42 ± 1.39	2.08 ± 1.08	0.53
Exit Velocity, m/s	1.45 ± 0.87	1.79 ± 0.79	0.32
Temperament Score	1.97 ± 0.94	2.01 ± 0.92	0.92

Temperament Evaluation

The Brahman heifers used in this experiment were evaluated for temperament at weaning by PS, EV, and TS. Pen score (Table 1) was assigned by a trained evaluator on a scale of 1 to 5 to describe the animal's willingness to be approached by a human (Hammond et al., 1996). A lower PS indicates calmer or more docile temperament, while a higher PS indicates a reactive or aggressive animal. The same evaluator assigned a PS to each animal at weaning.

Exit velocity was measured as the velocity at which an animal travels 1.83 m immediately after exiting a squeeze chute (Burrow et al., 1988; Curley et al., 2006). It was calculated from the time elapsed as the animal traverses 1.83 m after exiting the chute. Infrared sensors were used to start and stop a timer (FarmTek Inc., North Wylie,

TX) that measured the time elapsed. A faster EV indicates a more temperamental animal, while a slower EV indicates a calmer animal.

Temperament score was calculated as the numerical average of EV and PS at weaning (Curley et al., 2006, 2008; King et al., 2006). A higher TS indicates a more temperamental animal while a slower EV indicates a calmer animal. For the purpose of assigning animals to blocks using temperament, a TS ≥ 2 was considered "Temperamental" and TS ≤ 2 was considered "Calm" for this experiment.

Blood Collection Procedures

Heifers were kept in a dry lot with access to water, but no feed, for 12 h prior to cannulation. On the morning of each challenge day, heifers (n = 12) were fitted with jugular cannulas to allow for blood collection. A sterile 14-gauge thin-walled stainless steel biomedical needle (o.d. 2.11 mm) was inserted into the jugular vein and approximately 15 to 20 cm of PTFE (o.d. 1.66 mm; Cole-Palmer, Vernon Hills, IL) tubing was passed through the needle and into the vein. Approximately 15 cm of additional tubing was left outside of the animal. This tubing that extended from the vein was secured to the heifer's neck using stock glue (Santa Cruz Biotechnology, Santa Cruz, CA) and porous surgical tape. The cannula was then fitted with approximately 2 m of sterile plastic Tygon tubing (i.d. 1.59 mm, o.d. 3.18 mm; VWR Scientific, West Chester, PA) to allow for blood sampling in the stanchions. An 18-gauge needle with a 10 mL syringe was used to cap the end of the Tygon tubing. Before releasing the animal from the chute, the cannula was flushed with heparin solution (2 IU/mL) to ensure

patency and the Tygon tubing was secured to the back of the animal using Vetrap Bandaging Tape (3M, St. Paul, MN) and surgical porous tape. Immediately following these procedures, the heifers were released from the chute and placed in a stanchion.

Once all heifers were placed in the stanchions, a period of at least 15 min was given as time for the animals to acclimate. Blood samples were collected into a 10 mL no additive Vacutainer tube (BD Biosciences, Franklin Lakes, NJ) and a 10 mL EDTA-coated Vacuatainer tube (BD Biosciences, Franklin Lakes, NJ) at each time point during the IVGTT. Following each sample collection during the IVGTT, heifers were dosed with 20 mL of physiological saline solution (0.9%) and 7 mL of heparin solution (2 IU/mL). These solutions were administered in order to replace fluid volume and prevent blood clotting in the cannulas.

Blood samples collected into the EDTA-coated tubes were immediately centrifuged at 4˚ C to separate the plasma. The plasma was then aliquoted into 2 polyethylene storage tubes and flash-frozen in liquid nitrogen. Plasma samples were stored at -80˚ C. The blood samples collected into the no additive tubes were immediately placed on ice and then stored at 4˚ C for 12 h. Following this 12-h period, the samples were centrifuged at 4˚ C to separate the serum. The serum was then aliquoted into 2 polyethylene storage tubes and stored at -20˚ C.

Challenge Timeline

Blood samples (n = 16) were taken at the following time intervals in relationship to dextrose dose administration at 0 min: -120, -90, -60, -30, -10, 0, 10, 20, 30, 40, 60,

80, 100, 120, 140, 160, and 180 min. A 50% dextrose solution (Vedco, Inc., Saint Joseph, MO) was administered at time 0 at a dose of 0.5 mL/kg BW via the cannulas.

Glucose Enzymatic Assay

Serum glucose concentrations were determined from duplicate samples taken during the IVGTT. The samples were assayed using the commercially available enzymatic Autokit Glucose kit (Wako, Diagnostics, Richmond, VA) (see Appendix B) as described by Bernhard et al. (2012). Only the samples from the following time points were analyzed: -10, 0, 10, 20, 30, 40, 60, 80, 100, 120, 140, 160, and 180 min. The interassay and intraassay CV were 9.07% and 7.74%, respectively.

Insulin Immunoassay

Serum insulin concentrations were determined from duplicate samples taken during the IVGTT. The samples were assayed using the commercially available Insulin (Bovine) ELISA kit (ALPCO Diagnostics, Salem, NH) (see Apprendix A) as described by Bernhard et al. (2012). Only the samples from the following time points were analyzed: -10, 0, 10, 20, 30, 40, 60, 80, 100, 120, 140, 160, and 180 min. The interassay and intraassay CV were 4.32% and 5.60%, respectively.

Cortisol RIA

Serum cortisol concentrations were determined from duplicate samples taken during the IVGTT. The samples were assayed using a single antibody RIA procedure

(see Appendix C) as described by Curley et al. (2008). The procedure utilized rabbit anti-cortisol antiserum (Pantex, Div. of Bio-Analysis Inc., Santa Monica, CA) diluted 1:2,500, standards made by serial dilution (8,000 pg /100 µL to 3.9 pg/100 µL) of 4-pregnen-11β,17,21-triol-3,20-dione (Steraloids Inc., Newport, RI), and radio-labeled cortisol 3H-Hydrocortisone (1,2-3H, NEN, Boston, MA). Unknown cortisol concentrations were calculated using Assay Zap software (Biosoft, Cambridge, UK). The CPM were obtained from a liquid scintillation spectrophotometric beta-counter (Beckman Coulter LS 6500, Beckman Coulter, Inc., Brea, CA). The cortisol antiserum cross-reactivity was as follows: corticosterone, 60%; deoxycorticosterone, 48%; progesterone, 0.01%; and estradiol, 0.01%. Only the samples from the following time points were analyzed: -10, 0, 10, 20, 30, 40, 60, 80, 100, 120, 140, 160, and 180 min. The interassay and intraassay CV were 6.29% and 12.44%, respectively.

Statistical Analysis

Once glucose, insulin, and cortisol concentrations were determined at each time point, separate repeated measures ANOVA models were used to analyze the concentration of each marker over time using the MIXED procedure in SAS 9.3 (SAS Inst. Inc., Cary, NC). The fixed effects of time and treatment, with time being repeated, were analyzed for significance. Both spatial power and spatial exponential covariance structures were tested for model fit due to the uneven spacing of the time points. A spatial power covariance structure was selected for the repeated measures models due to its lower Akaike's information criterion and corrected Akaike's information criterion

values. The IGR was also calculated at each time point and analyzed similarly. Other measures of insulin sensitivity such as the IIND at 30 min post-challenge, peak insulin concentration, time to peak insulin concentration, time to return to basal glucose concentration, and AUC for both insulin and glucose concentrations post-challenge were calculated and analyzed for treatment differences using the MIXED procedure with treatment as the fixed effect. The IIND was calculated as $\Delta[I]/\Delta[G]$, where I is the serum insulin concentration and G is the serum glucose concentration and the change in concentrations was the difference between the basal and that determined at 30 min post-challenge. The AUC for both glucose and insulin was calculated using the trapezoidal rule (Yeh, 2002). The day of the challenge (1 or 2) and animal were random effects in all models.

Results and Discussion

Cortisol Response

Serum cortisol concentrations were analyzed between -10 and 180 min relative to glucose infusion (Figure 9) in order to account for possible differences in stress responsiveness between the 2 treatment groups. Over this period, there was no difference in cortisol concentration over time between treatments ($P = 0.18$) and the interaction between treatment and time was also non-significant ($P = 0.13$). Time was significant ($P < 0.01$), which would typically indicate that a stressor disturbed the animals around the time of glucose infusion. However, mean cortisol concentrations for both treatment groups remained below 10 ng/mL for the duration of the sampling period, and therefore

the animals were not considered stressed to the point that serum glucose or insulin concentrations would be altered.

Insulin Response

Both treatment groups responded to the glucose infusion with increased serum insulin concentrations (Figure 10). Over time, the PNS heifers had a lower serum insulin concentration compared to the C heifers ($P = 0.03$).

Time also influenced serum insulin concentration ($P < 0.01$), which was expected because insulin increased in response to the glucose infusion and then gradually decreased toward basal concentrations. However, the interaction between treatment and time was not significant in the model ($P = 0.58$). Therefore, both treatment groups followed the same pattern of insulin secretion over time.

The insulin total AUC, the time taken to reach peak insulin concentration following glucose infusion, and the time to return to basal insulin concentration were all influenced by prenatal treatment (Table 5) ($P < 0.01$). Neither the peak (C = 56.80 ± 5.62, PNS = 47.88 ± 5.62; $P = 0.13$), nor the basal (C = 11.94 ± 1.16, PNS = 11.04 ± 1.16; $P = 0.58$), insulin concentration was influenced by treatment (Table 5). The PNS heifers took more time to reach peak insulin concentration ($P < 0.01$), but returned to basal concentrations in less time ($P < 0.01$) (Table 5). Their peak insulin concentration, although not statistically different from the C heifers, was numerically lower. This resulted in a smaller total area under the insulin response curve for the PNS heifers. This

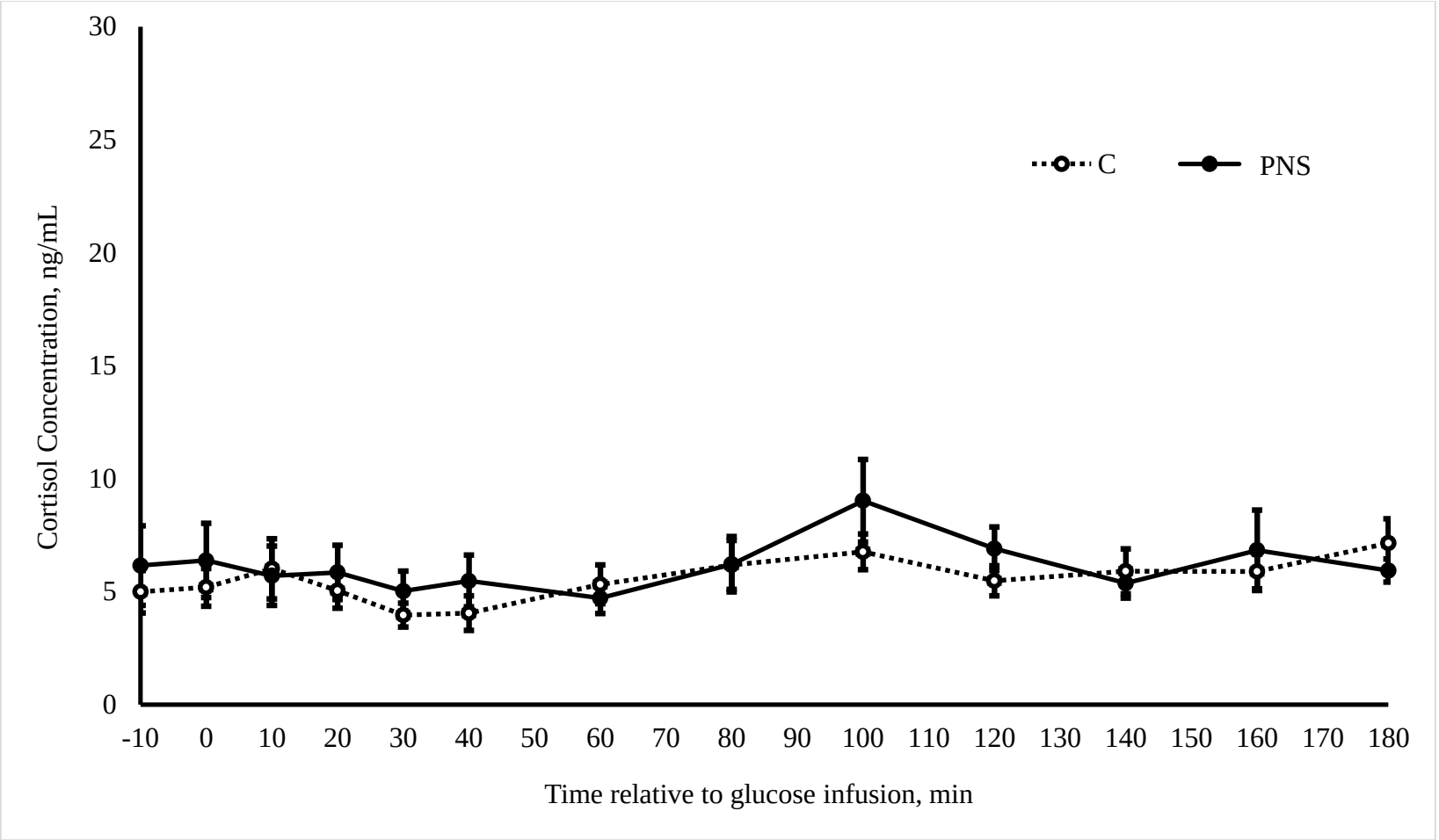

Figure 9. Mean serum cortisol concentrations over time in both control (C; open circle) and prenatally stressed (PNS; closed circle) heifers in response to an intravenous glucose tolerance test. A 50% dextrose solution was administered at Time 0 at a dose of 0.5 mL/kg BW. Time affected serum cortisol concentrations ($P < 0.01$). Neither treatment ($P = 0.18$), nor the interaction of time and treatment ($P = 0.13$), influenced serum cortisol concentrations.

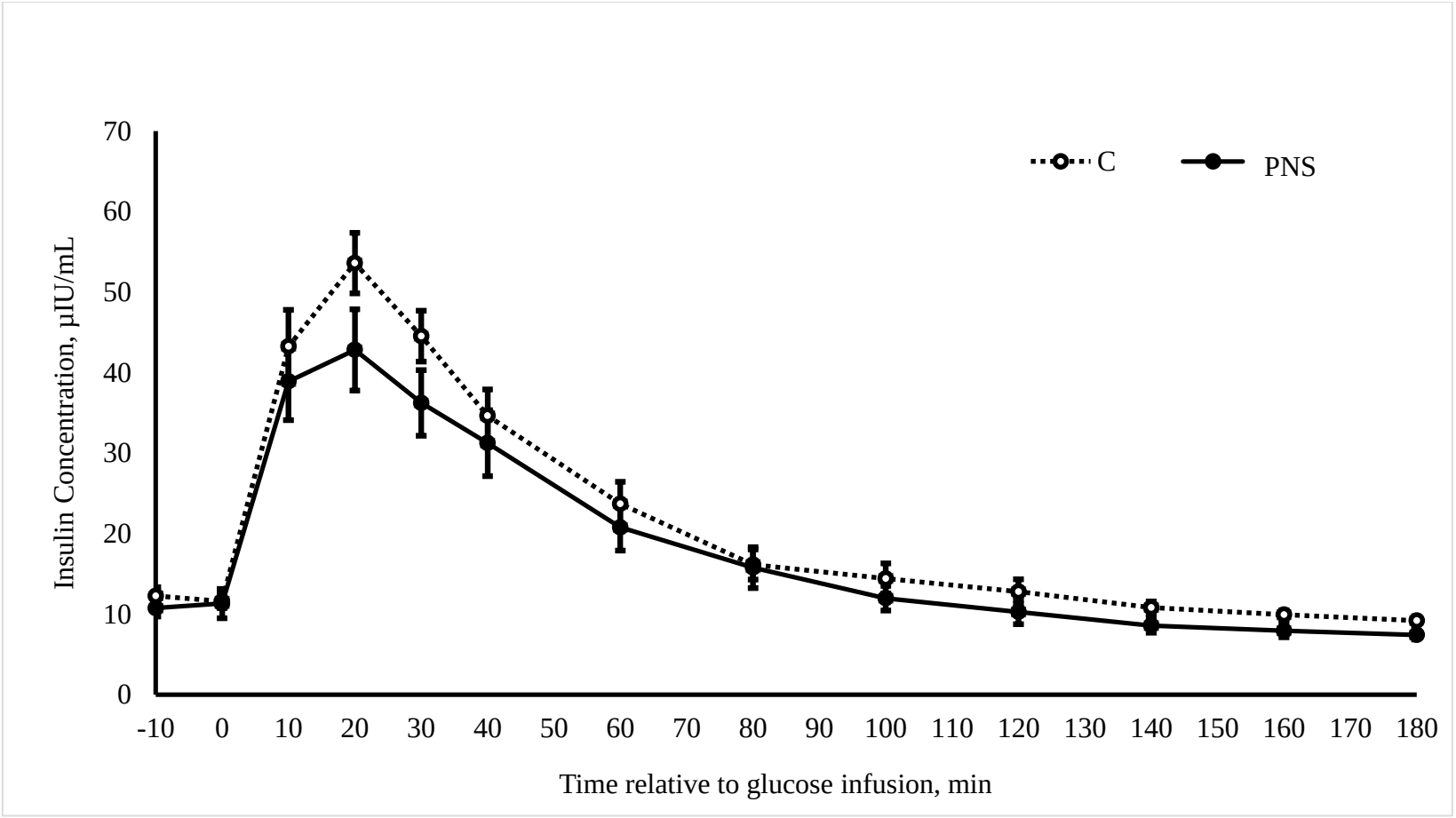

Figure 10. Mean serum insulin concentrations over time in both control (C; open circle) and prenatally stressed (PNS; closed circle) heifers in response to an intravenous glucose tolerance test. A 50% dextrose solution was administered at Time 0 at a dose of 0.5 mL/kg BW. Both treatment ($P = 0.03$) and time ($P < 0.01$) affected serum insulin, with the prenatally stressed heifers having lower concentrations over time. The interaction of time and treatment did not influence serum insulin concentrations ($P = 0.58$).

indicates that the PNS heifers had a decreased insulin response to the glucose challenge, compared to the C heifers.

The basal insulin concentrations observed in this study are slightly elevated, but the insulin total AUC is markedly smaller than those figures reported in a control group of Holstein heifers administered the same glucose dose (Bunting et al., 1994). Interestingly, the insulin AUC observed in both the C and PNS heifers was more comparable to that exhibited by Holstein heifers treated with chromium picolinate in order to improve insulin sensitivity and glucose tolerance (4,561 μIU • min • mL-1). However, these differences may be due to breed or diet.

Table 5. Glucose and insulin response variables to a glucose challenge in prenatally stressed and control yearling Brahman heifers. Variables with a P-value less than 0.05 were influenced by treatment group.

Variable	Treatment Group		P-value
	Control	Prenatal Stress	
Basal Insulin, μIU/mL	11.94 ± 1.16	11.04 ± 1.16	0.58
Time to return to basal insulin, min	126.7 ± 10.9	111.7 ± 10.9	< 0.01
Basal Glucose, mg/dL	82.89 ± 4.21	84.16 ± 4.21	0.81
Time to return to basal glucose, min	142.0 ± 2.51	125.0 ± 2.80	< 0.01
Peak Insulin, μIU/mL	56.80 ± 5.62	47.88 ± 5.62	0.13
Time to peak insulin, min	17.36 ± 2.29	22.01 ± 2.26	< 0.01
Total area under the insulin response curve, $\mu IU \cdot min \cdot mL^{-1}$	4,429 ± 123	3,543 ± 116	< 0.01

Glucose Response

As expected, serum glucose concentrations increased in all heifers following the glucose infusion (Figure 11). Over time, treatment did not influence the concentration of serum glucose ($P = 0.61$).

Time; however, did influence serum glucose concentration ($P < 0.01$), which was expected because glucose concentrations increased in response to the infusion and then gradually decreased toward basal concentrations. The interaction between treatment and time was not significant in the model ($P = 0.42$). This means that both treatment groups followed the same pattern in their glucose concentrations over time.

Basal serum glucose concentrations (C = 82.89 ± 4.21 mg/dL, PNS = 84.16 ± 4.21 mg/dL) were not influenced by prenatal treatment ($P = 0.81$) (Table 5). These values were elevated when compared to those reported in fasted Holstein heifers (64.9 ± 2.5 mg/dL) by McCann and Hansel (1986). However, the heifers in the mentioned study were fasted for a longer period of time than the C and PNS heifers. The time to return to basal glucose (C = 142.0 ± 2.51, PNS = 125.0 ± 2.8); however, was influenced by treatment ($P < 0.01$) (Table 5). Despite having a muted insulin response to the glucose challenge, the PNS heifers were able to clear the glucose from the blood and return to basal concentrations in less time than the C heifers. This indicates that the PNS heifers may have greater tissue sensitivity to insulin than the C heifers, making them more glucose tolerant.

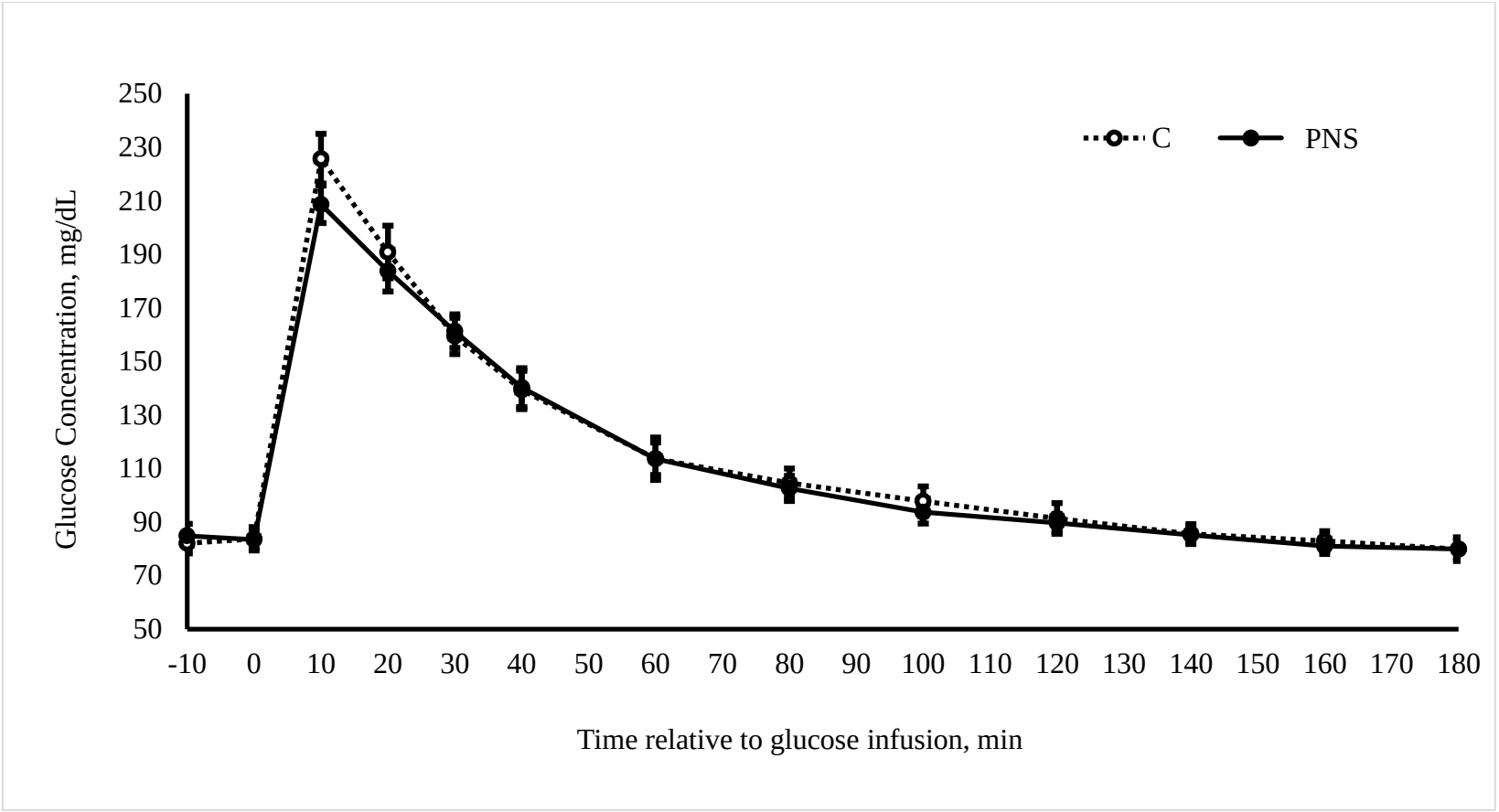

Figure 11. Mean serum glucose concentrations over time in both control (C; open circle) and prenatally stressed (PNS; closed circle) heifers in response to an intravenous glucose tolerance test. A 50% dextrose solution was administered at Time 0 at a dose of 0.5 mL/kg BW. Time affected serum glucose concentrations ($P < 0.01$). Neither treatment ($P = 0.61$), nor the interaction of time and treatment ($P = 0.42$), influenced serum glucose concentrations over time.

The Relationship between Insulin and Glucose

Human metabolic studies utilize several types of calculations in order to create indices of insulin sensitivity. Two of these are the ratio of insulin to glucose, or IGR, and the IIND. The IGR is calculated by dividing the concentration of serum insulin (µIU/mL) by the concentration of serum glucose (mg/dL) as described by Bradbury (2011). The IIND is calculated at individual time points as IIND = ΔI/ΔG, where ΔI is the change in the concentration of serum insulin (µIU/mL) from time 0 to the targeted time point and ΔG is the change in the concentration of serum glucose (mg/dL) from time 0 to the targeted time point (Cohn et al., 1999; Abdelmannan et al., 2010). In human and veterinary studies, it is common to calculate IIND at the 30-min time point to gauge insulin sensitivity. A higher IIND indicates insulin resistance, while a lower IIND indicates insulin sensitivity.

The IGR was calculated for each heifer in this study at each time point (Figure 12). Over time, the C heifers had a higher IGR than the PNS heifers ($P = 0.01$), which indicates that the PNS heifers were more sensitive to insulin. The interaction between treatment and time was non-significant ($P = 0.62$), but time did influence IGR ($P < 0.01$). In both treatment groups, the IGR increased following the glucose infusion and peaked at approximately 20 min post-infusion. The increase was caused by the large influx of glucose, which triggered increased insulin secretion. As glucose decreases, insulin secretion also decreases, and the IGR gradually falls towards basal levels.

This ratio has been associated with RFI in cattle. A low RFI indicates a more efficient animal, while a higher RFI indicates a less efficient animal. Shafer (2011)

found that Brahman heifers with a low RFI had a lower IGR over time following a glucose challenge than high RFI animals. The low RFI heifers also had a decreased insulin response to the glucose challenge, similar to the PNS heifers in this study. This suggests that the PNS heifers may have enhanced efficiency, compared to C heifers, and therefore prenatal stress may have actually benefited their productivity.

The IIND was calculated for each heifer at 30-min post-infusion. The PNS heifers tended to have a lower IIND than the C heifers (C = 0.44 ± 0.05, PS = 0.33 ± 0.05; P = 0.08). This trend is another indicator that the PNS heifers were less insulin resistant than the C heifers.

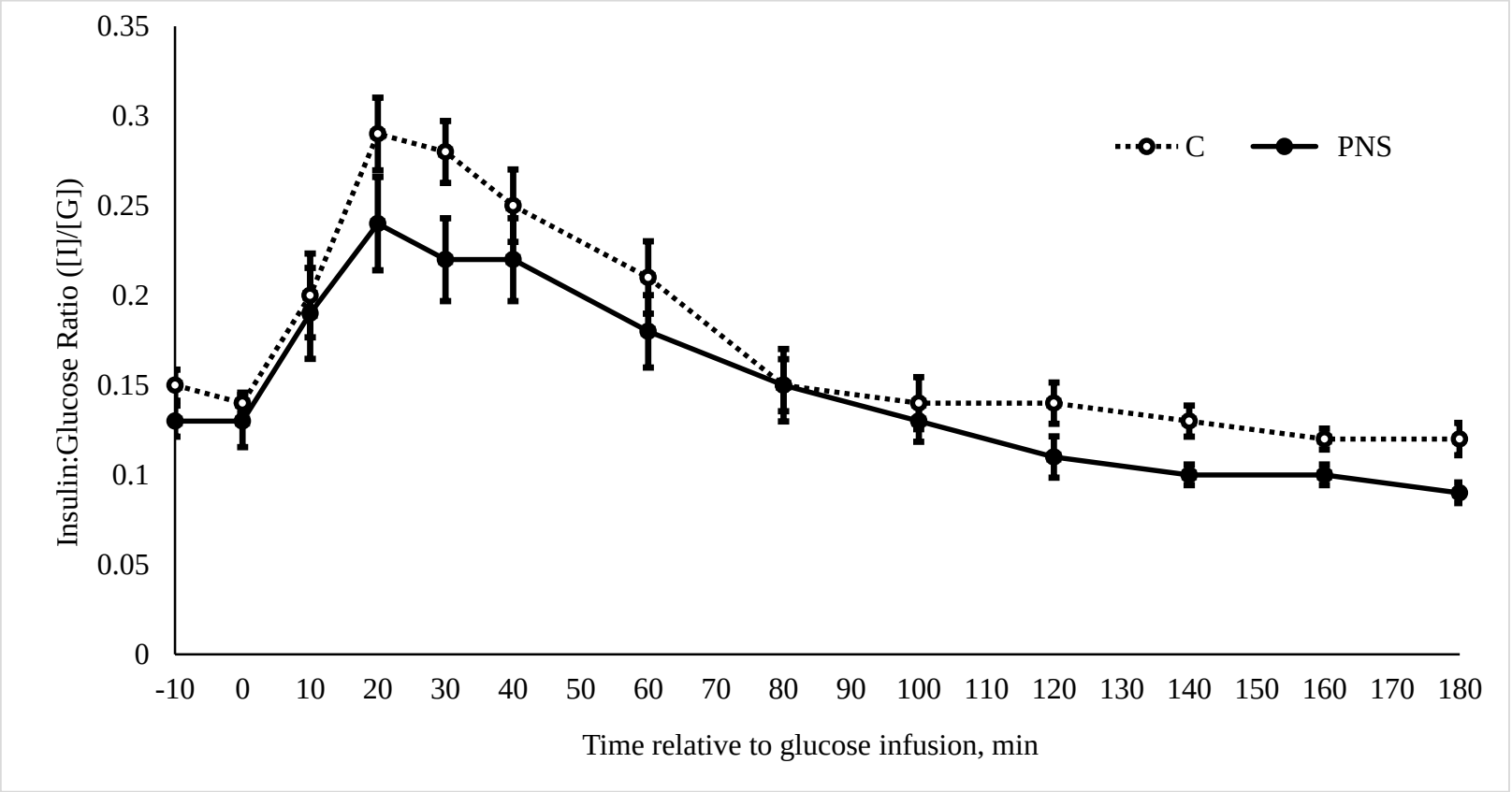

Figure 12. Mean Insulin:Glucose Ratio ([I]/[G]) over time in both control (C; open circle) and prenatally stressed (PNS; closed circle) heifers in response to an intravenous glucose tolerance test. A 50% dextrose solution was administered at Time 0 at a dose of 0.5 mL/kg BW. Both treatment ($P = 0.01$) and time ($P < 0.01$) affected the Insulin:Glucose Ratio, with the prenatally stressed heifers having a lower Insulin:Glucose Ratio over time. The interaction of time and treatment did not influence the Insulin:Glucose Ratio ($P = 0.58$).

Conclusion

This study evaluated whether prenatal stress affects postnatal sensitivity to insulin, and thus an animal's ability to tolerate glucose. It appears that the PNS heifers have increased insulin sensitivity compared with the C heifers. The majority of studies in the literature report the opposite finding (Entringer et al., 2008), but at least one other also observed an increase in insulin sensitivity in prenatally stressed animals (Franko et al., 2010). However, it is important to consider that in the present study, temperament was excluded as a factor. Littlejohn et al. (2013a,b) reported that this particular population of PNS heifers is more temperamental than C heifers. Furthermore, temperament has been shown to influence the response to a glucose challenge in Brahman heifers (Bradbury et al., 2011). Therefore, if heifers were randomly selected from the larger C and PNS population, it is not known if a similar increase in insulin sensitivity in PNS heifers would be observed.

Previous research has indicated a relationship between the response to a glucose tolerance test and feed efficiency in beef cattle (Shafer, 2011). If the IGR following a glucose challenge is used as an indicator of RFI, it appears that the PNS heifers may have a lower RFI than the C heifers, thus making them more feed-efficient.

Additional research should be conducted in order to ascertain whether these differences in insulin sensitivity between treatment groups manifest as differences in efficiency and productivity. Prenatally stressed bulls should also be evaluated due to the tendency for prenatal stress effects to be sex-specific.

CHAPTER IV

EFFECTS OF PRENATAL STRESS ON BIRTH WEIGHT, GROWTH, AND TEMPERAMENT

Introduction

In both humans and non-human species, studies have found a relationship between fetal insult (either stress or undernutrition) and alterations in disease patterns, behavior, stress responsiveness, and metabolism (Barker et al., 2002; O'Connor et al., 2005; Yehuda et al., 2005; Kapoor et al., 2006; Franko et al., 2010). If this phenomenon is present in livestock, then the welfare and nutrition of pregnant animals should be given careful consideration in order to maximize the productivity of their offspring.

In the beef industry, common management practices such as dehorning, transportation, and social mixing impose stress on cattle (Mench et al., 1990; Stafford and Mellor, 2005; Buckham Sporer et al., 2008). If it is found that prenatal stress negatively affects the productivity or efficiency of cattle, then this knowledge can be used to justify either avoiding submitting pregnant cows to stressful management practices altogether or during critical times during gestation.

It has already been established that calves born to dams who were transported at 60, 80, 100, 120, and 140 d of gestation have altered growth rates, stress responsiveness, and temperaments (Littlejohn et al., 2013a,b). There is also evidence that repeated stress during this period influences the morphological development of the HPA axis and the

rate of cortisol clearance (Lay et al., 1997a,b). However, it is not known if a stressor applied at a later stage of gestation will have similar effects.

The Callicrate Band ™ method of dehorning adult cattle is possibly a viable strategy to induce a low level of chronic stress in the cow during the second trimester of pregnancy (Neely, 2013). It can take between 20 and 50 d, depending on the size of the horns, to fully remove the horns. This period of horn tissue death may be stressful to the animal, and therefore could cause a significant chronic stress and possibly decreased feed intake resulting in weight loss.

The objective of this study was to determine if there are differences in birth weight, growth, and temperament of Brahman calves whose dams were stressed by a dehorning procedure during the second trimester of gestation and those whose dams were not.

Materials and Methods

All processes required to complete this project were approved by the Texas A&M University IACUC.

Animals and Experimental Design

Parous cows (n = 13) and nulliparous heifers (n = 20) will be dehorned using the Callicrate banding method between d 93 and 168 of gestation. Parous cows (n = 27) and nulliparous heifers (n = 5) at similar stages of gestation served as a control group (C).

The dehorned cattle (STRESS) had a mean d of gestation of 139 ± 20 on the d the bands were applied. The bands were applied to all STRESS cattle on November 1st, 2012.

All cows and heifers were weighed and assigned a BCS (1-9) at 28-d intervals starting on December 13, 2012 and continuing until the beginning of the calving season on March 5th, 2013. During this time period, cattle were kept on rye-ryegrass pastures and supplemented daily with ground corn, soybean hulls, and a mineral/vitamin premix. Within 24 h following calving, each cow was weighed and a BCS was assigned. The calf was also weighed. At 14 and 28 d post-calving, both the dam and calf were similarly weighed and scored. The temperament of the calf was also evaluated at these time points by both PS and EV.

Dehorning Procedure

Cattle were dehorned using the Callicrate Bander (No-Bull Enterprises LLC, St. Francis, KS) while restrained in a squeeze chute. The device was used to apply a high tension rubber band, secured with a metal clip, to the horn as close to the base of the head as possible. The procedure described on the No-Bull Enterprises LLC product website (www.nobull.net/bander/SBhornRemoval.htm) was used to apply the bands.

Body Condition Scoring

Cows were assigned a BCS (Table 6) in order to visually compare subcutaneous fat storage. The cows were scored on the 1-9 scale, where 1 indicates an extremely

emaciated animal and 9 indicates an extremely obese animal, traditionally used in beef cattle production (Wiltbank et al., 1962).

Temperament Evaluation

The calves used in this experiment were evaluated for temperament using PS, EV, and TS. Pen score (Table 1) was assigned by the same trained evaluator on a scale of 1 to 5 to describe the animal's willingness to be approached by a human (Hammond et al., 1996). A low PS indicates calmer or more docile temperament, while a higher PS indicates a reactive or aggressive animal. The same evaluator assigned a PS to each animal at weaning.

The EV was measured as the velocity at which an animal travels immediately after exiting a squeeze chute (Burrow et al., 1988; Curley et al., 2006). It was calculated from the time elapsed as the animal traverses 1.83 m after exiting the chute. Infrared sensors were used to start and stop a timer (FarmTek Inc., North Wylie, TX) that measured the time elapsed as the animal travelled 1.83 m. A faster EV indicates a more temperamental animal, while a slower EV indicates a calmer animal.

Temperament score was calculated as the numerical average of EV and PS at weaning (Curley et al., 2006, 2008; King et al., 2006). A higher TS indicates a more temperamental animal while a lower TS indicates a calmer animal.

Table 6. Body condition scoring system adapted from Wagner et al. (1988).

Score	Description
1	SEVERELY EMACIATED. All ribs and bone structures easily visible and physically weak. Animal has difficulty standing or walking. No external fat present by sight or touch.
2	EMACIATED. Similar to 1, but not weakened.
3	VERY THIN. No palpable or visible fat on ribs or brisket. Individual muscles in the hindquarter are easily visible and spinus processes are very apparent.
4	THIN. Ribs and pin bones are easily visible and fat is not apparent by palpation on ribs or pin bones. Individual muscles in the hindquarter are apparent.
5	MODERATE. Ribs are less apparent than in 4 and have less than 0.5 cm of fat on them. Last two or three ribs can be felt easily. No fat in the brisket. At least 1 cm of fat can be palpated on pin bones. Individual muscles in hindquarter are not apparent.
6	GOOD. Smooth appearance throughout. Some fat deposition in brisket. Individual ribs are not visible. About 1 cm of fat on the pin bones and on the last two to three ribs.
7	VERY GOOD. Brisket is full. Tailhead and pin bones have protruding deposits of fat on them. Back appears square due to fat. Indentation over spinal cord due to fat on each side. Between 1 and 2 cm of fat on last two to three ribs.
8	OBESE. Back is very square. Brisket is distended with fat. Large protruding deposits of fat on tailhead and pin bones. Neck is think. Between 3 and 4 cm of fat on last two to three ribs. Large indentation over spinal cord.
9	VERY OBESE. Description of 8 taken to greater extremes.

Statistical Analysis

The calf growth and temperament data were analyzed using the MIXED procedure of SAS 9.3 (SAS Institute, Inc., Cary, NC) to perform ANOVA with fixed and random effects. Calf response variables tested included weight at birth, 14, and 28 d of age, ADG from birth to 28 d of age, adjusted 180-d WW, and PS, EV, and TS at 14 d of age, 28 d of age, and at weaning. The fixed effects of prenatal treatment, calf sex, and dam parity, and the random effect of sire were included in the models. The dam weight and body condition data were analyzed using a repeated measures ANOVA model with treatment, parity, time, and their interactions as fixed effects and animal as a random effect. The subject was the animal nested within treatment and parity. Variance components, compound symmetry, and first-order autoregressive covariance structures were tested for model fit. The first-order autoregressive structure had both the lowest Akaike's information criterion and corrected Akaike's information criterion values, and thus it was selected for use in the repeated measures models.

Results and Discussion

Cow Body Weight and BCS

Both the BW (Figure 13; Figure 15) and the BCS (Figure 14; Figure 16) of the pregnant cattle in both treatment groups were analyzed over the time period between 42 d post-banding and 126 d post-banding. Treatment ($P = 0.02$), time ($P < 0.01$), and parity ($P = 0.01$) significantly affected BW, while treatment ($P = 0.03$) and time ($P < 0.01$) affected BCS. Over time, STRESS dams had lower BW than C dams. This was expected

because the stress associated with dehorning likely caused a decrease in feed intake over the period following the procedure. However, this treatment difference may also be attributed to the much larger proportion of nulliparous heifers present in the STRESS group, compared to the C group. Without measuring feed intake, it is not clear which factor contributed more significantly to the lower BW seen in the STRESS animals. Parity was associated with BW and not BCS over time because the nulliparous heifers had not yet reached their mature BW.

Nulliparous pregnant dams weighed less than parous pregnant dams at 70, 98, and 126 d post-banding ($P < 0.01$) and tended to weigh less at 42 d post-banding ($P = 0.07$; Figure 15). Furthermore, nulliparous dams had a higher BCS than parous dams at 42 d post-banding, but not at 70, 98, or 126 following banding (Figure 16). This may have been due to the heifers' increased energy requirements for both their own growth and the rapid growth of the fetus during late gestation. At 42, 70, and 98 d post-banding, STRESS dams had a lower BCS than C dams ($P \leq 0.04$), but the treatment groups did not differ in BCS at 126 d post-banding (Figure 14). During the time points closer to the banding procedure, cattle were more likely to have reduced feed intake due to increased stress at this time. By 126 d post-banding, the STRESS cattle may have recovered from the procedure and compensated for their previously lowered energy intake.

Body condition scores averaged between 5.9 and 6.6 within both treatments for each time period. This indicates that the cows generally maintained an acceptable level of condition prior to calving, regardless of treatment (Spitzer et al., 1995).

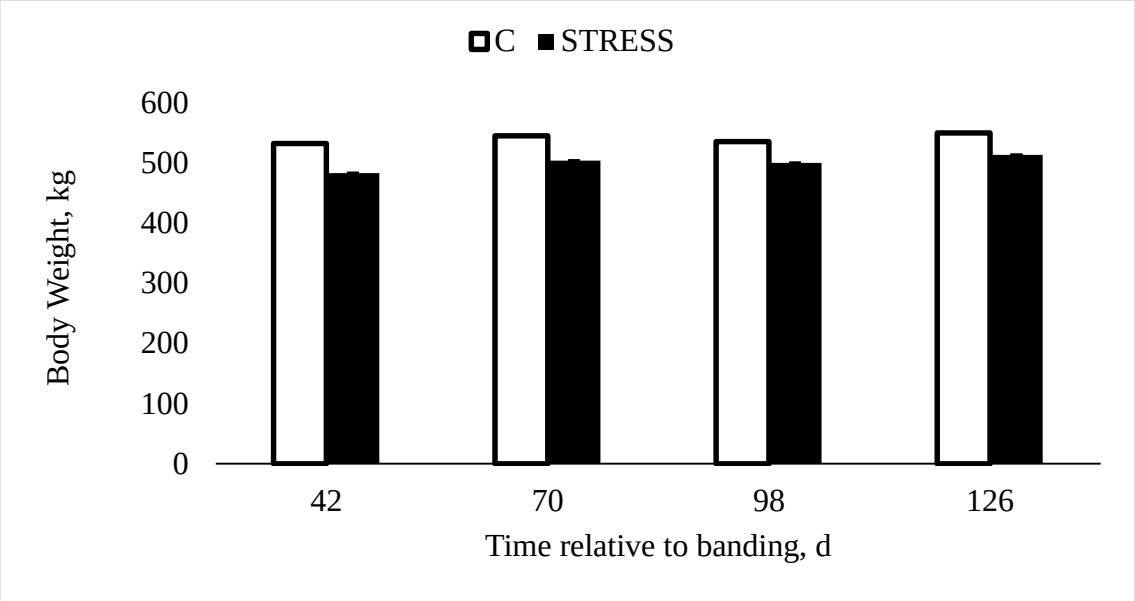

Figure 13. Body weights in dehorned (STRESS; black) and control (C; white) pregnant cattle following the application of Callicrate Bands to the horn base. The bands were applied on Day 0. Both treatment (P = 0.02) and time (P < 0.01) affected BW, with the dehorned cattle having lower BW than the control cattle over time.

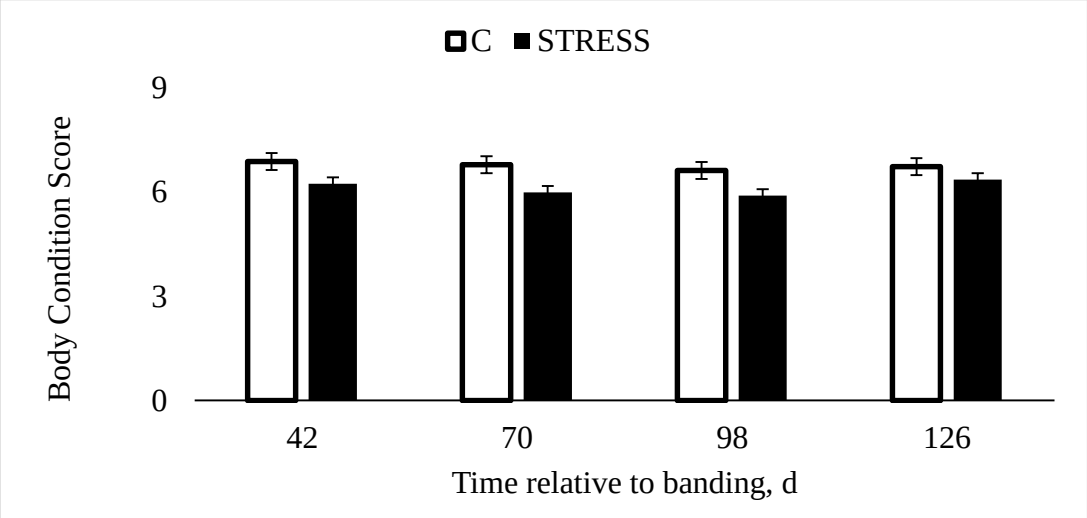

Figure 14. Body weights in dehorned (STRESS; black) and control (C; white) pregnant cattle following the application of Callicrate Bands to the horn base. The bands were applied on Day 0. Both treatment (P = 0.03) and time (P < 0.01) affected BCS, with the dehorned cattle having lower BCS than the control cattle over time.

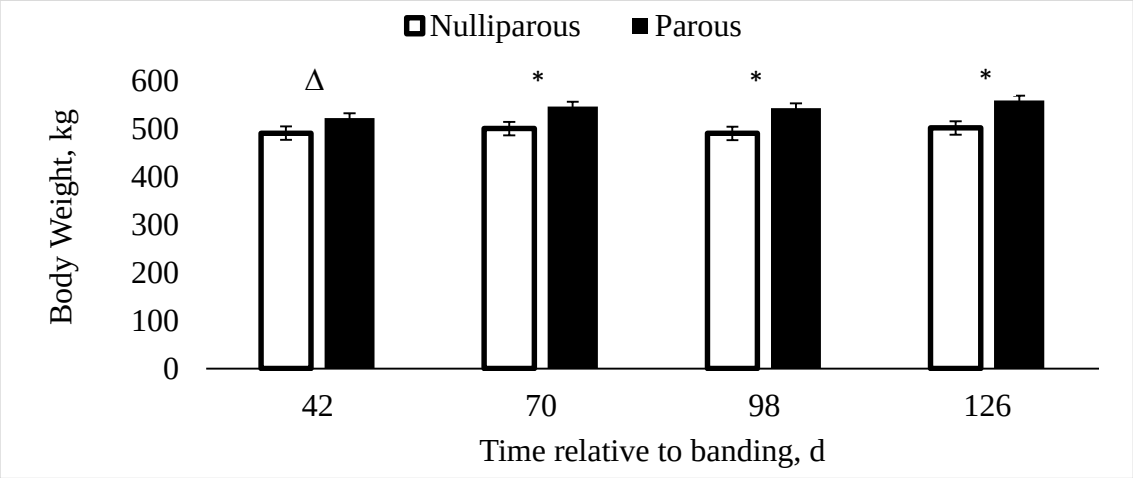

Figure 15. Body weights over time in pregnant parous cows (black) and pregnant nulliparous heifers (white) that were either dehorned or not. Callicrate Bands were applied to the dehorned cattle on Day 0. Parity (P = 0.01), time (P < 0.01), and the interaction between parity and time (P < 0.05) influenced BW. *Nulliparous cattle weighed less than parous cattle at 70, 98, and 126 d post-banding (P < 0.01). ΔNulliparous cattle tended to weigh less than parous cattle at 42 d post-banding (P = 0.07).

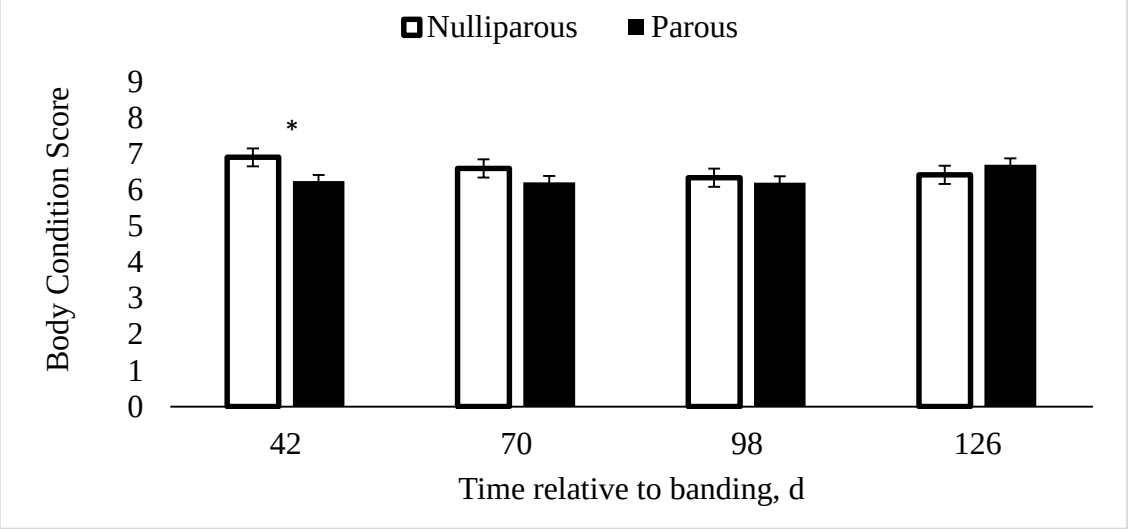

Figure 16. Body condition scores over time in pregnant parous cows (black) and pregnant nulliparous heifers (white) that were either dehorned or not. Callicrate Bands were applied to the dehorned cattle on Day 0. *The interaction between parity and time influenced BCS, with the nulliparous cattle having a higher BCS than the parous cattle at 42 d post-banding (P < 0.05).

Calf Temperament

At both 14 and 28 d of age, treatment did not influence PS across bull and heifer calves born to primiparous and multiparous dams (Figure 17; $P > 0.10$). However, at 14 d of age, bull calves tended to have a lower PS than heifer calves ($P = 0.06$). Bull calves born to C primiparous dams had lower PS than heifer calves born to C primiparous dams ($P = 0.04$). Heifer calves born to primiparous dams in the STRESS group tended to have a lower PS than heifer calves born to primiparous dams in the C group ($P = 0.06$). Heifer calves in the C treatment group were calmer when born to multiparous dams, compared with primiparous dams ($P = 0.03$). By 28 d of age, C heifer calves born to multiparous dams continued to have a lower PS than those born to primiparous dams ($P = 0.04$). Bull calves born to C primiparous dams tended to have lower PS than C heifer calves born to primiparous dams ($P = 0.07$).

At 14 d of age, calf EV was not influenced by treatment, dam parity, or calf sex (Figure 18; $P \geq 0.24$). At 28 d of age, EV was not influenced by treatment across both bull and heifer calves born to primiparous and multiparous dams (Figure 18; $P > 0.10$). However, at 28 d of age, bull calves tended to have a slower EV than heifer calves, regardless of treatment or dam parity ($P = 0.06$). Heifer calves born to primiparous dams in the C treatment group had a faster EV at 28 d of age than those in the STRESS treatment group ($P = 0.02$). The high mean EV observed in this group (3.17 ± 0.56 m/s) was consistent with the increased PS (4.50 ± 0.93) observed at the same age.

At both 14 and 28 d of age, TS was not influenced by treatment across both bull and heifer calves born to primiparous and multiparous dams (Figure 19; $P > 0.10$). At 14

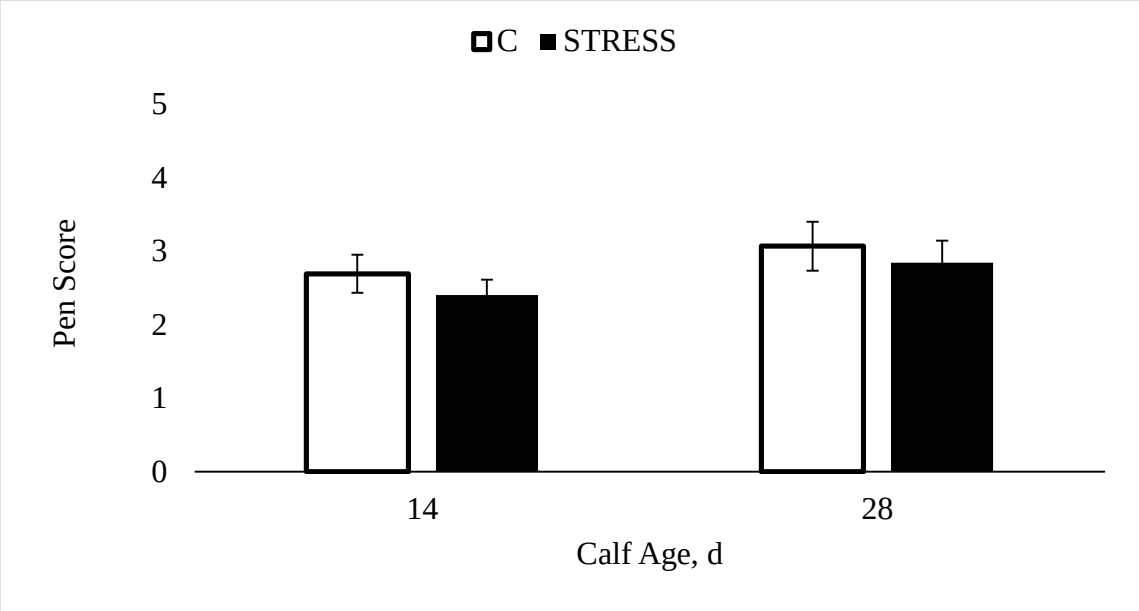

Figure 17. Pen scores of prenatally stressed calves (STRESS; black) and control calves (C; white) at 14 and 28 days of age. Treatment did not influence PS at 14 or 28 d of age ($P > 0.10$).

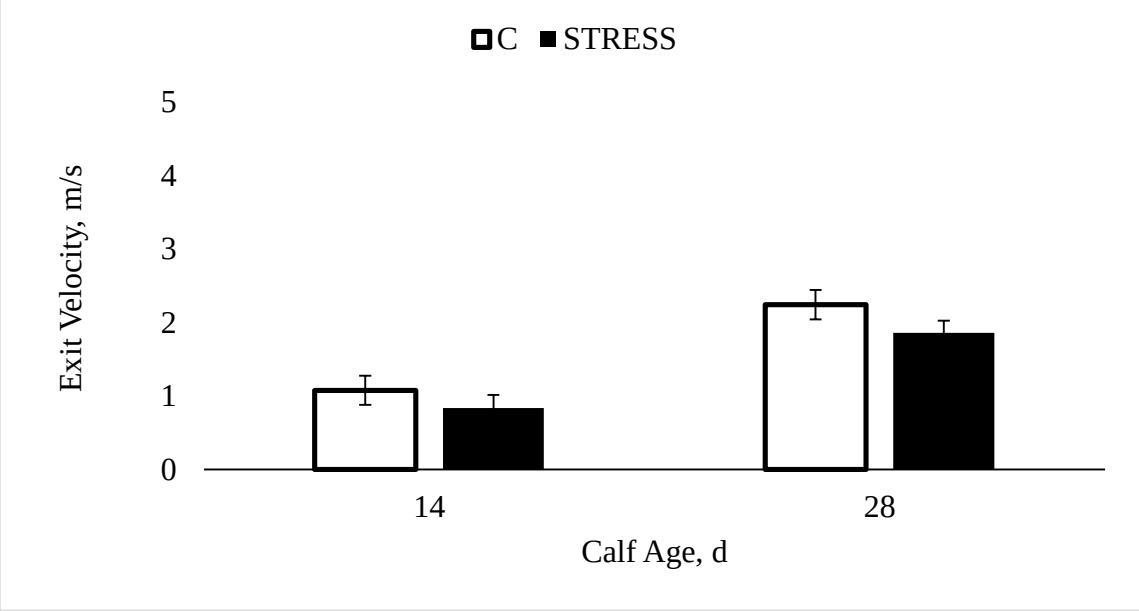

Figure 18. Exit velocities of prenatally stressed calves (STRESS; black) and control calves (C; white) at 14 and 28 days of age. Treatment did not influence EV at 14 or 28 d of age ($P > 0.10$).

Figure 19. Temperament scores of prenatally stressed calves (STRESS; black) and control calves (C; white) at 14 and 28 days of age. Treatment did not influence TS at 14 or 28 d of age (P > 0.10).

d of age, the C heifer calves born to primiparous dams tended to have higher TS than the heifer calves born to primiparous dams in the STRESS group (P = 0.08) and the heifer calves born to multiparous dams in the C group (P = 0.07). By 28 d of age, the differences were significant (P = 0.02; P = 0.04). Furthermore, the C heifer calves born to primiparous heifers also had higher TS than the STRESS heifer calves born to multiparous dams (P = 0.05). Once again, the C heifer calves born to primiparous heifers displayed more temperamental behavior than the other groups.

Temperament evaluation, when performed at weaning, has been shown to be more reflective of post-weaning behavior than when it is performed at younger ages (Burdick et al., 2011). Across all calves, neither PS, EV, nor TS were influenced by

treatment across (Figure 20; $P > 0.10$). However, the PS recorded at weaning tended to be lower in the STRESS treatment group, compared to the C treatment group, in calves born to multiparous dams (Figure 21; $P = 0.09$). Weaning TS tended to be lower in bull calves than in heifer calves, regardless of treatment or dam parity (Figure 22; $P = 0.06$). However, EV at weaning was not affected by treatment, calf sex, or dam parity ($P \geq 0.12$).

The bull calves in this study displayed calmer temperament; as measured by multiple measurements and at multiple ages. This is consistent with previous work (Burdick et al., 2009; Littlejohn et al., 2013a,b). Furthermore, the C heifer calves born to primiparous dams were markedly more temperamental than other groups, which resulted in an interaction of the effects of treatment, calf sex, and dam parity in many of the models. The mean PS, EV, and TS in this group were well above those reported in other studies (Littlejohn et al., 2013a,b) and therefore indicated an unusually temperamental group of calves. Furthermore, the small size of this group ($n = 2$) does not allow us to make any valid conclusions concerning these interactions. Therefore, it appears that temperament was largely unaffected by treatment in this study.

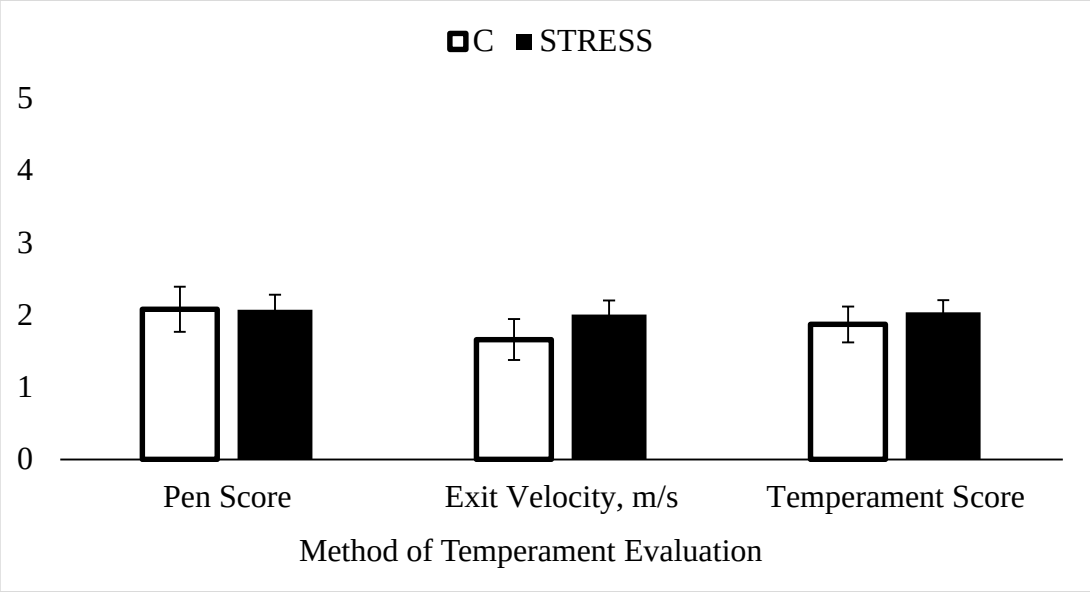

Figure 20. Pen scores, exit velocities (m/s), and temperament scores of prenatally stressed calves (STRESS; black) and control calves (C; white) at weaning. Treatment did not influence PS, EV, or TS at weaning (P > 0.10).

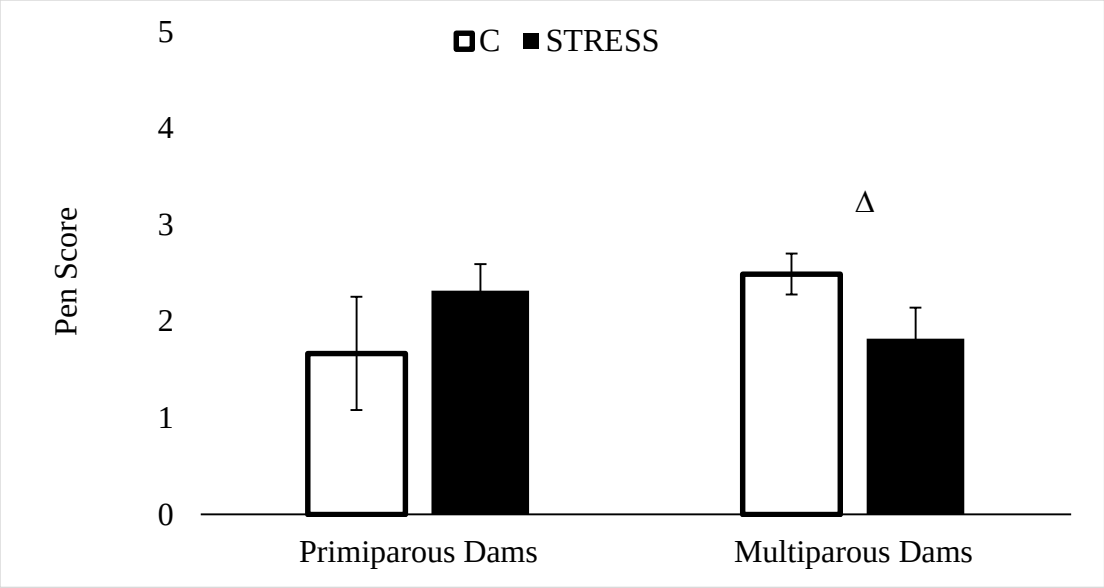

Figure 21. Weaning pen scores in prenatally stressed (STRESS; black) and control (C; white) calves that were born to primiparous and multiparous dams. ΔThe STRESS calves born to multiparous dams tended to have lower PS than C calves born to multiparous dams (P = 0.09).

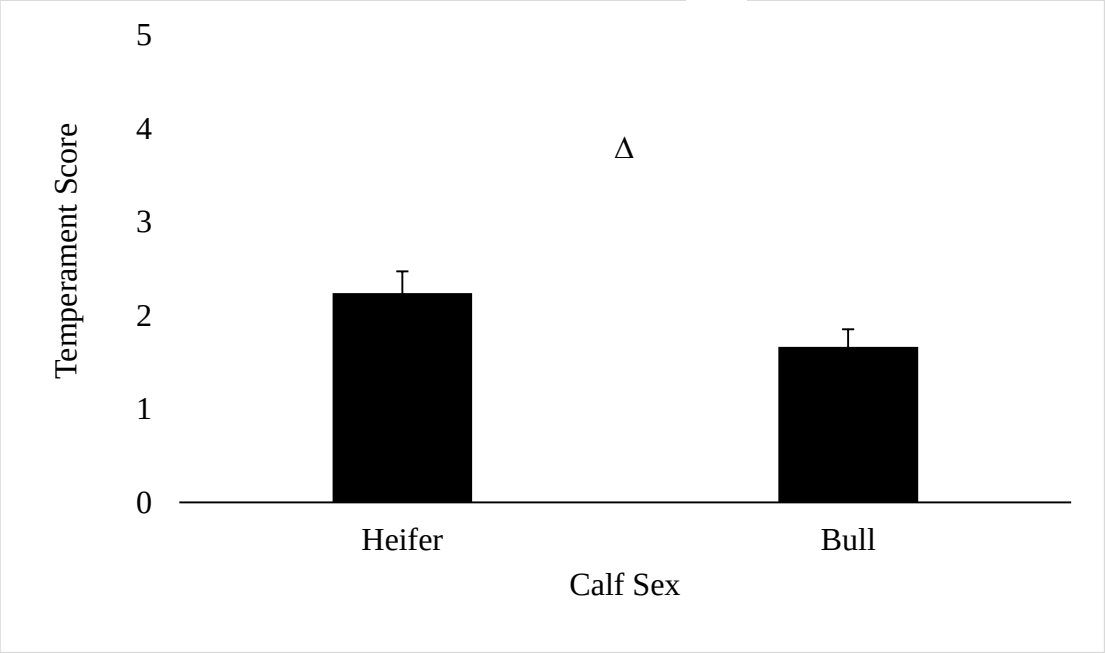

Figure 22. Temperament scores in bull and heifer calves at weaning that experienced prenatal stress or not. ΔHeifer calves tended to have higher TS at weaning than bull calves across treatment groups (P = 0.06).

Calf Weight

Birth weights were influenced by treatment group and calf sex, with the STRESS calves having a lower BW at birth than the C calves (Figure 23; P = 0.04) and bull calves being heavier at birth than heifer calves (P < 0.01). Dam parity did not affect birth weights (P = 0.45). The influence of treatment group on birth weight, in combination with the observed lowered BW and BCS of the STRESS dams, could indicate that intrauterine growth restriction occurred in these calves due to under nutrition during late gestation. This could continue to stunt long-term calf growth, as described by Wu et al. (2006).

Although our statistical analysis showed that dam parity did not influence birth weights, this may be confounded by the fact that the treatment groups were unbalanced. The proportion of primiparous dams in the STRESS group was much larger than that in the C group, and primiparous dams typically give birth to smaller calves (Tudor, 1972; Browning et al., 1995). Therefore, the treatment effect may have been exaggerated in the model.

At 14 and 28 d of age, calf sex influenced BW ($P < 0.01$), but treatment and dam parity did not ($P \geq 0.21$). Bull calves continued to be heavier than heifer calves through 28 d of age. Furthermore, STRESS bull calves born to multiparous cows tended to be heavier at 28 d of age than STRESS bull calves born to primiparous heifers (0.09). Heifer calves born to multiparous cows tended to be heavier in the C group than in the STRESS group at 28 d of age ($P = 0.10$). From birth to 28 d of age, the ADG of bull calves was greater than that of heifer calves ($P = 0.02$). However, neither treatment, nor dam parity, affected ADG during this time period.

The 180-d adjusted WW of calves was not affected by treatment or dam parity (Figure 24), but was increased in bull calves, compared to heifer calves ($P = 0.01$). This was expected because Browning et al. (1995) reported that sex differences in BW persist through weaning in Brahman calves. That study also reported that dam parity did not influence 205-d adjusted WW, which is consistent with our findings. It appears that although STRESS calves had lower birth weights, their growth through weaning was compensatory.

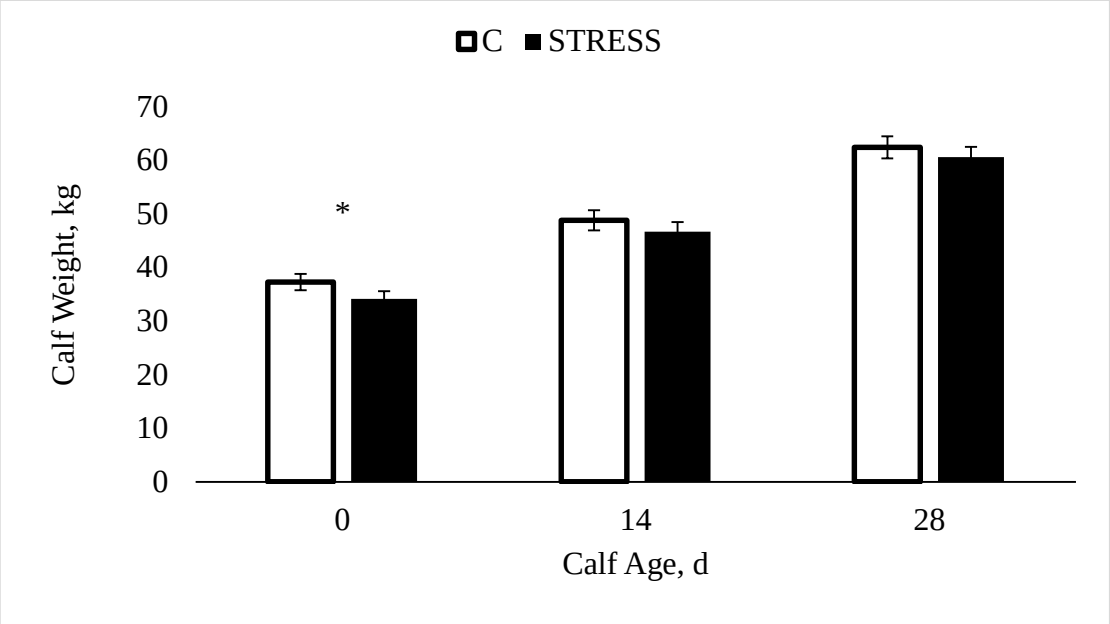

Figure 23. Body weights of prenatally stressed calves (STRESS; black) and control calves (C; white) at 0, 14, and 28 days of age. *At birth, STRESS calves weighed less than C calves (P = 0.04).

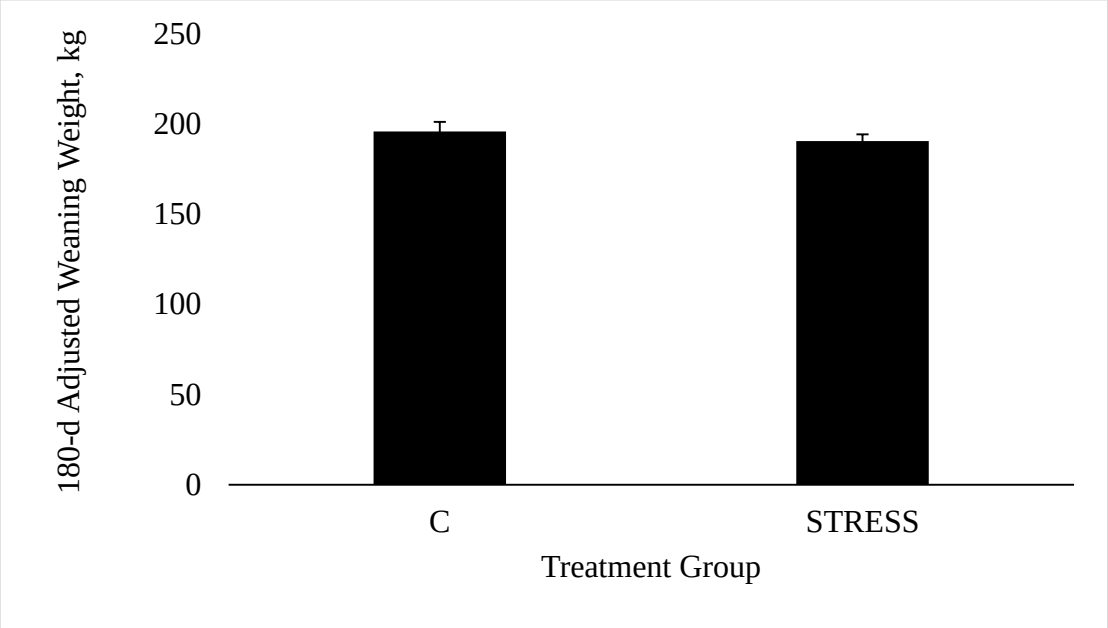

Figure 24. 180-d adjusted weaning weights for prenatally stressed (STRESS) and control (C) calves. Treatment did not influence the 180-d adjusted weaning weights (P > 0.10).

Conclusion

It appears that the dehorning procedure resulted in a decreased BCS and BW in the STRESS group of nulliparous heifers and multiparous cows. This decrease was most evident between 42 and 98 d post-banding, and had lessened by 126 d post-banding. This may indicate that banded cattle had decreased feed intake during this time, but it could also be due to the fact that the treatment groups were not balanced by parity.

While temperament was largely unaffected by treatment, birth weight was decreased in the calves born in the STRESS treatment group. This may have been due to either under nutrition during gestation, or it could simply be a reflection of the large proportion of primiparous dams in the STRESS group. Maternal under nutrition during the final trimester has been associated with intrauterine growth restriction and decreased birth weights in various species (Wu et al., 2006). If the STRESS group existed on a lower plane of nutrition for a portion of the third trimester, it would be expected that birth weights were affected by nutrient restriction.

Therefore, a dehorning procedure using a Callicrate Bander during late gestation has the potential to decrease the growth of fetal calves. However, growth and temperament of the calves through weaning was not influenced by the dehorning during the second trimester using the banding method.

CHAPTER V

CONCLUSION

The prenatal environment has been show by numerous studies to affect the growth and development of offspring; some even suggesting epigenetic effects that persist generation-to-generation. The long-term effects of stress during gestation on the offspring appear to be highly dependent on the type of stressor, the timing and duration of the stress, the species, and the sex of the offspring. Because of this specificity, more research should be done in livestock species in order to determine if performing stressful management practices during gestation negatively affects the productivity of the offspring.

Although the results in this thesis suggest that prenatal stress did not alter the HPA axis function of Brahman heifers, independent of temperament, it is likely that the PNS population of heifers actually does exhibit differential HPA axis characteristics. This is likely due to the increased proportion of temperamental animals in the population (Littlejohn et al., 2013a,b) and the knowledge that temperament is associated with HPA axis function (Curley et al., 2008).

While prenatal stress may not influence HPA axis function independent of temperament, it does appear to enhance insulin sensitivity. This is significant for producers because markers of enhanced insulin sensitivity, such as decreased IGR and IIND following a glucose tolerance test, are also associated with decreased RFI. However, the results of this study should be repeated before making any

recommendation because they contrast with those found in other published research. Furthermore, from a welfare perspective, it is not advisable to inflict stress on pregnant animals in order to produce more efficient offspring.

Dehorning pregnant cattle during late gestation appears to decrease the birth weight of their offspring. This is possibly due to decreased feed intake caused by the stressor. Under nutrition during late gestation is associated with intrauterine growth restriction, which may explain the results seen in this thesis. However, it is important to note that the results of this study should be repeated in groups of cattle that are balanced by parity before conclusions can be made. Furthermore, it appears that any differences in the BW of prenatally stressed calves at birth had disappeared by 14 d of age.

Similar to findings in other species, it appears that prenatal stress also affects the physiology and productive traits of calves. The findings in this thesis also suggest that these effects may be long-lasting or permanent. More research should be done in this field that examines the effects of stressors at different stages of gestation and examines the results of stress in both heifers and bulls.

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APPENDIX A

INSULIN IMMUNOASSAY PROCEDURES

(ALPCO™ Insulin (Bovine) ELISA, 80-INSBO-E01)

Materials Supplied in the Kit:

1. Insulin Microplate (coated with mouse monoclonal anti-insulin antibody)
2. Zero Standard (0 ng/ml)
3. Standards
   Five vials labeled A through E

   (.25, .5, 1.0, 3.0, 6.0 ng/ml)

4. Mammalian Insulin High and Low Controls
5. Conjugated Stock (HRP labeled monoclonal anti-insulin antibody)
6. Conjugate Buffer
7. Wash Buffer Concentrate
8. TMB Substrate
9. Stop Solution
10. Plate Sealers

Materials Required But Not Supplied

1. Micropipettes capable of dispensing 25 µL, 75 µL, and 100 µL
2. Repeating or multi-channel pipette capable of dispensing 75 µL and 100 µL
3. Volumetric containers and pipettes for reagent preparation
4. Distilled (deionized) water
5. Horizontal microplate shaker capable of 700-900 rpm
6. Microplate reader ith 450 and 620-650 nm filter

Immunoassay Procedure:

1. Bring all reagents and micropplates strips to room temperature.
2. Designate enough microplate strips for the standards, controls, and desired number of samples.
3. Prepare all reagents

Dilute the Conjugate stock with 10 parts Conjugate Buffer.

Reconstitute the Mammalian Insulin High and Low Controls by add **X** volume of deionized water, close the vial with the rubber stopper, gently swirl and then allow it to stand for 30 minutes prior to use.

Dilute the Wash Buffer Concentrate with 20 parts distilled water.

3. Pipette 25 µL of each standard, reconstituted control or sample into its respective well.
4. Pipette 75 µL of Working Strength Conjugate into each well.
5. Incubate on a horizontal microplate shaker, shaking at 700 - 900 rpm, at roomtemperautre for 2 hours.
6. Decant the contents and remove all visible content by inverting wells. Wash the microplate 6 times with at least 350 µL Working Strength Wash Buffer.
7. After the final wash remove the residual Wash Buffer and bubbles from the wells by inverting and firmly tapping the microplate on absorbant paper towels.
8. Pipette 100 µL of TMB Substrate to each well.
9. Incubate on a horiztonal microplate shaker, shaking at 700 – 900 rpm, at room temperature for 15 minutes.
10. Pipette 100 µL of Stop Solution to each well, and gently shake the microplate.
11. Remove bubbles before reading with the microplate reader.
12. Place the microplate in a microplate reader capable of reading the absorbance at 450 nm with a reference wavelength of 620-650 nm.

APPENDIX B

GLUCOSE COLORIMETRY PROCEDURES

(WAKO Autokit Glucose Protocol, 439-90901)

Materials Supplied:

1. Buffer Solution 2 x 150 mL
   60mmol/L Phosphate buffer (pH 7.1) containing 5.3 mmol/l Phenol

   Store at 2-10 °C

1. Color Reagent (When reconstituted) 2 x 150 mL
   Containing 0.13 U/mL Mutarotase, 9.0U/mL Glucose oxidase, 0.65 U/mL Peroxidase, 0.50 mmol/L 4-Aminoantipyrine, 2.7 Ascorbate oxidase

   Store at 2 – 10 °C

2. Standard Solution I 1 x 10 mL
   Containing 200 mg/dL Glucose

   Store at 2 – 10 °C

3. Standard Solution II 1 x 10 mL
   Containing 500 mg/dL Glucose

   Store at 2 – 10 °C

Materials Required But Not Supplied:

1. Micropipettes
2. Water bath that can hold constant 37 °C
3. Spectrophotometer

Protocol:

1. Dissolve the contents of one bottle of Color Reagent Soluton by dissolving the whole contents of one botle of Color Reagent powder into one, 150 mL, bottle of Buffer Solution.
2. Dilute Standards in distilled water as described below in microcentrifuge tubes:

Standard	**Amount Stock in µL (500 mg/dL stock)**	**Amount H_2O in µL**	**Final Concentration (mg/dL)**
1	200	--	500
2	100 # 1	100	250
3	100 # 2	100	125
4	100 # 3	100	62.5
5	100 # 4	100	31.25
6	--	100	0

3. Turn on the plate reader and set incubator to 37 °C.
4. Pipette 2 µL of the sample into each level of calibrator and each well. Add 300 µL of Working Solution to each well.
5. Tap the plate to mix the plates and incubate the plates at 37 °C for 5 minutes.
6. Read the plate at 505 nm, and copy the absorbance data into the 'Glucose Assay Template' to calculate standard curve and sample concentrations.

APPENDIX C

CORTISOL RADIOIMMUNOASSAY PROCEDURES

(^{3}H-Based Radioimmunoassay for Serum Cortisol Analyses)

Reagent Preparation

1. Charcoal-Dextran
0.60 g Activated Charcoal (Sigma C-5260)
0.06 g Dextran (Sigma D-4271)
500 mL PBSG

Mix charcoal and dextran into 500mL PBSG. Store at 4ºC for up to 1 month.
Mix well before each use.

2. PBSG (1 L)
0.070 g Monobasic Sodium Phosphate (Sigma, S-9638; FW 138.0)
1.350 g Dibasic Sodium Phosphate (Sigma, S-0876; FW 142.0)
8.812 g Sodium Chloride (Sigma, S-9888; FW 58.44)
1.000 g Sodium Azide (Sigma, S-2002; FW 65.01)
0.372 g Disodium EDTA: dehydrate (Sigma; ED2SS, FW 372.2)
1.000 g Gelatin (J. T. Baker, 2124-01)

Into 900 mL double-distilled water, weight out and add all reagents except EDTA and gelatin. Mix and adjust to pH 7.5 using 1.0 M HCl or NaOH. Bring to final volume in calibrated 2 L beaker or volumetric flask. Add EDTA and gelatin with continuous stirring over low heat until dissolved (approximately 1 h).

Store at 4˚ C.

3. Tracer Preparation
NET 396 (1,2,6,7-3H(N)-Hydrocortisone (PerkinElmer)
PBSG

Introduce tracer stock into 25 mL PBSG, mix for 5 min on stir-plate and let stand for 10 min at 4˚ C. Calculate appropriate dilution and add appropriate volume of PBSG.

Mix well and incubate overnight at 4˚ C.

Standard Preparation

Cortisol STD, ng/mL
STD 1 0.500
STD 2 1.000
STD 3 2.000
STD 4 4.000
STD 5 8.000
STD 6 16.000
STD 7 32.000
STD 8 64.000

Radioimmunoassay Procedure:

1. Allow samples, standards, controls, and PBSG to warm to room temperature.
2. Pipette PBSG into the T tubes, 900 μL; NSB tubes, 200 μL; and Ø tubes, 100 μL.
3. Add charcoal striped serum to all but the T tubes.
4. Pipette pools (50 or 100 μL) of each pool (High, Low Composite)
5. Add 100 μL of PBSG to each of the pools.
6. Pipette 100 or 50 μL of samples
7. Add 100 μL of PBSG to each of the samples
8. Incubate all but the T tubes in 70 °C water bath for an hour.
9. After removing the tubes from the water bath, allow them to cool for 30 minutes and integrate all tubes into test-tube racks according to tube number.
10. Pipette 100 μL of radio-label hydrocortisone to all tubes, and pipette 100 μL of cortisol antibody to all but the T and NSB tubes.
11. Shake tubes and incubate at 4° C overnight
12. Pipette 500 uL of charcoal-dextran to all tubes except the T tubes
13. Shake the tubes and load them into the centrifuge.
14. Allow the tubes to site with the charcoal-dextran for 15 minutes
15. Run the centrifuge for 25 minutes at 4° C and 2700 relative centrifugal force (RCF).
16. Decant the samples into scintillation vials.
17. Cap and shake the samples and then incubate them at room temperature overnight.
18. Shake the samples and load them into the beta-counter.

APPENDIX D

ACTH RADIOIMMUNOASSAY PROCEDURES

(MP Biomedicals ImmunChem^{TM} hACTH DA, 07-106101)

Materials Supplied in the Kit:

1.	Anti-ACTH	1 x 5.5 mL
2.	ACTH Standards	8 x 1 mL
3.	ACTH Controls	2 x 1 mL
4.	^{125}I hACTH	1 x 5.5 mL
5.	Precipitant Solution	1 x 27 mL
6.	ACTH Water	1 x 30 mL

Reagent Preparation:

1. ANTI-ACTH
   Prior to use, reconstitute with 5.5 mL of water and allow to stand 15 minutes at 4 °C ± 2 °C. After use, store at -20 °C ± 2 °C. Thaw only once
2. ACTH Standards
   Prior to use, reconstitute with 1.0 mL (2.0 mL for the 0) of water and allow to stand 15 minutes at 4 °C ± 2 °C. Afer use, store at -20 °C ± 2 °C. Thaw only once.
3. ACTH Controls
   Prior to use, reconstitute with 1.0 mL of water and allow to stand 15 minutes at 4 °C ± 2 °C. After use, store at -20 °C ± 2 °C. Thaw only once.
4. hACTH-^{125}I
   Prior to use, reconstitute with 5.5 mL of water and allow to stand 15 minutes at 4 °C ± 2 °C before use. After use, store at -20 °C ± 2 °C. Thaw only once.
5. Precipitant Solution
6. ACTH Water

Materials Required But Not Supplied

1. Pipettes that can accurately and precisely deliver the required volumes (100 µL and 500 µL)
2. A test-tube rack
3. Ice bath large enough to accommodate test-tube rack
4. Refrigerated Water Bath or a Refrigerator capable of maintaining 4 °C ± 2 °C

5. Centrifuge
6. An aspiration or decanting device
7. A gamma counter calibrator for ^{125}I

Assay Procedure:

1. Reconstitute lyophilized reagents and mix gently.
2. Let the reagents sit for 15 minutes at 4 °C ± 2 °C. Then take out the vial of precipitant solution and let it warm to room temperature.
3. Set the assay in consecutively numbered polystyrene tubes. Then place the test tube rack on ice. All reagents and samples should be placed on ice. Add the solution in the order indicated in the protocol. Pipet all reagents directly from shipping vials.
4. Add diluent water, hACTH standards, controls, samples, anti-serum and hACTH-^{125}I to the test tubes as indicated in the protocol. Shake test tube rack for 30 seconds, vortex tubes thoroughly and incubate at 4 °C ± 2 °C for at least 16 hours.
5. After incubation, locate the bottle of precipitant solution, at room temperature, and mix well with shaking. Add 0.5 mL of this solution to all tubes.
6. Shake the tube rakc for 30 seconds and vortex tubes thoroughly until one homogeneous color is seen in the tube. Centrifuge at 950 – 1050 x g for 10 to 15 minutes.
7. Aspirate or decant the supernatant. Blot the rim of the test tube on absorbent paper. Count the precipitate remaining in the tubes in a gamma counter. A counting time of at least 2 minutes per tube is suggested.