INFLUENCE OF NUTRITION DURING THE JUVENILE PERIOD ON GENE EXPRESSION WITHIN THE HYPOTHALAMIC ARCUATE NUCLEUS AND ON AGE AT PUBERTY IN HEIFERS

A Thesis

by

CAROLYN C. ALLEN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2010

Major Subject: Physiology of Reproduction

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Approved by:

Co-Chairs of Committee,	Gary L. Williams
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ABSTRACT

Influence of Nutrition during the Juvenile Period on Gene Expression within the Hypothalamic Arcuate Nucleus and on Age at Puberty in Heifers. (August 2010)

Carolyn C. Allen, B.S., University of Connecticut

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Developmental changes within the hypothalamus are necessary for maturation of the reproductive neuroendocrine axis. Recent reports have implicated several neuronal networks in this process, but genes involved in their regulation have not been elucidated. Using a well-established model for nutritional induction of precocious puberty, objectives were to 1) use microarray technology to examine changes in gene expression within the arcuate nucleus (ARC) of the hypothalamus in pre-pubertal heifers fed high or low-concentrate diets, and 2) determine if high-concentrate diets are required for nutritional induction of precocious puberty. In Experiment 1, early-weaned, cross-bred heifers were fed either a high-forage/low-gain (HF/LG; 0.45 kg/d) or a high-concentrate/high-gain (HC/HG; 0.91 kg/d) diet for 91 d. Analysis of microarray data indicated that 346 genes were differentially expressed ($P < 0.05$) between HC/HG and HF/LG heifers. Expression of three key metabolic genes [neuropeptide Y (NPY), agouti-related protein (AGRP), and growth hormone receptor (GHR)] observed to be differentially expressed in the microarray analysis was investigated further by

quantitative PCR. Real-time RT-PCR indicated that expression of *NPY*, *AGRP* and *GHR* was lower ($P < 0.05$) in HC/HG compared to HF/LG heifers. In contrast, concentrations of insulin ($P < 0.05$), IGF-1 ($P < 0.002$) and leptin ($P = 0.1$) were greater in HC/HG compared to HF/LG. For Experiment 2, 48 heifers were used in 2 replicates (24 heifers/replicate) in a 2 x 2 factorial design to examine the roles of diet type (HF vs HC) and rate of gain (LG, 0.45 kg/d vs HG, 0.91 kg/d) on age at puberty. Heifers were fed HC/HG, HC/LG, HF/HG or HF/LG (n = 12/group) for 14 wk, and then switched to a common growth diet (0.68 kg/d) until puberty. Heifers in both HG groups reached puberty at a younger age (54.5 ± 1.8 wk) than heifers in both LG groups (60.2 ± 1.9 wk; $P < 0.04$). A marked increase ($P < 0.01$) in serum concentrations of leptin occurred in HC/HG heifers between 24 and 30 wk of age. This increase in circulating leptin was not observed in other groups. Overall, results indicate that nutritional regulation of reproductive neuroendocrine development involves the control of *NPY, AGRP* and *GHR* expression. The abrupt increase noted for circulating leptin in heifers fed HC/HG diets, if timed and sustained appropriately, could represent an important temporal cue for activation of the neuroendocrine system and the onset of puberty.

DEDICATION

To Elizabeth,

Christopher,

Rachael,

and Anne

ACKNOWLEDGEMENTS

I would first like to express my sincere appreciation and thanks to Dr. Gary Williams for providing support, mentorship, knowledge and motivation during my training. It was a privilege to have spent a year working with you in Beeville, and an experience I will never forget. Without your guidance, understanding, and endless patience I never would have made it this far. I sincerely thank you for constantly challenging me and for helping me to become more than I thought possible.

I would like to express equal appreciation and thanks to Dr. Marcel Amstalden. Amidst the multitude of lengthy discussions and hours spent in the lab, you have not only provided constant support and dedication to my success, but you have provided encouragement and understanding on days when I felt the world was coming to an end. Thank you for your mentorship, your patience with me, for challenging me, and for helping me to become confident with my work.

Thank you to Dr. Luis Tedeschi, for not only serving as a committee member, but for his willingness to collaborate on my project. Additionally, I would like to thank Dr. Robert Burghardt, for serving as a committee member and for offering advice and support with developing our methodology for tissue collection. I would also like to extend my sincere appreciation and thanks to Dr. Xianyao Li for his help and guidance with my microarray work; Kelli Kochan for the hours spent teaching me how to design primers and for her assistance with qRT-PCR; Dr. Ulisses Braga-Neto for his most excellent analysis of our microarray data; Dr. Penny Riggs for her willingness to

collaborate and assist with our qRT-PCR and microarray studies; Dr. Tom Spencer for assistance with microarray and for collaborating on my project; Bruna Alves for her assistance with histology, and all other members of the Williams/Amstalden coalition that have helped make my project a success.

A special thank you to the Beeville crew: Ray Villarreal, Randle Franke, Dr. Randy Stanko, Phani Potulla, Cathy Cardwell, Marsha Green, and Gary Hart, for their endless help and support with my heifer work, last minute ordering, and broken centrifuges.

I would also like to acknowledge those close to my heart that have provided constant support, guidance, and encouragement. My parents, Crane and Laurie Allen, and siblings, Elizabeth, Chris, Rachael and Anne for providing me with the tools for my success, for helping me in every way possible, and supporting my decisions regardless of what they might be, and to Rick and Denise Bouchard for their encouragement, support, motivation and inspiration. I would also like to take the opportunity to thank Heather Lord and Audra Leach for providing constant encouragement, optimism and friendship from 2,000 miles away. My acknowledgements would not be complete without expressing my thanks to Becky Simmons who has not only provided constant support and advice, but has been a true friend and partner in crime beginning the very first day I arrived in Texas. Finally I would like to acknowledge Jeremy Redmond, Megan Minten, Nicole Burdick, Mike Peoples, Lisa Caldwell, Anna Poovey, Sarah Black and Melissa Mahan for being my support system during my time at Texas A&M. Without you all, none of this would have been possible.

NOMENCLATURE

AGRP	Agouti-related protein
AVPV	Anteroventral periventricular nucleus
ARC	Arcuate nucleus
FSH	Follicle-stimulating hormone
DMH	Dorsomedial hypothalamus
GnRH	Gonadotropin-releasing hormone
GH	Growth hormone
GHR	Growth hormone receptor
ICV	Intracerebroventricular
LCM	Laser capture microdissection
LH	Luteinizing hormone
ME	Median eminence
MBH	Mediobasal hypothalamus
NPY	Neuropeptide Y
PeN	Periventricular nucleus
POA	Pre-optic area
VMH	Ventromedial hypothalamus
VFA	Volatile fatty acid

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CHAPTER I

INTRODUCTION

Puberty is the consequence of developmental changes within the brain that lead to the attainment of reproductive capacity. In the events leading up to the first ovulation, gonadotropin-releasing hormone (GnRH) released into the hypothalamic-hypophyseal portal circulation mediates secretion of luteinizing hormone (LH) from the anterior pituitary gland. As maturation progresses, there is a decrease in hypothalamic sensitivity to estradiol negative feedback, which results in an increase in the pulsatile release of GnRH, and subsequently, LH. These events lead to development of a dominant, estrogen-active follicle, increased concentrations of circulating estradiol, and ultimately the pre-ovulatory surge of LH and first ovulation [1]. In heifers, the luteal phase following the initial ovulatory surge of LH is usually associated with lower fertility compared to those that follow [2]. Consequently, it is helpful for beef heifers to become pubertal well in advance of first breeding in order to increase the probability of conception early in their first breeding season, and to maximize lifetime productivity [2-4]. Puberty is a complex process and is dependent not only on the physiological changes directly related to the reproductive neuroendocrine axis, but also is influenced by a multitude of other cues emanating from the somatotropic axis and metabolism.

This thesis follows the style of Biology of Reproduction.

Although it is known that developmental changes within the hypothalamus underlie the processes driving the onset of puberty, the nature of these changes and the genes regulating them have not been elucidated. The hypothalamus plays a central role in the regulation of reproduction, and does this in part by integrating metabolic and reproductive signals within the arcuate nucleus (ARC). The ARC, also known as the infundibular nucleus, is located in the mediobasal hypothalamus (MBH) adjacent to the third ventricle and median eminence (ME). Neurons within the ARC are highly heterogeneous and project internally to act within the ARC, as well as projecting to other regions of the hypothalamus such as the ME [5] and pre-optic area (POA) [6], to act upon various other neurons. Neurons expressing neuropeptide Y (NPY), agouti-related protein (AGRP), proopiomenalocortin (POMC), and cocaine- and amphetamine-regulated transcript (CART) are of particular interest as they relate to the study of hypothalamic control of puberty. These neurons respond to local and peripheral metabolic signals and subsequently influence neuroendocrine events related to reproductive function [7].

One currently accepted viewpoint is that a critical window exists during early calf-hood development in heifers during which sensitivity to dietary quality and quantity is heightened [8, 13-15]. As a result, dietary manipulations during this period can have a profound influence on the timing of puberty. Specifically, it is believed that increasing dietary energy intake hastens morphological and physiological changes in the hypothalamus, and allows neuroendocrine events imperative to attainment of puberty to occur at a younger age. Conversely, restrictions to dietary energy intake delay the onset

of puberty [9, 10] and it is thought to delay the morphological and physiological events in the hypothalamus necessary for puberty to occur.

Ruminant and monogastric species derive dietary energy from ingested feedstuff via different mechanisms. All mammalian species require adequate blood glucose concentrations for survival. However, unlike monogastric species, ruminants do not depend entirely on glucose absorption in the small intestine to maintain blood concentrations of glucose for normal bodily function, but rather depend primarily on utilization of volatile fatty acids (VFA). Volatile fatty acids are the end-products of anaerobic microbial fermentation, and are utilized by ruminants as the primary source of metabolizable energy [11]. Although acetate is produced in larger quantities in the rumen, it does not exhibit the gluconeogenic properties of propionate; thus in ruminants, propionate is the primary substrate utilized in gluconeogenesis [11, 12]. It is well documented [8, 13-16] that a combination of early-weaning and the feeding of high-starch, propionate-favoring diets to pre-pubertal heifers results in a high incidence of precocious puberty. In ruminants, an increased starch content of the diet would be expected to increase the ruminal propionate to acetate ratio, and to provide a greater amount of energy to the animal for immediate use, as well as for accumulation of body

mass during growth and development. It is unknown if the reduction in age at puberty in heifers fed high-starch diets is due to the gluconeogenic nature of the diet, or if the same effect can be created with a high-forage, acetate favoring diet that promotes a similar rate of body weight gain.

The objectives of experiments described herein were to 1) examine gene expression within the ARC of the hypothalamus in pre-pubertal heifers fed diets previously shown to hasten or delay maturation of the reproductive neuroendocrine axis, and 2) to determine if propionate-favoring highly gluconeogenic diets are required for nutritional induction of precocious puberty.

CHAPTER II

LITERATURE REVIEW

Puberty in Heifers

Puberty is the consequence of a series of morphological and physiological changes that lead to the maturation of the hypothalamic-hypophyseal complex and reproductive capacity. Fertility in beef heifers progressively increases from the first to third ovulation [2], so it is important to initiate reproductive cyclicity well in advance of the first breeding season in order to increase the probability of conception early in that breeding season. This in turn maximizes lifetime productivity [2, 3]. The age at which heifers reach puberty can be affected by a variety of factors including body weight [17], age [18], subspecies [19], pre-weaning body weight gain [20], post-weaning body weight gain [21, 22], dietary restriction [23-25], and dietary intake [8, 13-16, 25].

In order for puberty to occur, a disinhibition of the hypothalamic-hypophyseal complex through a decline in sensitivity to estradiol negative feedback must be realized. This results in increased secretion of GnRH, LH and follicle stimulating hormone (FSH), which will in turn stimulate the gonads and the initial ovulation. Pre-pubertal heifers contain adequate stores of LH and FSH in the anterior pituitary gland to stimulate follicular growth and ovulation. In response to stimulation of gonadotropes by repeated injections of GnRH, LH was released in a fashion similar to that observed directly before puberty [26]. Thus, adequate hypothalamic stimulation of the adenohypophysis to allow synthesis of the gonadotropins necessary for reproductive function is present well before

puberty. However, a lack of high-frequency pulses of GnRH appears to be the limiting factor for pubertal development [1]. During growth and development of prepubertal heifers, circulating concentrations of LH increase from birth until 3 mo of age, after which they decline until 6 mo of age [27]. Heifers exhibit a linear increase in concentrations of LH during the 5 mo period preceding puberty [1]. Frequency of LH pulses increases with a concomitant decline in pulse amplitude during the month before puberty [1, 27]. As noted earlier, as heifers progress towards puberty, sensitivity of the hypothalamus to estradiol negative feedback declines. Research in sheep indicates that there is an increase in the frequency of GnRH pulses. This promotes an increase in secretion of LH and FSH, and in follicular growth, which in turn stimulates increased secretion of estradiol. A peak in circulating estradiol ultimately stimulates a preovulatory surge of LH which results in ovulation [1]. It is unclear what causes the decline in sensitivity to estradiol; however, Day and co-workers have demonstrated a decline in the number of cytosolic estrogen receptors (ER) in the anterior hypothalamus, MBH and anterior pituitary gland during sexual maturation in heifers. The number of ER in the POA and ME did not change during the same period of time [28]. Initially, it was thought that since GnRH neurons do not express estrogen receptor-alpha (ERα) [29], estradiol could mediate GnRH secretion indirectly; however, studies in mice [30], rats [29, 31, 32], sheep [33] and humans [34] have demonstrated the presence of estrogen receptor-beta (ERβ) in a subpopulation of GnRH neurons. In cattle, GnRH perikarya are located primarily in the medial and lateral pre-optic areas [35], which according to the experiments by Day et al., did not exhibit a change in ER number during the pre-pubertal

period [28]. Collectively, these findings suggest that, although a decline in ER receptor number may be involved with the pre-pubertal decline in estradiol negative feedback, estradiol directly affects the GnRH neuronal system.

Influence of Nutrition on Age at Puberty in Ruminants

Appropriate nutritional balance is a prerequisite for allowing juvenile animals to proceed through pubertal development and to insure continuing reproductive capacity in adulthood. In cattle, the onset of puberty can be hastened or delayed by modifying dietary energy intake [8, 13-16]. In particular, energy balance has been linked to sexual maturation of the hypothalamus through timing of a developmental decline in estradiol negative feedback [15]. It is well documented that both heifers and ewes subjected to restrictions in dietary energy intake reach puberty at an older age than those fed diets containing adequate energy content [9, 10]. Relatively low circulating concentrations of LH, a decreased frequency of LH pulses, and decreased responsiveness to GnRH have been demonstrated in heifers subjected to restrictions in dietary energy intake [36]. This phenomenon has also been demonstrated in sheep. The frequency of GnRH pulses is reduced in growth-retarded lambs [37]; however, administration of hourly injections of the neurotransmitter N-Methyl-D, L-Aspartate (NMA), a GnRH secretagogue, results in the anterior pituitary gland secreting pulses of LH that are similar in size to those stimulated by physiological pulses of GnRH [38]. Additionally, adequate amounts of LH and FSH are present in the anterior pituitary of growth-retarded lambs as well as prepubertal heifers, and physiological doses of GnRH stimulate the release of

gonadotropins [39, 40]. These findings suggest that gonadotropin secretion is reduced, not because of a decrease in anterior pituitary function, but due to reduced secretion of GnRH. This also indicates that animals must be in an appropriate energy balance for puberty to occur. If a decrease in dietary energy intake delays the onset of puberty, it would be logical to speculate that an increase in dietary energy intake would hasten the onset of puberty. Increasing energy intake has been shown to decrease the age at which puberty occurs in heifers. Heifers weaned at 7 mo of age and fed a high starch diet starting at 60 d before onset of the breeding season reached puberty at slightly younger ages than heifers fed a high starch diet starting 30 d before the breeding season [16]. This implies that increasing energy balance in heifers earlier in life is more effective at stimulating the onset of puberty than later in life. Heifers weaned early, and subsequently fed a high concentrate diet until puberty, had a high incidence of precocious puberty, while early-weaned heifers fed a low-energy diet did not exhibit precocious puberty. Additionally, heifers weaned at 3 mo of age and fed a high concentrate diet from 126-196 d of age to gain body weight at a high rate, or weaned at 3 mo of age and fed a high concentrate diet from 126 d of age until puberty was reached, had a high incidence of precocious puberty. Interestingly, heifers fed a low concentrate diet from 126 d of age and then switched to a high concentrate diet on day 196 had a low incidence of precocious puberty. This indicates that both rate of gain and timing of the gain are important factors in stimulating the earlier onset of puberty, and suggests that neuroendocrine mechanisms modified by dietary energy intake are occurring before 6 mo of age [8]. Precocious puberty induced by earlier weaning and feeding of a high-

concentrate diet is associated with an enhanced secretion of LH, similar to that observed in older heifers developed on normal-growth diets. This indicates that mechanisms underlying precocious puberty are comparable to those described in heifers that reach puberty at a more conventional age but are functioning at a much earlier age [13-15].

Influence of Nutrition on Follicular Growth

Heifers weaned early and fed a high concentrate diet also demonstrated increased follicular development [14]. Follicle stimulating hormone and LH secreted from the anterior pituitary gland are the primary gonadotropins associated with follicular growth [41] and act on the granulosa and theca cells, respectively, of the growing follicle to stimulate folliculogenesis and steroid hormone production. Estradiol synthesis requires an androgen precursor; however, granulosa cells lack the ability to convert cholesterol to androgens. Theca cells convert cholesterol to androgens under the influence of LH, and granulosa cells aromatize androgens to estradiol via FSH-mediated aromatase activity [42]. Ovarian follicles have been observed in heifers as young as 2 wk of age, but they lack the ability to spontaneously ovulate and form corpora lutea. The number of small and medium follicles increases steadily from 2 to 14 wk of age, then remains static until heifers reach approximately 8 mo of age. The number of large follicles has been observed to increase steadily from 2 wk of age until approximately 8 mo of age. Neither growth nor regression rate of follicles changed as heifers matured [43]. In prepubertal heifers, there is a rapid increase in follicular growth during the first mo after onset of a high energy diet [44] and dominant follicle diameter was larger in animals with greater

dietary energy intake than heifers of the same age with a lesser intake of dietary energy [45]. Heifers weaned early and fed a high-concentrate diet exhibited an increase in maximum follicle diameter compared to control-fed heifers [14]. Heifers exhibiting precocious puberty had greater dominant follicle diameters and longer follicular waves then heifers that did not reach precocious puberty. Although dominant follicle diameter was increased at a younger age in heifers that reached precocious puberty, the number of follicles was decreased [15]. This relates to the observation of an increasing number of follicles between 2 wk of age to approximately 4-6 mo, followed by a decrease in number until puberty [14, 46]. In the foregoing experiments, serum estradiol did not differ at 126 d of age between heifers that eventually did or did not exhibit precocious puberty. However, serum estradiol concentrations started to increase earlier in heifers reaching precocious puberty [15], and this was related to the coincident increase in the frequency of LH pulses [14].

The Arcuate Nucleus

The hypothalamus is essential for regulation of appetite and energy balance, as well as being the central modulator of reproductive function. Within the hypothalamus, there are vast numbers of neuronal populations located in various nuclei that function alone as well as in a synergistic manner to integrate local and peripheral signals. In particular, the ARC plays a vital role in integration of metabolic and neuroendocrine signaling pathways. The two primary neuronal populations within the ARC that respond directly to metabolic cues result in opposite responses. A subpopulation of neurons in the ARC co-express *NPY* and *AGRP*, both of which are orexigenic neurotransmitters that

respond during times of low energy balance and stimulate appetite and feeding behavior [47, 48]. The second subpopulation of neurons in the ARC co-express *POMC* and *CART*, both of which are anorectic compounds that respond to signals of excess energy by suppressing appetite and feeding behaviors. The ARC is conveniently located close to the ME, which has a compromised blood brain barrier and allows for the hypothalamus to respond to various hormones in the peripheral circulation such as leptin and insulin. Both NPY/AGRP and POMC/CART neurons respond to leptin; however, they do so in opposing directions. Although the ARC responds primarily to metabolic signaling, the neurons project outward to exert stimulatory or inhibitory effects on various other nuclei of the hypothalamus to maintain energy homeostasis, as well as to modulate reproductive function. For example, NPY/AGRP neurons project from the ARC to GnRH perikarya and fibers in the POA and the ME [5] and in response to peripheral and local metabolic signaling, mediate release of GnRH [49]. There is thought to be a critical window of growth and development during early calf-hood that predetermines the age at which heifers become pubertal. It is believed that modifying calf-hood nutrition during that critical period will positively or negatively influence the age at which puberty is reached [8]. It is also thought that the ARC will respond to modifications in nutrition during the proposed "critical window" and aid in hastening maturation of the reproductive axis.

Roles of Signaling Peptides in Pubertal Development and Energy Homeostasis

Onset of puberty and energy homeostasis are influenced by various metabolic hormones, growth factors, neuropeptides, and neurotransmitters including leptin, insulin, insulin-like growth factor-1 (IGF-1), NPY, AGRP, POMC, and kisspeptin.

Leptin

Leptin is a potent satiety hormone synthesized from the *LEP* gene, reflects body-fat mass, influences energy intake and expenditure, and integrates metabolic signaling with reproductive function. Leptin is secreted primarily by white adipocytes; however, it has also been found to be secreted from the various other cell types in the placenta [50], stomach [51], and pituitary [52]. The leptin receptor (LEPR) is structurally similar to other cytokine receptors [53, 54] and functions via janus-kinase (JAK)/STAT (signal transducers and activators of transcription), mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3K) pathway activation [55, 57]. Due to alternative splicing of the leptin receptor gene (*LEPR*), there are at least 5 different isoforms of the leptin receptor including one long form, and several short forms [58, 59]. The extracellular and transmembrane domains are identical; however, the number of cytoplasmic amino acid residues differs between isoforms [58]. The long form of the receptor is expressed in high abundance within the hypothalamus, specifically in the ventromedial hypothalamus (VMH) [60-62] and the ARC [61, 62]. In the VMH, leptin receptor is differentially expressed depending on energy state. Dyer and co-workers found that in the VMH and ARC, leptin receptor is expressed in greater abundance in ovariectomized adult ewes subjected to feed restriction than in well-fed ovariectomized

adult ewes [61]. Similar results were found in feed-restricted wethers compared to normally-fed wethers; however, differences in leptin receptor gene expression were observed only in the ARC and not the VMH [63]. The long form of the leptin receptor is also expressed in the lung, kidney, liver, adipose tissue, and pancreatic β-cells [59, 64].

Within the hypothalamus, leptin plays an important role in the integration of metabolic signaling and reproductive function. Leptin appears to communicate nutritional status to specific regions of the hypothalamus including whether energy balance is sufficient for initiation/continuation of reproductive function via interactions with various neuronal populations. Furthermore, leptin is necessary for maintaining normal body weight. Individuals exhibiting homozygous mutations of the leptin gene are obese and lack the ability to reproduce [65, 66]. In mice with mutations for *Lep* (*ob/ob*), leptin is absent and results in obesity [65] and impaired reproductive function in both males and females [66]. Although male *ob/ob* mice are able to gain reproductive function by reducing body weight through energy restriction [67], females lack the ability to do so. Interestingly, sterility is reversed in female *ob/ob* mice treated with leptin, and ovulation, pregnancy and parturition are able to occur [66] through activation of the hypothalamic-hypophyseal axis [68]. Since treatment with leptin allows for normal reproductive function to occur, it was thought that perhaps leptin plays an imperative role in the initiation of puberty through maturation of the hypothalamic-hypophyseal complex. Although prepubertal heifers exhibit a steady linear increase in serum leptin as puberty approaches [69], leptin alone does not accelerate the maturation

of the hypothalamic-hypophyseal complex in heifers [70] or rats [71]. However, leptin is believed to have a permissive effect on the initiation of puberty.

Neuroendocrine responses to leptin differ depending on nutritional balance and reproductive status. During the peripubertal period, heifers are highly sensitive to short-term energy restriction as reflected by decreased frequency of LH pulses, and decreased concentrations of insulin and IGF-1 [72]. However, subcutaneous treatment with leptin prevents fasting-induced reductions in frequency of LH pulses, not only in peripubertal heifers [73], but also in rats [74] and wethers [75]. In contrast, LH pulse frequency is not affected by short-term fasting in mature cows. Short-term fasting causes a decrease in mean concentrations of leptin and leptin mRNA in mature cattle [76]; however, treatment with leptin stimulates an increase in GnRH secretion into third ventricle cerebral spinal fluid [70], and increases mean concentrations and pulse amplitude of LH in cattle [76] and sheep [75]. Treatment with exogenous leptin does not elicit the same effect in full fed sheep and cattle. In fact, central infusion of leptin had no effect on mean concentration, or the frequency or amplitude of pulses of LH in well-nourished sheep [75, 77] or cattle [76].

Neuropeptide Y and leptin receptor mRNAs are co-expressed in neurons within the ARC [78], suggesting that leptin directly regulates NPY. In fact, it appears that leptin negatively regulates NPY [79]. In diabetic rats, NPY expression within the ARC was increased; however, once insulin concentrations were corrected, concentrations of leptin increased rapidly, NPY expression was greatly reduced, and reproductive function resumed. Additionally, NPY mRNA expression is increased in fasted rats, but is

reduced following treatment with leptin. Interestingly, NPY mRNA expression in the ARC is also reduced when normally fed rats are treated with leptin [71]. These findings are interpreted to indicate that leptin has a direct inhibitory effect on NPY neurons, and that suppression of NPY allows for release of GnRH and subsequent secretion of LH.

Collectively, it appears that although leptin does not act as a trigger for the onset of puberty, its presence is required and acts passively during sexual development to activate and maintain function of the reproductive neuroendocrine axis, possibly through a reduction of NPY inhibitory tone on GnRH neurons.

Insulin-like Growth Factor-1

Insulin-like growth factor-1 is a 70 amino acid, single-chain peptide that is linked to somatic growth, glucose metabolism, and survival, proliferation and differentiation of a variety of cell types [80]. In prepubertal heifers, concentrations of serum IGF-1 vary greatly depending on breed, diet, and rate of gain. Crossbred *Bos taurus* heifers fed a diet formulated to promote a high rate of gain exhibited greater serum concentrations of IGF-1 in the 10 weeks prior to puberty than heifers fed a diet formulated to promote a low rate of gain. As the low gain heifers approached puberty, serum IGF-1increased to concentrations similar to high gain heifers which could be attributed to the increase in nutrient intake during the 4 weeks prior to initiation of puberty [25]. Crossbred *Bos indicus*-influenced heifers fed a high concentrate diet had greater serum concentrations of IGF-1 than heifers fed a high fat diet; however both groups demonstrated a decrease in concentrations as puberty approached [81]. Prepubertal bulls fed a diet designed to yield a moderate rate of gain exhibited an

increase in IGF-1 and a decrease in IGF-1 binding protein-2 (IGFBP-2) as they approached puberty. In contrast, bulls fed a restricted-energy diet exhibited a delayed rise in IGF-1 and an increased concentration of IGFBP-2 [82]. These findings agree with previous research in prepubertal heifers [25, 81], young steers [83], and young bulls [84], and indicate that IGF-1 is influenced by nutrient intake. Secretion of GnRH from isolated ME of the hypothalamus is induced by exposure to IGF-1 [85]. In studies conducted in juvenile rats, third ventricle injections of IGF-1 were capable of stimulating a release of LH, while third ventricle administration of GnRH antiserum plus IGF-1 blocked the pituitary response to IGF-1 [86]. It is believed that IGF-1 does not act directly on GnRH neurons [87], but acts via glial intermediate pathways since glial cells in the ME contain IGF-1 receptor (IGF-1R) mRNA [88, 89].

Neuropeptide Y

Neuropeptide Y is a 36-amino acid orexigenic neurotransmitter involved in food intake, thermoregulation, and reproductive function [90, 91]. Although widely distributed throughout the peripheral and central nervous system (CNS), NPY is expressed in the greatest abundance in the hypothalamus, particularly in the ARC. Since NPY neurons project from the ARC to the ME where they are in close proximity to GnRH fibers [5] as well as with GnRH neurons in the POA [6] it appears that NPY has a dual regulatory effect on GnRH neurons in response to local and peripheral metabolic signaling. The effects of NPY are mediated by six receptor subtypes and are denoted Y_1-Y_5 and y_6 [92-94]. The Y receptor subtypes belong to the G protein-coupled receptor superfamily and inhibit adenylyl cyclase [95, 96]. Subtypes Y_1, Y_2 and Y_5 preferentially

bind NPY and peptide YY (PYY) [92]; however, Y_1 and Y_5 appear to be largely involved with metabolic and reproductive functions. The Y_1 subtype is highly conserved between *Xenopus laevis* and mammals [92], and has been localized in the brain, heart, kidney and gastrointestinal tract of humans, rats and mice [97-99]. It appears that the Y_1 receptor is a key regulator of GnRH release and feeding behavior. Intact female pre-pubertal rats exhibited a significant increase in release of LH when the Y_1 receptor antagonist was administered intracerebroventricular (icv) [93]. Castrated male rhesus monkeys also exhibited an immediate release of LH following Y_1 receptor blockade [100]. Gonzales and co-workers [101] studied NPY inhibition of the reproductive axis during poor nutritional balance, and in particular the involvement of the Y_1 receptor. They utilized both male and female Y_1 knock-out ($Y_1^{-/-}$) mice fed either restricted or ad libitum diets, and examined food intake, body-weight gain, and the onset of puberty. They found that $Y_1^{-/-}$ mice were lighter than wild-type ($Y_1^{+/+}$) mice at weaning. There were no differences in age at puberty between full-fed Y_1 wild-type and $Y_1^{-/-}$ mice; in contrast, onset of puberty was significantly delayed in feed-restricted $Y_1^{+/+}$ mice as compared to feed-restricted $Y_1^{-/-}$. Circulating concentrations of LH was also greatly reduced in feed-restricted Y_1 wild-type compared to full-fed Y_1 wild-type mice. These results were interpreted to indicate that the NPY Y_1 receptor is vital in sensing metabolic conditions by the hypothalamic-hypophyseal axis, and that Y_1 is a key mediator of the effects of NPY on reproductive function [101]. As mentioned previously, the Y_1 receptor subtype is not the only mediator of NPY effects on reproductive function. The Y_5 receptor subtype also appears to integrate NPY signaling and reproductive function.

In fact, the Y_5 receptor subtype has been found on GnRH neurons in the POA [6] and it appears to not only mediate NPY-stimulated feeding behavior, but also modulates release of GnRH [49].

Hypothalamic NPY is responsive to metabolic hormones, such as leptin and insulin. This is reflected during changes in metabolic state by increases in NPY mRNA expression and peptide release during fasting or undernourishment, and by decreases after feeding or during positive energy balance [7]. Within the ARC, mRNAs for NPY and leptin receptor (LEPR) are co-expressed [78, 102]. Additionally, concentrations of NPY, NPY mRNA [103] and feed intake were reduced [71] with leptin treatment. This indicates that leptin has a direct regulatory effect on NPY. Insulin undoubtedly regulates NPY as well but, since it is unclear if NPY neurons express insulin receptor (INSR), it is possible that this regulation occurs through an indirect pathway. Cultured murine hypothalamic tissue sections treated with estradiol for 24 h displayed a significant decrease in both NPY and AGRP mRNA expression, indicating that estradiol exerts an inhibitory effect on NPY/AGRP neurons. However, since NPY/AGRP neurons in the mouse do not express estrogen receptor-α (ERα), it is possible that estradiol exerts its inhibitory effects indirectly [79].

It is evident that reproductive function is directly correlated with metabolic state since sexual maturation is delayed [104] and reproductive function is stalled when metabolic conditions are not adequate. Hypothalamic NPY is important, not only for its involvement in regulation of food intake and appetite in response to energy balance, but also for integration of metabolic and reproductive signaling and control of the

reproductive neuroendocrine axis. Hypothalamic NPY has been reported to have both stimulatory and inhibitory effects on reproductive function depending on species, physiological state, duration of exposure (e.g. chronic or acute exposure), and steroid hormone environment.

Chronic infusion of NPY into the lateral ventricle of the brain disrupts reproductive cyclicity in intact adult female rats, and compromises reproductive ability in intact male rats [91, 105]. Intact juvenile female rats, chronically exposed to NPY exhibit delayed sexual maturation [105].

In contrast, a single dose of exogenous NPY administered icv to pre-pubertal female rats stimulated precocious puberty [106]. This could be via a mechanism similar to that observed in earlier studies that reported a simultaneous surge of GnRH/NPY the day prior to vaginal opening in intact juvenile female rats [107]. Neuropeptide Y expression patterns within the hypothalamus and POA in intact growing female rats mimicked the expression patterns of GnRH in the same areas [107].

Release of GnRH and LH was inhibited or reduced in ovariectomized (OVX) ewe lambs [108, 109], rhesus monkeys [110], and rabbits [111] treated icv with exogenous NPY which indicates an inhibitory effect of NPY in the absence of gonadal steroids. In contrast, estradiol treated OVX rats and rabbits exhibited a transient increase in release of GnRH and LH when administered exogenous NPY icv [112, 113]. However, these observations were restricted to rats and rabbits, since estradiol-treated OVX rhesus monkeys did not display any change in mean concentration, pulse frequency, or pulse amplitude of LH when treated with NPY [114], Moreover, OVX ewe

lambs treated with estradiol exhibited a reduction in release of GnRH and LH following icv administration of NPY [108]. In cattle, NPY appears to play an inhibitory role in both OVX and OVX estradiol-treated cows as both exhibited a reduction in mean concentrations of LH following icv administration of exogenous NPY [115, 116]. Neuropeptide Y expression patterns have not yet been characterized in prepubertal heifers.

Agouti-related Protein and the Hypothalamic Melanocortin System

Agouti-related protein and POMC, in concert with NPY, regulate energy homeostasis through intricate signaling mechanisms in response to metabolic hormones (e.g. insulin) and various signals from adipose tissue (e.g. leptin) and the gastrointestinal tract (e.g. cholecystokinin) [117]. Agouti-related protein is a small orexigenic peptide found primarily in the hypothalamus and adrenal gland [118, 119]; however, it is found less abundantly in the subthalamic nucleus, testis and lung [118]. Within the hypothalamus, *AGRP* expression is restricted to the ARC and the inner palisade zone of the ME. In humans there appears to be several splice variants of *AGRP*; the larger transcript is localized in the hypothalamus, while the smaller transcript is expressed in the peripheral tissues [118]. Hypothalamic POMC is a large anorexigenic polypeptide precursor synthesized primarily in the ARC [35] and is involved in regulation of energy homeostasis [119]. Although the mature form of POMC is biologically inactive, during posttranslational processing it is cleaved into several biologically active peptides including adrenocorticotropic hormone (ACTH), α- β- and γ-melanocortin-stimulating hormones (α-MSH, β-MSH and γ-MSH, respectively), and the endogenous opioid β-

endorphin. Tung and co-workers conducted elaborate studies with *Pomc* knock-out mice (*Pomc*$^{-/-}$) to determine the effects of melanocortins on food intake and body weight. By icv administration of α-, β-, γ-MSH to *Pomc*$^{-/-}$ mice and subsequent evaluation of food intake, body weight gain, and fat and lean body mass, it was determined that α-MSH is the primary POMC derivative involved in food intake and energy homeostasis [121]. These findings are in agreement with previous research demonstrating significant weight loss in *Pomc*-null mice subjected to treatment with a stable α-MSH agonist [119]. Melanocortins mediate their effects via 7-transmembrane domain G-coupled protein receptors located on various neurons in the hypothalamus. Although five different subtypes of the melanocortin receptors exist (MC-R), only melanocortin 3 receptor (MC3R) and melanocortin 4 receptor (MC4R) are expressed in the hypothalamus, both which bind α-MSH [122]. Interestingly, AGRP binds to the MC-R with high affinity and blocks the actions of α-MSH [119].

During times of adequate energy balance, α-MSH is released from POMC neurons and subsequently binds to the MC4R on secondary neuronal populations, primarily in the paraventricular nucleus (PVN). Activation of the MC4R leads to decreased food intake and increased use of energy [123]. Conversely, AGRP selectively antagonizes MC3R and MC4R [119] and blocks the anorexigenic effects of α-MSH. Concurrent with the antagonistic actions of AGRP on MC3R and MC4R, NPY exerts orexigenic effects via the aforementioned Y receptors on various secondary neuronal populations. Collectively, in an effort to increase energy balance, the combined actions of AGRP and NPY lead to an increase in appetite. Neurons expressing AGRP/NPY and

those expressing POMC/CART mediate energy homeostasis by responding to a plethora of metabolic signals. They are regulated primarily by leptin and insulin; however, they respond to glucose, fatty acids, and various other nutrients as well. Leptin signaling from adipose tissue is reflective of energy stores and inhibits AGRP neurons, while stimulating POMC neurons. Activation of the LEPR triggers the JAK/STAT second messenger signaling pathway and subsequent phosphorylation, activation and nuclear translocation of the signal transducer and activator of transcription 3 (STAT3) in both neuronal populations. Following nuclear translocation, STAT3 binds to the promoter region of both genes. However, binding to *POMC* causes recruitment of histone acetylases, increases transcription and subsequent protein synthesis, while binding to *AGRP* causes recruitment of histone deacetylases and decreases mRNA and protein synthesis [123]. This transcriptional regulation of *POMC* and *AGRP* by leptin is imperative for attaining and maintaining adequate energy stores. Additionally, both leptin and insulin mediate cellular function in AGRP and POMC neurons via the PI3K signaling cascade, and ATP-dependent potassium channels [123].

As previously mentioned, the majority of AGRP neurons co-express NPY and project from the ARC to the PVN and POA [47]. Studies in OVX rats [124] and rhesus monkeys [125] suggest an inhibitory role of AGRP on secretion of GnRH, and subsequent gonadotropin secretion. Schioth and co-workers demonstrated in OVX steroid-primed rats that central administration of human AGRP completely abolished the surge of LH [124]. Furthermore, continuous icv administration of human AGRP to OVX adult rhesus monkeys results in suppression of LH pulses, and decreased

concentrations of LH [125]. Conversely, central administration of AGRP to intact male rats causes an increase in GnRH and gonadotropin secretion, suggesting that the actions of AGRP at the level of the hypothalamus are sexually dimorphic [126]. Additionally, AGRP had no effect on gonadotropin secretion in cultured pituitary cells. This suggests that AGRP regulates gonadotropin secretion via mediation of GnRH neurons [126]. However, although MC4R is expressed in the ARC, PVN, medial POA [127] and immortalized hypothalamic GT1-1 cells [128], it is unclear if melanocortin receptors are present on GnRH neurons. Clearly, AGRP is vital in regulating GnRH secretion; however, it is unclear if AGRP directly or indirectly regulates GnRH neurons.

Kisspeptin

Recent studies have suggested a role for the neuropeptide, kisspeptin in stimulating GnRH/LH release [129-131]. Kisspeptins are a neuropeptide family derived from the *KISS1* gene. The initial product of *KISS1* is a 145-amino-acid peptide, which is cleaved into a 54-amino-acid peptide commonly known as kisspeptin-54. Three shorter peptides, kisspeptin-14, kisspeptin-13 [132] and kisspeptin-10 [133] have also been identified; all three are terminated by a common RF-amide motif and have a C-terminus identical to kisspeptin-54 that is involved mainly in ligand/receptor binding [132-134].

Kiss1 is expressed in the placenta, testes, pancreas, liver, small intestine [134] and the brain [135-138]. *Kiss1* mRNA has been localized to the ARC of the hypothalamus, an area of the hypothalamus important for regulation of GnRH secretion in mice [135, 139], rats [136, 140], primates [130, 137], hamsters [141], and female sheep [138, 142]. It is also expressed in the anteroventral periventricular nucleus

(AVPV) in mice and rats [135, 139-141], the periventricular nucleus (PeN) [139] and dorsomedial hypothalamus (DMH) in mice [143], and the POA and DMH in sheep [138, 142, 144]. *Kiss1* appears to be regulated primarily by gonadal steroids [136, 138, 139]. In the absence of estradiol, *Kiss1* mRNA in the ARC was greatly increased in ewes [138], female non-human primates and the human female [137]. In the presence of endogenous estradiol or estradiol replacement, abundance of *Kiss1* mRNA in the ARC was decreased [137, 138]. However, estradiol exerts both positive and negative effects on Kiss1 neurons in rodents. In the absence of estradiol due to ovariectomy, mice exhibit greater expression of *Kiss1* mRNA in the ARC but decreased expression in the AVPV. Estradiol replacement caused *Kiss1* mRNA in the ARC to decrease, and *Kiss1* mRNA in the AVPV to increase [139]. This indicates a dual role for regulation by estradiol in the rodent brain. Although many *Kiss1* neurons express both ERα and ERβ [139, 140, 144], based on elaborate studies using ERα and ERβ knock-out mice, it is apparent that estradiol mediates its effects on *Kiss1* neurons via ERα [139]. It also appears that *Kiss1* is regulated by progesterone, but not the extent of regulation by estradiol [138].

Evidence indicates that kisspeptin directly regulates secretion of GnRH [129, 131, 135, 136]. Following ICV administration of kisspeptin, a subsequent release of LH has been detected in rats [136], mice, ewes [129] and primates [131]. Administration of a highly-specific GnRH antagonist prior to kisspeptin treatment abolished the aforementioned LH response [135]. This confirms that kisspeptin mediates the release of LH from the adenohypophysis by stimulating release of GnRH from the

hypothalamus. The effects of kisspeptin are mediated by the kisspeptin receptor, GPR54 [129], which is a G protein-coupled receptor similar to the galanin receptor [145]. It is highly conserved between rats and humans, differing greatly only in the C and N termini, and the third extracellular loop of the C-terminal intra-cellular domain [132]. Messenger RNA for GPR54 is expressed in great abundance in a variety of organs and tissues including the pituitary, placenta, spinal cord, pancreas [132, 134] and hypothalamus [129, 136, 145]. The mRNA for GPR54 is found in lesser abundance in small intestine, thymus, spleen, lung, testis, kidney and liver [132]. Within the hypothalamus, GPR54 mRNA is expressed in GnRH neurons in the rat [136], rhesus monkey [146], and mouse [129].

Recently, it has become apparent that kisspeptin/GPR54 signaling must be functional in order for normal sexual maturation to occur. In normally-developing female primates, mRNA for both Kiss1 and GPR54 increase progressively from the juvenile to the pubertal periods [131]. Furthermore, humans [130, 147] and mice [130, 148] with homozygous mutations in GPR54 did not attain normal puberty, displayed hypogonadotropic hypogonadism, and exhibited low concentrations of gonadotropins and gonadal steroids. Interestingly in GPR54 knock-out ($GPR54^{-/-}$) mice, adequate amounts of GnRH, LH and FSH were present, and injection of GnRH stimulated release of both LH and FSH from the anterior pituitary [130]. This indicates that normal anterior pituitary function is present, and that GPR54 is required to initiate and maintain normal sexual maturation at puberty. However, all mutations in the GPR54 gene do not result in obliteration of sexual development. In humans, a single point-mutation in the

GPR54 gene sequence can cause central (gonadotropin-dependent) precocious puberty [149]. Additionally, there is evidence that GPR54 expression is affected by nutritional restriction [150].

Microarray Technology and Its Use in Neuroendocrine Research

Microarray technology has been utilized to examine the expression patterns of thousands of genes in various tissues and cells from a multitude of mammalian species. Many microarray platforms are somewhat costly and require special expertise to utilize; however, the ability to efficiently assess the expression profiles of a large number of genes in one assay has had a profound impact on neuroendocrine research. This has been particularly true for identification of hormonally-regulated genes expressed in the hypothalamus and pituitary gland that respond to changes in various physiological conditions [151, 152]. To maximize success with microarrays, it is essential that tissues or cells are properly harvested, and that RNA samples are appropriately extracted, are of high-quality, and are collected in enough quantity to detect changes in expression patterns between treatment groups. The method by which tissue is collected prior to RNA extraction is usually determined by the type of tissue in question. Homogeneous tissue is easily collected; however, many neuroendocrine tissues consist of highly heterogeneous cell populations. In particular, it is difficult to harvest tissue from specific regions of the hypothalamus (e.g ARC), or to harvest a specific cell type (e.g. GnRH neuron) without contamination from adjacent regions (e.g. VMH) or surrounding cells [153]. In recent years, use of laser capture micro-dissection (LCM) technology has alleviated that problem since it allows for dissection of specific areas of tissue or specific

cell types. In particular, LCM has been utilized in studies designed to identify and examine gene profiles in specific hypothalamic regions in the rat [154, 155], hamster [156], and mouse [157]. Although this is an exceptional tool, it is not a suitable option for all experiments since many LCM protocols require tissue to be sectioned at a thickness of no more than 10 μm (Ambion LCM staining protocol). In cattle, many hypothalamic cell types exceed 10 μm and sectioning tissue at that thickness will result in fragmentation of the cellular membrane and subsequent loss of cell content. Another method for extracting tissue is to collect a punch sample; however, in some species it is difficult to validate the exact location the sample was taken from. In addition, this approach is not optimal for experiments that require precise isolation of specific cell types or tissues, and it is apparent that other methods must be developed to successfully harvest uncontaminated tissue and cells from specific regions of the bovine hypothalamus. In addition to appropriate tissue extraction, microarray technology is optimized with high-quality RNA samples. To maximize RNA quality, selection of an appropriate extraction method is a critical step. The method used for RNA extraction depends on the type of sample (i.e. cells or tissue) and how much sample is available. Total cellular RNA is often isolated by using a methodology that incorporates a reagent containing phenol and guanidine isothiocyanate which work to maintain the integrity of the RNA, while breaking down cell walls to release cellular content. This method is widely used; however, it may not be optimal for all experiments, especially in those lacking a large quantity of sample tissue. Several commercially-available RNA isolation kits have been used to prepare samples prior to microarray analysis. A variety of studies

have been conducted to examine the commercially-available RNA extraction kit that yields the largest amount and highest quality RNA from both whole tissue and cells. In studies involving bovine blood and whole tissue to compare total yield and quality of RNA between several extraction kits, it was found that although the TRIzol (Invitrogen) extraction kit produced the greatest yield of RNA, the RNAqueous kit (Ambion) produced RNA of the greatest quality [158]. Similar conclusions were deduced from studies utilizing cultured cells; however, it was found that although the TRIzol method produced the greatest yield, samples were contaminated by proteins and genomic DNA [159]. The presence of both proteins and genomic DNA are unfavorable in RNA samples that are to be used in microarray experiments. Following extraction, RNA integrity must be confirmed. A simple method to determine RNA quality is to obtain 260/280 absorbance ratios from a spectrophotometer which indicates possible RNA degradation and chemical or genomic DNA contamination. However, for use in highly sensitive microarray experiments, it is more favorable to measure RNA quality using a chip-based assay such as the Bioanalyser 2100 (Agilent Technologies, Inc., Palo Alto, CA, USA). This assay has been utilized extensively to confirm RNA integrity prior to microarray analysis [159, 160]. Finally, the type of microarray must be determined. Usually microarray samples are labeled with two fluorophores, however, up to four can be used. One drawback of utilizing two and four color arrays is that fluorophore uptake by the samples is not equal, and differences in fluorophore intensity during analysis must be taken into consideration. A simple solution is to use a dye-swap array design. In a dye-swap array, an equal amount of sample is labeled with each color and hybridized

accordingly so that any "dye bias" is compensated for. Currently there are no previous reports of utilizing a dye-swap technique to examine differential gene expression within the ARC of the hypothalamus in cattle, although a bovine oligo microarray is commercially available (Agilent Technologies, Santa Clara, CA).

CHAPTER III

GENE EXPRESSION IN THE ARCUATE NUCLEUS OF HEIFERS IS AFFECTED BY CONTROLLED INTAKE OF HIGH- AND LOW-CONCENTRATE DIETS

Introduction

Age at onset of puberty is largely dependent upon rate of growth during the prepubertal period. Nutrient restriction during postnatal development delays puberty [9-10] by inhibiting the release of GnRH [37]. Many factors have been implicated in signaling nutritional status to the control of reproduction, including hormones (insulin [161], IGF-1 [25, 85], GH [162] and leptin [81], and nutrients (glucose [161] and fatty acids [81]). These signals are believed to act on the hypothalamus to control various neuroendocrine functions, including the regulation of GnRH release [86, 70]. Major metabolic-sensing neurons are located within the arcuate nucleus of the hypothalamus and include NPY/AgRP neurons [47, 48]. Both are responsive to changes in nutrient status [7, 102] and appear to play a major role in mediating the effects of nutrition on reproductive function [49]. However, a host of complex interactions involving neuronal and glial networks influence this system [163]. The development of microarray technology and computational methods to analyze data derived from high-throughput approaches provide an opportunity to investigate these complex networks and the pathways that integrate the nutritional regulation of pubertal development.

In maturing heifers, accelerated growth during early calf-hood has a major influence on age at puberty. Heifers weaned at 3 mo of age and fed diets that promote high rates of body weight gain between 3 and 7 mo of age reach puberty much earlier and at lower body weight than heifers fed diets to gain weight at a lower rate during the same period [8, 13-15]. Elevated body weight gain later during calf-hood does not seem to have such impact. Therefore, a critical window for nutritional imprinting of neuroendocrine functions that regulate age at onset of puberty seems to exist early in development. We propose that this imprinting is a result of functional changes in neuronal networks that integrate energy expenditure and reproductive function. In the studies reported herein, early-weaned heifers fed diets that promote markedly differing weight gains during early calf-hood were used to examine the expression of genes that are differentially responsive to nutritional inputs. We investigated changes in gene expression in the arcuate nucleus because this region of the hypothalamus is an area populated by important metabolic-sensing neurons [47, 48], as well as neurons implicated in pubertal development [131].

Materials and Methods

All animal-related procedures used in this study were approved by the Institutional Agricultural Animal Care and Use Committee (IAACUC) of the Texas A&M University System.

Animal Procedures

Twelve Angus-sired heifers (½ Angus, ¼ Hereford, ¼ Brahman) were weaned at approximately 3 mo of age, stratified by date of birth, and assigned randomly to one of two dietary treatments (n = 6/treatment) in two replicates (n=6/replicate): 1) HF/LG [High-Forage/Low-Gain; average daily gain (ADG) of 0.45 kg/d]; 2) HC/HG [High–Concentrate/High-Gain; ADG of 0.91 kg/d]. Diets were balanced using the Large Ruminant Nutrition System for cattle [164]. Targeted ADG was attained by adjustments in dry matter intake (DMI) based on body weight changes determined weekly. Ingredients and diet chemical compositions are presented in Table 1.

Heifers were allocated to pens measuring 25.9 x 9.5 m^2 and fed an acclimation diet for 2 wk post-weaning. During the first week of the adaptation period, heifers in both treatments were fed the HF diet up to a maximum of 2.7 kg/head daily on a pen basis. During the second week of the adaptation period, heifers assigned to the HC/HG group were fed a diet consisting of 50% HF diet and 50% HC diet up to a maximum of 2.7 kg/head daily on a pen basis. Heifers assigned to the HF/LG treatment were fed the HF diet through the second week of the adaptation period. Following the 2-wk adaptation period, heifers were fed 100% of their respective treatment diets for 13 wk. Heifers were weighed once a week for the duration of the experiment.

Table 1. Ingredients and chemical composition of high-forage (HF) and high-concentrate (HC) diets fed to heifers during the study.

Ingredients	HF	HC
Cracked corn, % DM	39.03	50.75
Soybean meal, % DM	--	17.61
Chopped coastal Bermuda grass hay, % DM	3.47	26.95
Dehydrated alfalfa meal pellet, % DM	57.30	3.83
Calcium carbonate, % DM	--	0.86
Calcium monophosphate, % DM	0.20	--
Vitamin A/D/E premix, mg/kg of DM	71	73
Chemical composition		
Metabolizable energy, Mcal/kg	2.32	2.57
Crude protein, % DM	14.3	17.5
Digestible intake protein, % CP	66%	67%

At the completion of the experimental feeding period, heifers were humanely slaughtered after overnight fasting. A block of tissue containing the septum, preoptic area, and hypothalamus was dissected and frozen in liquid nitrogen vapor. Tissue blocks were stored at -80^{o} C until further processing. A rumen fluid sample from each heifer was collected immediately post-mortem and frozen at -20^{o} C for determination of volatile fatty acid profile. A single blood sample was collected and liver weights were obtained at slaughter. Blood samples were placed on ice immediately after collection. Serum was obtained from blood samples by centrifugation and stored at -20^{o} C.

Hormone Assays

Circulating concentrations of insulin, IGF-I and leptin were determined in serum samples collected at the time of slaughter. Concentrations of insulin were determined by a single phase RIA kit (Coat-A-Count; Siemens, Los Angeles, CA) reported previously for bovine serum [165, 166]. However, we used a bovine insulin preparation for standards (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, and 2.5 ng/ml) and references instead of the human insulin standards provided with the kit. Minimum detectable concentrations were 0.1 ng/ml and intra- and inter-assay coefficients of variation (CV) were 9.8 and 14.5%. Circulating concentrations of IGF-1 were determined in triplicate samples as reported previously [167], except that, for determinations reported herein, we used a rabbit anti-IGF-1 serum provided by the National Hormone and Pituitary Program (NHPP, Torrance, CA). Concentrations of IGF-1 in samples collected at slaughter were determined in a single assay and intra-assay CV was 19%. Circulating concentrations of

leptin were determined in a single RIA as described previously [168]. Sensitivity of the assays was 0.1 ng/ml and intra-assay CV averaged 13.3%.

VFA Analysis in Ruminal Fluid

Ruminal fluid collected from experimental animals at slaughter was mixed briefly, transferred to 15-ml conical tubes, centrifuged, and the aqueous portion was transferred to a fresh 15-ml conical tube. Samples were stored at -20^{o} C until analysis for fatty acid composition using gas liquid chromatography [169].

Tissue Processing

Frozen blocks of hypothalamic tissue were cut in coronal sections at a thickness of 20 μm using a cryostat. Tissue sections were thaw-mounted on Superfrost/Plus glass microscope slides (Fisher Scientific, Waltham, MA; Cat #12-550-15) and frozen immediately. Slides were then stored at -80^{o} C until processing.

RNA Isolation and Extraction

A single series of tissue sections 200 μm apart from each heifer was processed for Cresyl violet staining and observed using bright- and dark-field microscopy to determine the location of the ARC. Location of the ARC was based on identification of well established anatomical markers [170]. A separate series of sections containing the

ARC was used for tissue dissection and RNA isolation. Using a 25 G needle, an area of approximately 1 mm in diameter targeting the ARC was scraped from the slides (Fig 1). Scraped tissue was immediately placed in lysis solution (RNAqueous-Micro; Ambion, Austin, TX). Scrapes were repeated in 21-54 sections and all sections were maintained frozen during scraping. Total RNA was collected from tissue scrapes using RNAqueous-Micro (Ambion, Austin, TX) according to manufacturer instructions for the Laser-Capture Micro-Dissected (LCM) tissue protocol, except that RNA was harvested into 400 µl of Lysis Solution (RNAqueous-Micro), and precipitated with 200 µl of 100% ethanol. Samples were incubated in Elution Solution (RNAqueous-Micro®) for 5 min before eluted through the column. Total RNA isolated was treated with DNase to remove genomic DNA. Quantity of RNA was determined using the NanoDrop ND-1000 spectrophotometer (ThermoFisher Scientific, Wilmington, DE). Quality of RNA was determined using the RNA 6000 Pico Kit (Agilent Technologies, Santa Clara, CA), according to manufacturer instructions.

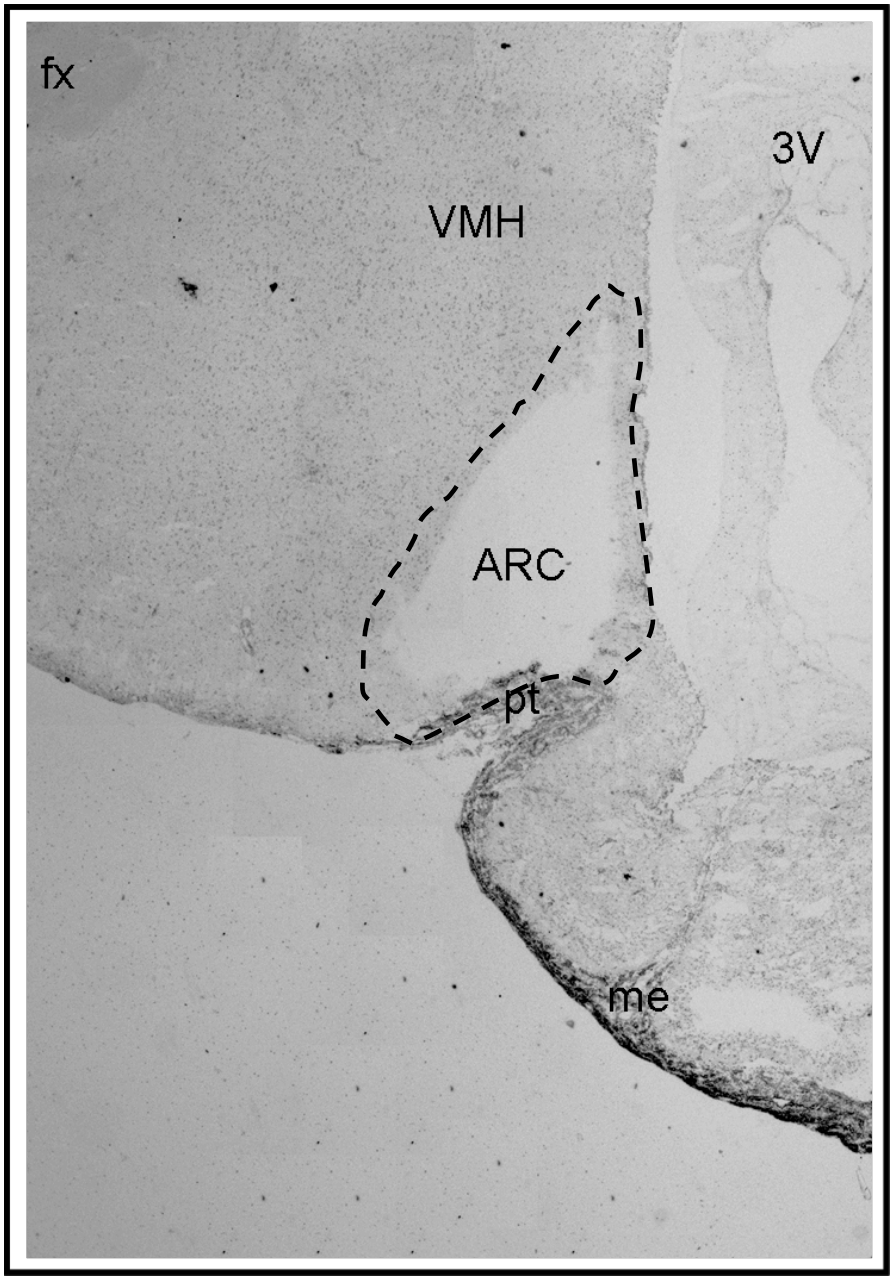

FIG. 1. Cresyl violet-stained section through the mediobasal hypothalamus illustrating area (within dashed line) of ARC tissue dissected and removed for RNA extraction. Dissection was based on anatomical landmarks: VHM, ventromedial hypothalamus; ARC, arcuate nucleus; fx, fornix; me, median eminence; pt, pars tuberalis.

Microarray Procedure and Analysis

cRNA labeling. Total RNA (120 ng) from each heifer was initially reverse transcribed to cDNA using the two-color Quick Amp Labeling Kit (Agilent). Labeled cRNA was synthesized by *in vitro* transcription of cDNA in presence of either cyanine-3 (Cy3) or cynanine-5 (RNA Spike-In Kit, Two-Color; Agilent) as per manufacturer's instructions. One-half of the samples from each dietary group were labeled with Cy3 and the other half with Cy5. Labeled cRNA was purified using RNeasy Mini columns (Qiagen, Valencia, CA). Yield and specificity of cRNA were determined based on fluorospectrometry (NanoDrop ND-1000, Thermo Fisher Scientific).

Microarray hybridization. Microarray hybridizations were conducted using the Agilent two-dye 4 x 44 K bovine gene expression array (Agilent Technologies, Santa Clara, CA). Each array contained 44,407 valid oligo probes, 1264 of which were positive control probes, and 153 were negative control probes. Each probe was replicated twice on each array; therefore, each array contained 21,495 unique oligos.

Hybridizations were performed using the Gene Expression Hybridization Kit (Agilent) following manufacturer instructions. Labeled cRNA samples from one heifer in each of the two dietary groups were hybridized to an array, resulting in total of 6 arrays. The dye swap design resulted in each array being hybridized to either Cy3-labeled HC/HG cRNA and Cy5-labeled HF/LG samples, or Cy5-labeled HC/HG and Cy3-labeled HF/LG samples. Arrays were incubated at 65^{o}C with rotation for 17 h in a microarray hybridization chamber. Following hybridization, arrays were washed according to the Agilent Two-Color Microarray protocol (Agilent).

Microarray imaging, data acquisition, normalization and analysis. Agilent arrays were scanned at 5-μm resolution on an Axon GenePix 4100 scanner (Molecular Devices Corporation, Sunnyvale, CA). Signal intensities were quantified using the GenePix pro 6.0 software (Molecular Devices Corporation, Downingtown, PA) and following procedures described previously [171]. Normalization of log-ratios was accomplished via intensity-dependent nonlinear location shift using Loess regression. Differential expression was assessed by fitting a linear model for each probe as follows:

$$Y_{ij} = D_i + (-1)^{c_j} A_i + \varepsilon_{ij}$$

Where Y_{ij} is the observed log-ratio for probe i on array j; D_i is the dye effect for probe i; A_i is the log-ratio between HF/LG over HC/HG for probe i, $c_j = 0$ for $j = 1,\ldots,3$ and $c_j = 1$ for $j = 4,\ldots,6$; and the ε_{ij} are assumed to be zero-mean Gaussian residual of constant variance. For each probe, differential expression was detected by testing for a nonzero coefficient A_i in the model, which is accomplished via a Bayesian test where the P-value is moderated by borrowing information from correlated probes, thereby increasing statistical power [172]. Power is also increased by taking into consideration in the analysis the estimated correlation between pairs of duplicated spots. The analysis is weighted such that any suspect probes (with negative quality flags) have zero weight, and the good probes (with zero quality flags) have weight 1. The multiple testing problem is addressed by adjusting the P-values according to the False-Discovery Rate (FDR) [173].

Quantitative RT-PCR

Three genes exhibiting differential expression in the micro array experiment were selected for quantitative real-time RT-PCR analysis. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ribosomal protein L19 (RPL19) were used as reference control genes. Primers used for real-time RT-PCR were designed using Oligo 6 software (Molecular Biology Insights, Inc., Cascade, CO) and sequences are shown in Table 2. Total RNA (20 to 200 ng) isolated from the ARC was reserve transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) and oligo $(dT)_{20}$ primer (Integrated DNA Technologies, Coralville, IA). Briefly, PCR reactions contained 2 µl cDNA (diluted 1:4 in 25 ng/µl yeast tRNA), 10 µl SYBR® GreenER™ PCR master mix (Invitrogen, Carlsbad, CA), 0.6 µl of each forward and reverse primers and RNA-free water to a final volume of 20 µl. Quantitative real-time PCR was carried out in 96-well plates using the ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA). Cycling conditions were 95^{o} C

for 10 minutes followed by 40 cycles of 95^{o} C for 15 seconds and 60^{o} C for 1 minute.

Analysis of qRT-PCR

Quantitative RT-PCR data were analyzed using the $2^{-\Delta\Delta CT}$ method [174]. Threshold cycle (CT) data for each gene was normalized for the mean CT values of the control genes GAPDH and RPL19 for each sample (ΔCT), and transformed to the average expression of HF/LG samples (ΔΔCT). Mean fold change in HC/HG group was compared to HF/LG group.

Statistical Analysis

Final body weight, overall ADG, liver weight, ruminal propionate to acetate ratio, hormone concentrations at slaughter, and mean fold change of selected differentially expressed genes were analyzed by ANOVA using the PROC MIXED procedure of SAS (Statistical Analysis System, Cary, NC). For all data, the model included dietary treatment, replicate, and treatment by replicate interaction.

Table 2. Gene name, symbol, primer sequence, and accession number for all genes used for qRT-PCR.

Gene name	Gene symbol	Sequence of primers	Accession No.
Agouti-related protein	*AGRP*	GAAGAGGATAACGAACAG CAGGGGTTCGTGGTGGGTA	NM_173983
Growth hormone receptor	*GHR*	ATCACCACAGAAAGCCTTACCACTA GACAGGTATCTCAGAACTTGGAAC	NM_176608
Neuropeptide Y	*NPY*	AAGCAGAGATACGGGAAACGA ATTGGGAGGACTGGCAGACT	NM_001014845
Glyceraldehyde-3- phosphate dehydrogenase	*GAPDH*	CAGCGACACTCACTCTTCTACCTT GAACTCTTCCTCTCGTGCTCCT	NM_001034034
Ribosomal protein L19	*RPL19*	ACCCCAATGAGACCAATGAA GCAGTACCCTTTCGCTTACCTAT	NC_007317

Results

Body Weight, ADG, and Ruminal VFA

Mean body weight at the beginning of the study did not differ ($P = 0.9$) between groups (HF/LG, 139.7 ± 6.4 kg; HC/HG, 127 kg ± 6.4 kg). Body weight increased linearly in both dietary groups, with greater ADG ($P < 0.0001$) in HC/HG heifers (0.98 ± 0.05 kg/d) than in HF/LG heifers (0.51 ± 0.06 kg/d) (Fig. 2).

Mean body weight, mean liver weight as a percentage of body weight, and ruminal fluid propionate:acetate ratio were greater ($P < 0.02$) in HC/HG heifers than in HF/LG heifers (Table 3). Mean circulating concentrations of insulin and IGF-1 were greater ($P < 0.05$) at the time of slaughter in HC/HG heifers than in HF/LG heifers, and circulating concentrations of leptin tended to be greater ($P = 0.1$) in HC/HG heifers (Table 3).

Microarray Gene Expression

After correction for FDR [173], a total of 346 genes exhibited a significant log-ratio between HF/LG and HC/HG treatment groups ($P < 0.05$). Among the 346 differentially expressed genes, 229 exhibited increased and 117 exhibited decreased

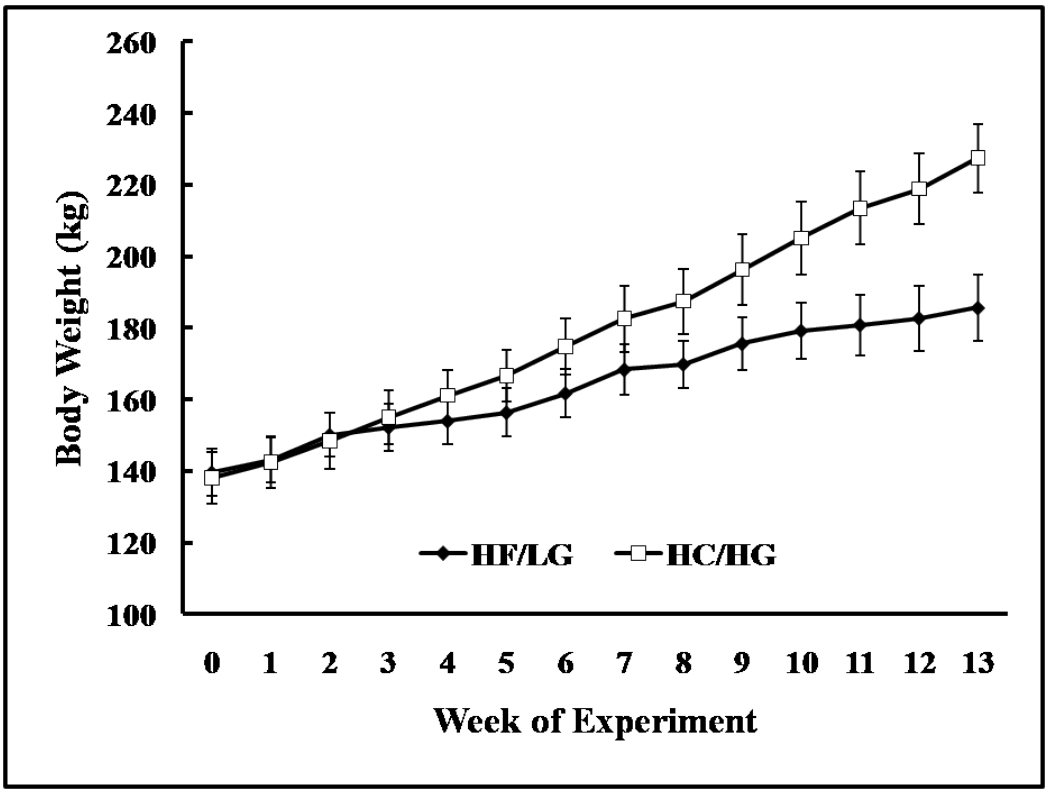

FIG. 2. Mean (± SEM) body weights of heifers weaned at 3 mo of age and fed either a high-forage/low-gain (HF/LG) or a high-concentrate/high-gain diet (HC/HG) for 13 wk. Body weight of HC/HG heifers was greater than HF/LG heifers beginning at week 10 ($P < 0.06$) and continuing through week 13 ($P < 0.03$).

Table 3. Mean (± SEM) body weight, liver weight, rumen propionate:acetate ratio, and serum concentrations of metabolic hormones at time of slaughter in heifers fed high-forage/low-gain (HF/LG) or high-concentrate/high-gain (HC/HG) diets for 14 wk beginning at 16 ± 1 wk of age.

Variable	HF/LG	HC/HG	*P*-value
Body weight, Kg	172.12 ± 9.19	218.49 ± 11.47	0.02
Liver weight, % of BW	1.07 ± 0.04	1.42 ± 0.04	0.003
Propionate:acetate	0.23 ± 0.01	0.31 ± 0.01	0.001
Insulin, ng/ml	0.94 ± 0.17	1.16 ± 0.26	0.05
IGF-1, ng/ml	52.01 ± 4.39	103.75 ± 9.9	0.002
Leptin, ng/ml	2.55 ± 0.35	3.62 ± 0.43	0.1

Table 4. Gene symbol, gene description and fold change of 10 genes exhibiting greater expression ($P < 0.05$) in heifers fed high-concentrate diets to gain body weight at a high-rate (0.91 kg/d) than heifers fed high-forage diets to gain body weight at a low rate (0.45 kg/d).

Gene Description	Gene Symbol	Fold Change
Transmembrane protein 149	*TMEM149*	3.06
General transcription factor IIH, polypeptide 5	*GTF2H5*	2.04
G protein-coupled receptor 45	*GPR45*	2.01
Agouti protein	*AGOUTI*	1.82
Similar to SEC14p-like protein TAP3, transcript variant 1	*LOC513294*	1.76
Similar to dedicator of cytokinesis 6	*LOC539711*	1.64
Dentin sialophosphoprotein	*DSPP*	1.58
Tensin 4	*TNS4*	1.48
Toll-like receptor 6 (tlr6)	*TLR6*	1.48
Ras-related associated with diabetes	*RRAD*	1.42

Table 5. Gene symbol, gene description and fold change of 10 genes exhibiting greater expression ($P < 0.05$) in heifers fed high-forage diets to gain body weight at a low rate (0.45 kg/d) than in heifers fed high-concentrate diets to gain body weight at a high-rate (0.91 kg/d).

Gene Description	Gene Symbol	Fold Change
Agouti related protein	*AGRP*	4.72
Alpha-1 acid glycoprotein	*ORM1*	4.18
Neuropeptide Y	*NPY*	3.3
Similar to KIAA0748 gene product, transcript variant 1	*LOC538993*	1.84
Similar to CRYM transcript variant 1	*CRYM*	1.8
Similar to collagen, type IX, alpha 3	*LOC504788*	1.76
Corticotropin releasing hormone	*CRH*	1.42
Similar to glutamate receptor interacting protein 1	*LOC519502*	1.38
Retinol binding protein-1	*RBP1*	1.14
Regulator of G-protein signaling 2, 24kDa	*RGS2*	1.4

expression in HC/HG heifers compared to HF/LG heifers. The top 10 genes exhibiting greater expression in HC/HG heifers compared to HF/LG heifers are represented in Table 4, and the top 10 genes exhibiting greater expression in HF/LG heifers compared to HC/HG heifers is represented in Table 5.

qRT-PCR of Selected Genes

NPY, AGRP and *GHR* genes were selected as specific genes of interest out of the 346 differentially-expressed genes. These genes are specifically involved in signaling metabolic status to the reproductive neuroendocrine axis. Quantitative RT-PCR analysis of these genes confirmed microarray observations and indicated that mean expression of NPY, AgRP and GHR was lower ($P < 0.02$) in HC/HG heifers than in HF/LG heifers (Fig. 3).

Discussion

The ARC is a major area of the hypothalamus that integrates complex signaling pathways that regulate metabolic and reproductive functions. Since the initiation of puberty is greatly influenced by nutritional balance and metabolic status, we sought to examine an array of genes within the ARC that may be responsive to, and/or mediate the

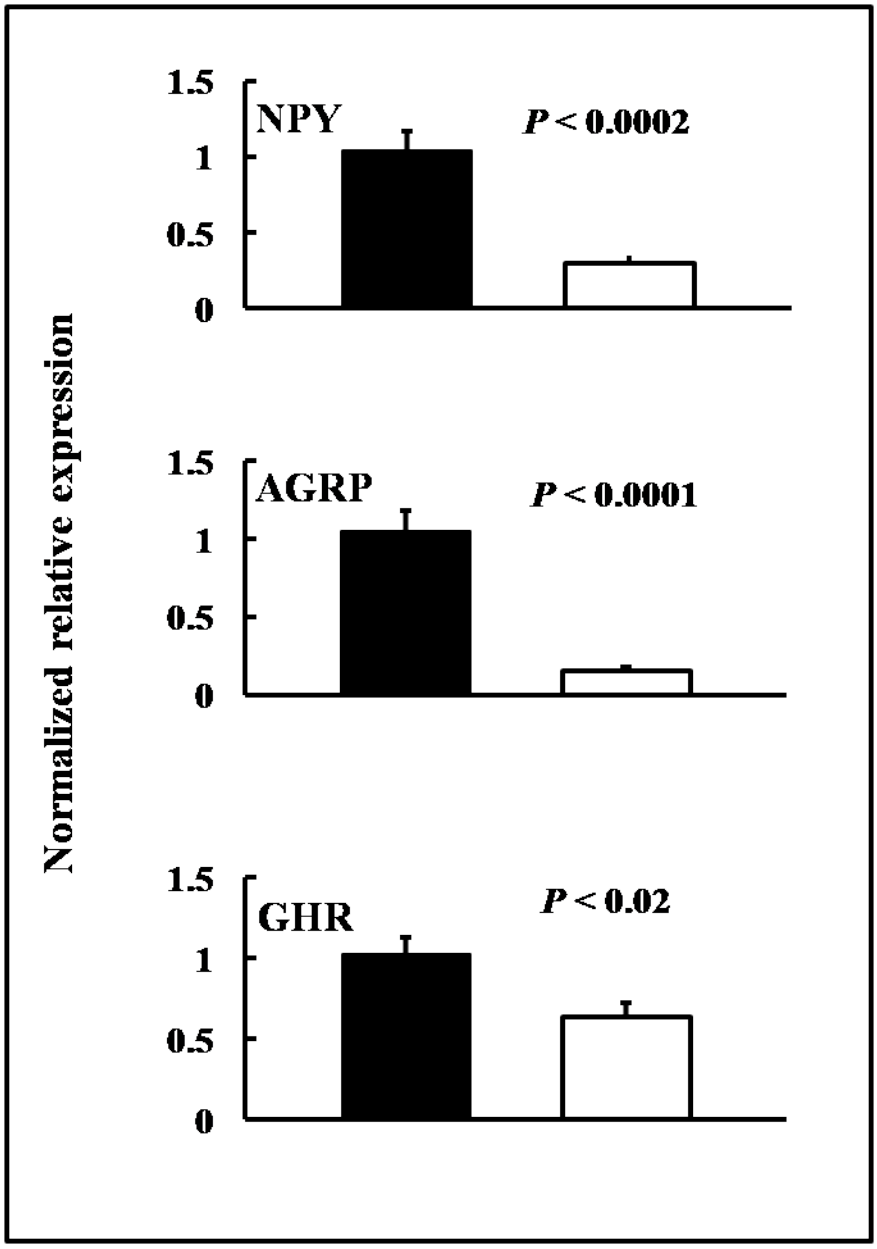

FIG. 3. Normalized mean (± SEM) expression of neuropeptide-Y (*NPY*), agouti-related protein (*AGRP*), and growth hormone receptor (*GHR*) genes in the arcuate nucleus of heifers fed a high-forage diet to gain body weight at a low rate (HF/LG) and heifers fed a high-concentrate diet to gain body weight at a high rate (HC/HG). Expression is relative to mean normalized expression of HF/LG samples in the experiment.

effects of, nutritional inputs known to affect the process of sexual maturation. The precocious puberty model in heifers described by Gasser et al [8, 13-15] provides an ideal approach for examining the involvement of the ARC in the nutritional regulation of neuroendocrine function during the early juvenile development. The current studies provided evidence that a considerable number of genes expressed within the ARC are regulated by nutrient intake concurrent with the development of distinct metabolic and hormonal states.

Using microarray gene expression analysis, we identified a total of 346 genes that were differentially expressed between HC/HG and HF/LG heifers at the end of the 13-wk feeding period (7 mo of age). This observation demonstrated that the heifer is exquisitely sensitive to nutritional input during early calf-hood. Importantly, nutrient requirements for maintenance were met, and both groups of heifers were in positive energy balance. However, source and amount of nutrients available for growth differed between treatment groups. Therefore, differences in gene expression within the ARC between heifers growing at distinct rates indicate that the state of nutrient sufficiency to support growth involves functional changes within the hypothalamus early in development. We propose that those changes may be important for early maturation of the reproductive neuroendocrine axis. In mice, neuronal projections originating in the ARC toward hypothalamic regions that regulate metabolism and feed intake are established during the first two weeks after birth [175]. This observation suggests that the early juvenile period of development is critical for development of hypothalamic pathways that control neuroendocrine functions during maturation and adulthood.

Of the differentially-expressed genes identified in the microarray study, expression of *NPY*, *AGRP*, and *GHR* genes were confirmed by qRT-PCR. Neuropeptide Y and AGRP are known to be intimately involved in signaling metabolic status to the central reproductive axis through neuronal pathways emanating from within the ARC [90, 91, 124, 125]. Neuropeptide Y projections are observed in close proximity to GnRH neurons and dendrites in the POA [6] and GnRH fibers in the ME [5]. Neuropeptide Y5 receptor has been localized in GnRH neurons [5, 6], indicating direct actions of NPY upon GnRH neurons. Furthermore, *NPY* expression increases during negative energy balance and decreases during adequate or excess energy. Therefore, because of its involvement in the control of reproductive function in sexually-mature animals [91, 105, 115, 116], NPY is a major focal point in our efforts to understand mechanisms associated with the metabolic control of sexual maturation. Importantly, treatment of female rats with NPY delays sexual maturation and disrupts reproductive cyclicity [105]. Furthermore, McShane and co-workers have reported that icv administration of NPY to estradiol-implanted, ovariectomized ewes reduced release of LH [108]. Others have reported that NPY administration suppresses circulating concentrations of LH by inhibiting pulsatile release of GnRH and LH in ovariectomized, estradiol-implanted cattle [115, 116]. Additionally, acute central treatment of NPY inhibits secretion of LH in mature, ovariectomized cattle pretreated with leptin [175], suggesting that NPY signaling may be downstream of leptin's actions. Based on previous reports [8, 13-15], the nutritional strategies employed in the current experiment were designed to promote a relatively high rate of body weight gain, which when

associated with high concentrate diets have been shown to markedly hasten puberty in heifers. Thus, our observation of decreased *NPY* expression in the ARC of HC/HG heifers compared to HF/LG support the hypothesis that NPY neuronal pathways may serve as metabolic integrators of pubertal process. Specifically, elevated *NPY* expression in the ARC may serve as a metabolic 'brake' for pubertal onset. However, the neuroendocrine mechanisms and pathways by which NPY exerts this function remain unclear, but may involve differential NPY projections that are established during early calf-hood development. Alterations in hypothalamic neuronal circuitry associated with nutritional inputs have been observed in during early postnatal development in rodents [177].

The majority of the NPY neurons in the ARC co-express *AGRP* [47] which function together to increase energy balance in times of inadequate nutrition. In the current study, differential expression of *AGRP* between HC/HG and HF/LG heifers were parallel to that of NPY. Agouti-related peptide is an antagonist of melanocortin receptors (MCR) and blocks the actions of α-melanocortin stimulating hormone (α-MSH) [119]. Hypothalamic α-MSH is cleaved from its polypeptide precursor, POMC, during post-translational processing and upon binding to MCR suppresses appetite and feeding behavior. It has been suggested previously that AGRP exerts negative modulatory effects on GnRH neurons [124, 125] and thus ultimately, secretion of gonadotropins from the adenohypophysis. The MC4R is expressed in the ARC, PVN, and medial POA [127]. Moreover, it is expressed in immortalized hypothalamic GnRH-secreting GT-1 cells [128], but it is unclear if they are expressed specifically on GnRH

neurons. Based on in vitro experiments, AGRP does not appear to affect gonadotropin secretion directly in cultured pituitary cells [126]. The apparent role of AGRP is to function in synchrony with NPY to increase appetite and feeding behaviors by blocking the actions of α-MSH. Similarly to *NPY*, *AGRP* expression was more abundantly expressed in HF/LG than in HC/HG heifers. Thus, AGRP may bolster NPY's actions as a metabolic 'brake' on pubertal onset by suppressing GnRH release. Previously, it has been demonstrated that nutritional inputs during early postnatal development affect neuronal development of AGRP projections in the hypothalamus [177].

The current study also revealed that *GHR* expression was decreased in HC/HG heifers relative to HF/LG. Growth hormone is the cognate ligand for GHR and GH secretion is regulated by an intricate interaction between growth hormone-releasing hormone (GHRH) and somatostatin actions in somatotrophs [178]. Growth hormone-releasing hormone neurons in the ARC express few GHR [179], however, the majority (95%) of NPY neurons in the ARC express GHR [180]. In addition, evidence indicates that NPY neurons act as an intermediate step in the regulation of GH [180]. In rats, hypophysectomy caused a significant reduction of *NPY* mRNA expression in the ARC, and treatment with GH restored mRNA levels to that of control animals [180]. This indicates a direct regulation of *NPY* expression by GH, likely via GHR on NPY neurons in the ARC. Concentrations of GH in circulation tend to decrease during juvenile growth and are low at puberty, particularly in heifers gaining weight at high rates [180]. Therefore, it is possible that the elevated nutritional status promoted in the HG/HG group supports alterations in the NPY system involving GH signaling. Nevertheless,

previous reports from our laboratory [115] and from others [182, 183] have demonstrated paradoxical but clear stimulatory effects of NPY on the release of GH in cattle and sheep. Thus, the interrelationships between NPY and GH release remain ambiguous, but seem to reinforce a metabolic status that may be permissive (if suppressed) or restrictive (if maintained elevated) to reproductive maturation.

The greater circulating concentrations of leptin and insulin in HC/HG compared to HF/LG heifers confirm the elevated nutritional status in that group, and the greater concentrations of IGF-1 in circulation support greater potential for increased somatic growth. The increase in mean circulating concentrations of leptin in HC/HG heifers is congruent with the observed decrease in expression of *NPY* and *AGRP*, because leptin has been shown to suppress *NPY* and *AGRP* gene expression and release [71, 79, 123]. Leptin also positively modulates GH secretion under fasting conditions by direct actions at the anterior pituitary [184] and attenuates the stimulatory effects of NPY on GH release in cattle [176]. In addition, numerous studies have demonstrated the close association between secretion patterns of leptin and insulin. Similar to leptin, insulin also has negative regulatory effects on *NPY* and *AGRP* expression.

The foregoing changes in metabolic hormones and in expression of key regulatory genes in the ARC are also congruent with the observed changes at slaughter of ruminal volatile fatty acid content. In ruminants, propionate is readily converted to glucose in the liver, whereas acetate is not [11]. Elevated hepatic function may explain increased liver weight in HC/HG heifers, supporting increased hepatic IGF-1 synthesis and maintaining greater concentrations of IGF-1 in circulation.

In summary, our results have confirmed marked differences in gene expression within the ARC in heifers nutritionally programmed to hasten or delay pubertal onset. Among the differently-expressed genes, *NPY*, *AGRP*, and *GHR* exhibited decreased expression under nutritional conditions that promoted a relatively high rate of gain. These genes are known to be involved in the neuroendocrine control of metabolic functions, and interact to regulate the reproductive neuroendocrine axis. Therefore, it is possible that nutritionally-induced changes in expression of these genes during juvenile development may also serve as key signals for regulating puberty.

CHAPTER IV

EFFECTS OF TWO LEVELS OF DIETARY CONCENTRATE AND BODY WEIGHT GAIN DURING THE JUVENILE PERIOD ON METABOLIC ENDOCRINE STATUS AND AGE AT PUBERTY IN HEIFERS

Introduction

Reproduction is an energy-demanding physiological process that is greatly influenced by nutrition. Thus, appropriate nutritional balance is a prerequisite for juvenile animals to proceed through pubertal development and to insure continuing reproductive capacity in adulthood. Puberty is the result of progressive developmental changes within the brain that lead to the attainment of reproductive capacity. In maturing heifers, it is important to attain reproductive capacity well in advance of the beginning of the first breeding season to increase the probability of conception early in that breeding season and to maximize lifetime productivity [2-4].

One currently accepted viewpoint is that a critical window exists during early calf-hood development in heifers in which sensitivity to dietary quality and quantity is heightened [8, 13-16]. Consequently, dietary manipulations during this period can have a profound influence on the timing of puberty. Specifically, heifers weaned at 3 mo of age and fed diets high in starch that promote high rates of body weight gain between 3 and 7 mo of age exhibit an increased tendency to reach puberty precociously (≤ 300 d) and at lower body weight than heifers fed to gain weight at a lower rate [13-15]. In ruminants, diet composition and quantity are critical determinants of the type and

amount of volatile fatty acids (VFA) produced during fermentation [11, 184]. Although acetate is produced in larger quantities in the rumen, it does not exhibit the gluconeogenic properties of propionate; thus in ruminants, propionate is the primary substrate utilized in gluconeogenesis [11, 12]. Increasing dietary starch in the diet promotes ruminal propionate production and increases propionate to acetate ratio. Therefore, greater amounts of highly-soluble carbohydrates in the diet provide greater energy substrates to the animal for immediate use, as well as for accumulation of body fat mass during growth and development. However, it is unclear if the cues driving precocious puberty in heifers fed high-starch diets are created as a result of the gluconeogenic nature of the diet, or if the same effect can be created with a high-forage, acetate- favoring diet that promotes a similar rate of body weight gain. In the studies reported herein, early-weaned heifers were utilized to investigate the interaction of dietary energy source (propionate or acetate-favoring diets) and rate of body weight gain on long-term secretion patterns of metabolic signaling hormones and age at puberty.

Materials and Methods

All animal-related procedures in this study were approved by the Institutional Agricultural Animal Care and Use Committee (IAACUC) of the Texas A&M University System. This experiment was conducted at the Texas AgriLife Research station, Beeville, TX.

Animals and Experimental Design

Forty-eight Angus-sired heifers out of Brahman x Hereford F_1 dams from the Texas AgriLife Research-Beeville herd were weaned at 14 ± 1 wk of age and stratified by date of birth. Heifers were allocated randomly within stratification into 1 of 4 treatment groups in a 2 x 2 factorial arrangement (2 diets and 2 rates of body weight gain) involving 2 replicates and 6 heifers/group. Treatment groups were: 1) High-Forage, Low-Gain (HF/LG; 0.45 kg/d); 2) High-Forage, High-Gain (HF/HG; 0.91 kg/d); 3) High-Concentrate, Low-Gain (HC/LG; 0.45 kg/d); 4) High-Concentrate, High-Gain (HC/HG; 0.91 kg/d). Diets were balanced using the Large Ruminant Nutrition System for cattle [164]. Heifers were fed their respective diets once daily in the morning.

Ingredients and chemical composition for the high-forage and high-concentrate diets are represented in Table 6. Targeted ADG was attained by adjusting to dry matter intake (DMI) based on ADG.

Heifers in each dietary treatment were placed in pens measuring 25.9 x 9.5 m and fed an acclimation diet for 2-wk post-weaning. During the first week of the acclimation period, heifers in HC and HF groups were offered the HF diet up to a maximum of 2.7 kg/head daily on a pen basis. During the second week of the adaptation period, heifers assigned to HC treatment groups were offered a diet consisting of 50% HF and 50% HC up to a maximum of 2.7kg/head daily on a pen basis. After the 2-wk adaptation period, heifers were fed 100% of their respective diets (HC or HF) for the following 14 wk. After the 14 wk dietary treatments, all heifers were switched to a common diet that included a corn-cottonseed meal-based supplement designed to promote a moderate (0.68 kg/d) rate of body weight gain, and free choice Coastal Bermuda grass hay, until puberty was confirmed.

Table 6. Ingredients and chemical composition of high-forage (HF) and high-concentrate (HC) diets fed to heifers during the 14-wk dietary treatment period.

Ingredients	HF	HC
Cracked corn, % DM	39.03	50.75
Soybean meal, % DM	--	17.61
Chopped coastal Bermuda grass hay, % DM	3.47	26.95
Dehydrated alfalfa meal pellet, % DM	57.30	3.83
Calcium carbonate, % DM	--	0.86
Calcium monophosphate, % DM	0.20	--
Vitamin A/D/E premix, mg/kg of DM	71	73
Chemical composition		
Metabolizable energy, Mcal/kg of DM	2.32	2.81
Crude protein, % DM	13.8	17.8
Digestible intake protein, % CP	61	69

Blood Collection and Determination of Puberty

Blood collection during the experiment was performed prior to feeding by caudal venipuncture and samples were placed immediately on ice. Blood samples were collected once a week during the dietary treatment period and common growth period until heifers reached approximately 35 wk of age, after which blood was collected twice weekly until puberty was confirmed. Plasma or serum was harvested and stored at -20^{o}C until analyses for circulating concentrations of hormones. Puberty was confirmed by observing serum concentrations of progesterone $\geq$ 2 ng/ml in at least two consecutive samples. Once puberty was confirmed, heifers were removed from the study.

Hormone Assays

Circulating concentrations of leptin, insulin, IGF-1, and glucose were determined in plasma samples collected during weeks 0, 2, 4, 6, 8, 10, 12 and 14 of the dietary treatments. Circulating concentrations of leptin, insulin, and IGF-1 from the common growth period were determined in serum samples in the 5 periods preceding puberty, and at puberty. "Period" consisted of samples collected every third week during the common growth period. Differences in age at puberty allowed only for analysis of the 5 periods preceding puberty without significantly decreasing sample size. Circulating concentrations of leptin were determined in a single RIA as described previously [168]. Minimum detectable concentration was 0.1 ng/ml with intra- and inter-assay CV of 1.8 and 3.8%. Concentrations of insulin were determined using a solid-phase ^{125}I-RIA Coat-A-Count kit developed for human serum (Siemens, Los Angeles, CA) and reported previously for bovine serum samples [165, 166]. However, we used a bovine insulin

preparation for standards (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, and 2.5 ng/ml) and references instead of the human insulin standards provided with the kit. Minimum detectable concentrations were 0.1 ng/ml with intra- and inter-assay CV of 9.2 and 23.6%, respectively. Circulating concentrations of IGF-1 were determined in triplicate samples as reported previously [167], except that, for determinations reported herein, we used a rabbit anti-IGF-1 serum provided by the National Hormone and Pituitary Program (NHPP, Torrance, CA). Minimum detectable concentrations were 0.3 ng/ml and intra- and inter-assay CV were 7.4 and 19.1%, respectively. Concentrations of glucose were quantified using an enzymatic kit assay (Wako Diagnostics, Richmond, VA).

Statistical Analysis

Effects of diet, rate of body weight gain, wk (14-wk dietary treatment period), period (common growth period), replicate, and all possible interactions on BW and hormone concentrations were analyzed by analysis of variance (ANOVA) using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC). The repeated measures model was:

$$Y_{ijklm} = \mu + D_j + G_k + h_{i:j:k} + T_l + R_m + (DG)_{jk} + (DT)_{jl} + (GT)_{kl} + (DR)_{jm} + (GR)_{km} + (DGT)_{jkl} + (DGR)_{jkm} + e_{ijk}$$

Y_{ijklm} is the observation of the i th heifer in the j th diet and k th gain group during the l th week/period in the m th replicate, μ = the overall mean, $D_j = j$ th diet, $G_k = k$ th gain, $T_l = l$ th week/period, $R_m = m$ th replicate, $h_{i:j:k}$ = random effect of the i th heifer within the j th diet and k th gain group, $(DG)_{jk}$ = diet x gain interaction, $(DT)_{il}$ = diet x time interaction, $(GT)_{kl}$ = gain x time interaction, $(DR)_{jm}$ = diet x replicate interaction,

$(GR)_{km}$ = gain x replicate interaction, $(GDT)_{jkl}$ = diet x gain x time interaction, $(GDR)_{jkm}$ = gain x diet x replicate interaction, an e_{ijk} = random residual effect.

Since circulating concentrations of insulin, IGF-1 and glucose differed ($P < 0.05$) among treatment groups at week 0, analysis of covariance using values at wk 0 as covariates was employed.

The effects of diet, ADG, and replicate on body weight at puberty and age at puberty were analyzed by ANOVA for a single observation using the MIXED procedure of SAS (Cary, NC) (model: $Y_{jkm} = \mu + D_j + G_k + (D_j \text{ x } G_k) + R_m + e_{jk}$, with notation as defined previously).

If replicate (R_m) was not significant ($P > 0.05$), it was excluded from the model and all heifers were treated as one replicate. Least squares means were compared using the PDIFF command incorporating the Tukey Honest Significant Difference (HSD) test for multiple comparisons. Statistical significance was accepted at $P < 0.05$.

Results

One heifer from the HC/LG dietary treatment group was removed during the 14-wk dietary treatment period due to lack of normal growth. A second heifer from the HC/LG dietary treatment group was removed from all analyses due to significant over performance. Thus, all analyses for the HC/LG dietary treatment group were conducted with only 10 heifers.

Body Weight, ADG, and Age at Puberty

Mean body weight at the beginning of the study did not differ between groups and averaged 140.22 ± 2.62 kg (Fig. 4). Although body weight increased linearly in all dietary groups, ADG was greater ($P < 0.04$) in HG heifers (0.81 ± 0.06 kg/d) than in LG heifers (0.44 ± 0.06 kg/d). Heifers in the HG dietary groups became significantly heavier after 8 wk of feeding (24 ± 1 wk of age) and remained heavier throughout the remainder of the dietary treatment ($P < 0.0001$).

During the common growth period, heifers gained 0.66 ± 0.14 kg/d, and mean body weight at puberty did not differ between dietary groups (332.75 ± 7.04 kg). Mean age at puberty did not differ between dietary treatment (HF or HC), however HG heifers reached puberty at a younger age (54.5 ± 1.8 wk) than LG heifers (60.2 ± 1.9 wk; $P < 0.04$) (Table 7.).

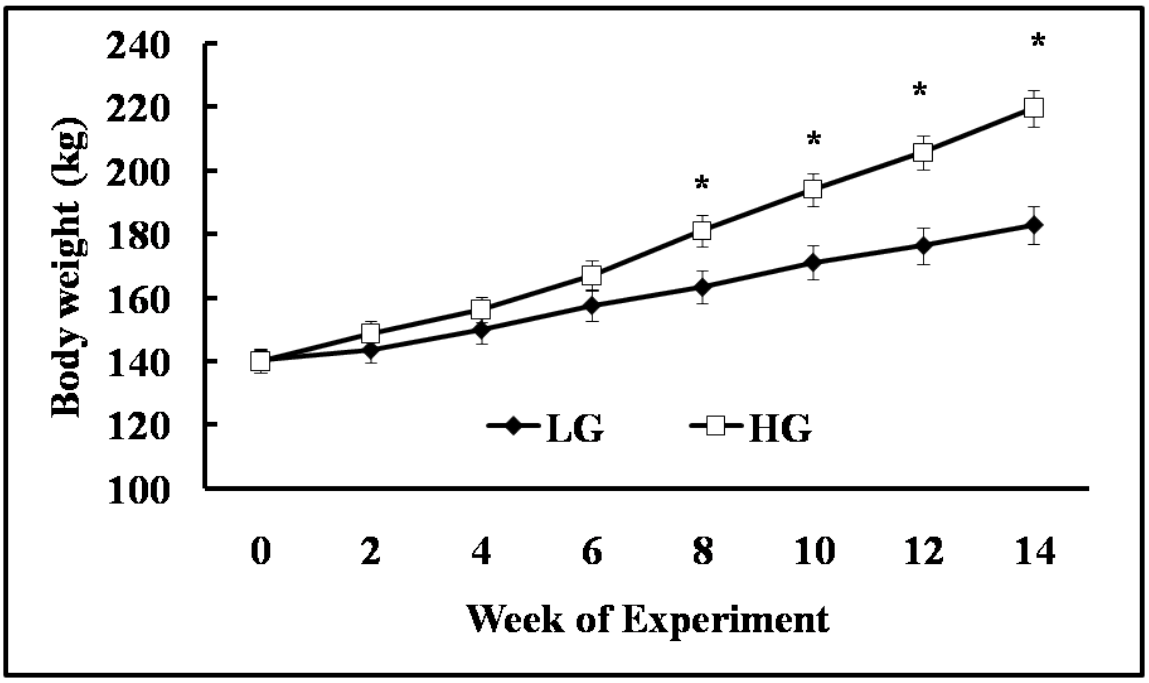

FIG. 4. Mean (± SEM) body weights of heifers weaned at 14 ± 1 wk of age and fed high-forage or high-concentrate diets for 14-wk to gain body-weight at a low (0.45 kg/d; LG) or high (0.91 kg/d; HG) rate. After 8 wk of feeding (24 ± 1 wk of age) HG heifers became significantly heavier than LG heifers, and remained so during the rest of the 14-wk dietary treatment period (*$P < 0.0001$) .

Table 7. Average daily gain (ADG), body weight at start of trial, body weight at end of 14-wk dietary treatment period, body weight at puberty, and age at puberty for heifers in all treatment groups.

	Dietary Treatment			
Variable	High-Forage Low-Gain (n=12)	High-Concentrate Low-Gain (n=10)	High-Forage High-Gain (n=12)	High-Concentrate High-Gain (n=12)
ADG	0.44 ± 0.05[a], kg/d	0.43 ± 0.06[a], kg/d	0.74 ± 0.06[b], kg/d	0.88 ± 0.06[b], kg/d
Body weight at start of trial	139.38 ± 5.34 kg/d	141.62 ± 5.97, kg/d	142.57 ± 5.34, kg/d	137.45 ± 5.34, kg/d
Body weight at end of 14-wk dietary treatment	182.35 ± 8.35[c], kg/d	184.13 ± 9.33[c], kg/d	215.35 ± 8.35[d], kg/d	223.83 ± 8.35[d], kg/d
Body weight at puberty	347.0 ± 13.5, kg/d	330.17 ± 15.1, kg/d	333.58 ± 13.5, kg/d	325.5 ± 13.5, kg/d
Age at puberty	60.3 ± 2.6, weeks	59.9 ± 2.9 weeks	54.33 ± 2.6 weeks	54.75 ± 2.6, weeks

Row means with different superscripts differ ([a, b] $P < 0.004$; [c, d] $P = 0.07$)

Circulating Metabolic Hormones

Heifers in the HC/HG treatment group exhibited greater concentrations of circulating leptin than HF/LG and HF/HG heifers ($P = 0.1$) after 8 wk of feeding (24 ± 1 wk of age), and remained elevated throughout the remainder of the dietary treatment period ($P < 0.01$) (Fig. 5).

Although concentrations of circulating insulin did not consistently among treatment groups during the 14-wk dietary treatment period, circulating concentrations of insulin were greater in HG heifers than in LG heifers after 4 wk (20 ± 1 wk of age; $P = 0.01$), 6 wk (22 ± 1 wk of age; $P = 0.1$) and 14 wk (30 ± 1 wk of age; $P < 0.04$) of feeding. No effect of dietary treatment was observed (Fig. 6). Mean concentrations of IGF-1 were not affected by dietary treatment or ADG during the 14 wk dietary treatment period. Overall mean concentrations of glucose were greater ($P < 0.006$) in HG heifers (64.78 ± 1.74 ng/ml) than LG heifers (57.35 ± 1.85 ng/ml) during the 14-wk dietary treatment period, but did not differ significantly by week (FIG. 7).

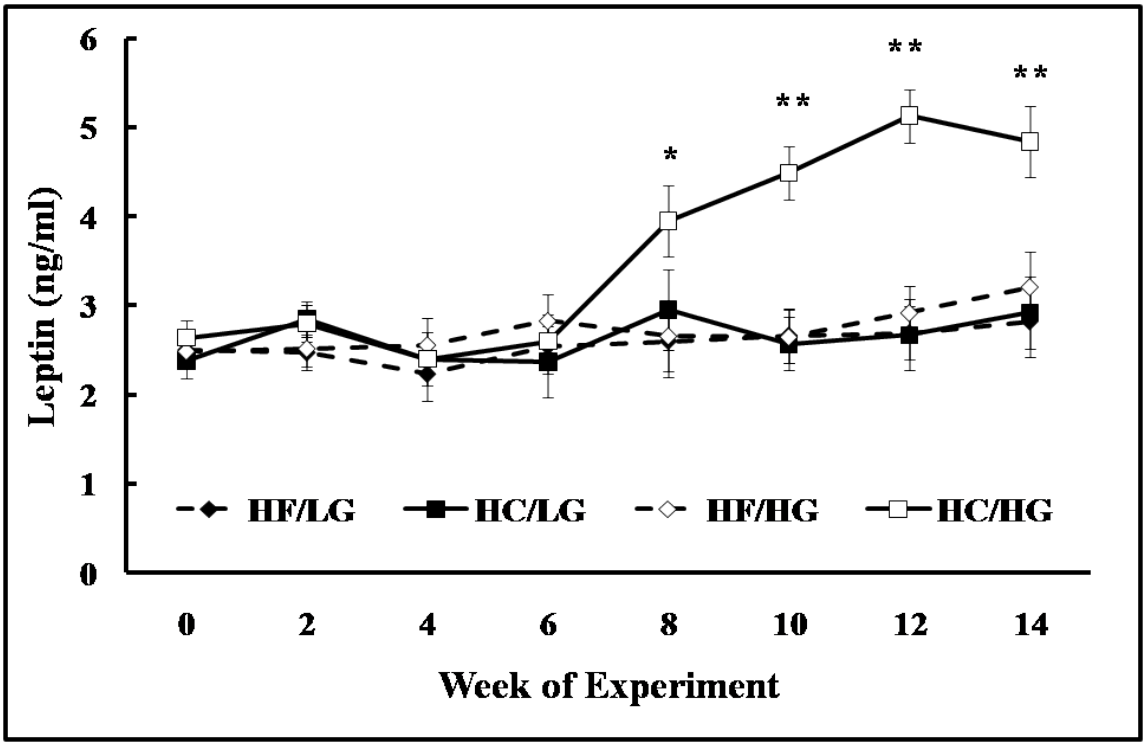

FIG. 5. Mean (± SEM) concentrations of circulating leptin in heifers fed high-forage (HF) and high-concentrate (HC) diets to gain body weight at low (LG) and high (HG) rates for 14 wk beginning at 16 ± 1 wk of age. Heifers in the HC/HG treatment group exhibited greater concentrations of circulating leptin than HF/LG and HF/HG heifers (*P = 0.1) after 8 wk of feeding (24 ± 1 wk of age), and remained elevated throughout the remainder of the dietary treatment period (**P < 0.01).

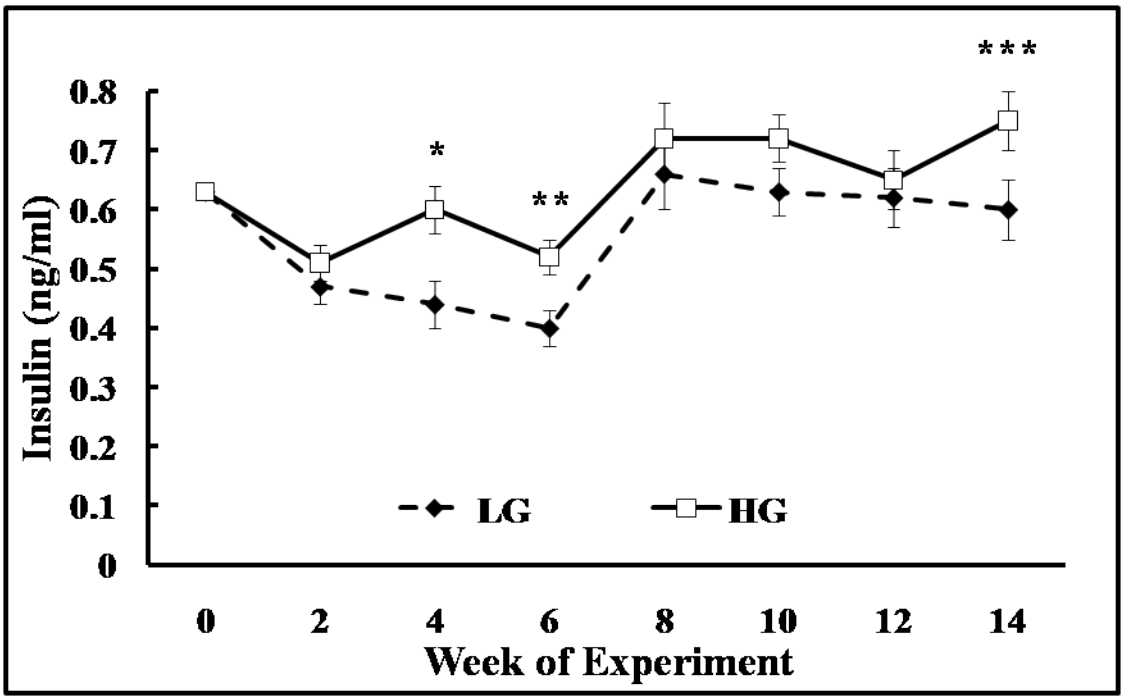

FIG. 6. Mean (± SEM) concentrations of circulating insulin in heifers fed high-forage and high-concentrate diets to gain body weight at low (LG) and high (HG) rates for 14 wk beginning at 16 ± 1 wk of age. Circulating concentrations of insulin were greater in HG heifers than in LG heifers after 4 wk (20 ± 1 wk of age; $P = 0.01$), 6 wk (22 ± 1 wk of age; $P = 0.1$) and 14 wk (30 ± 1 wk of age; $P < 0.04$) of feeding.

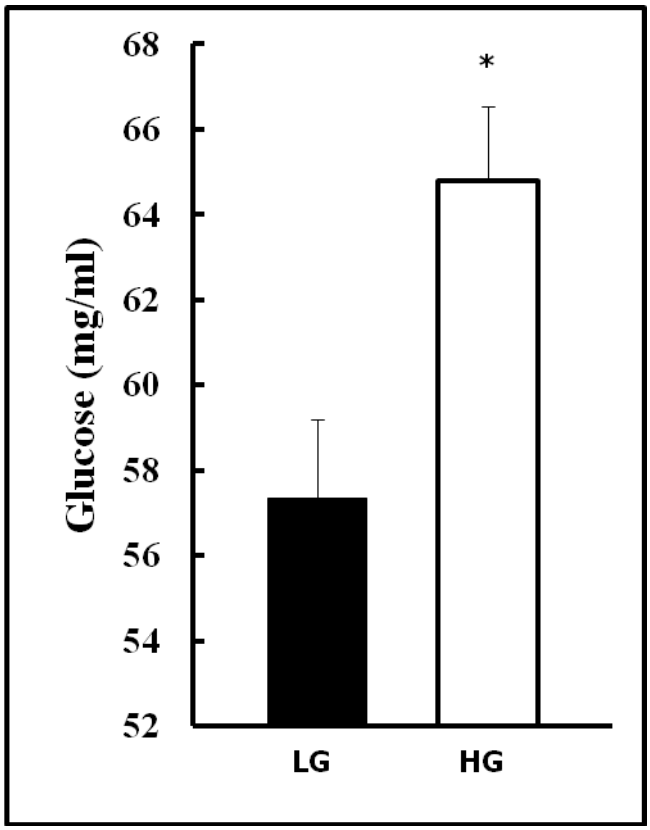

FIG. 7. Mean (± SEM) concentrations of circulating glucose in heifers fed high-forage and high-concentrate diets to gain body weight at low (LG) and high (HG) rates for 14 wk beginning at 16 ± 1 wk of age. Overall mean concentrations of glucose were greater (*$P < 0.006$) in HG heifers than LG heifers during the 14-wk dietary treatment period, but did not differ significantly by week.

Circulating concentrations of leptin were greater in HF heifers ($P < 0.03$) than in HC heifers at puberty (Fig. 8). Circulating concentrations of insulin differed between diet and replicate during the common growth period. High forage heifers in replicate 1 exhibited greater concentrations of insulin than HC heifers beginning at period 5 ($P < 0.03$) of the common growth period, and remained greater until puberty ($P < 0.02$) (Fig. 9a). Concentrations of insulin in replicate 2 heifers were not affected by diet, gain or period during the common growth period (Fig. 9b). Mean concentrations of IGF-1 differed by period ($P < 0.0004$) during the common growth period, however did not differ by diet or gain (Fig. 10).

Discussion

Feeding diets high in energy to early-weaned beef heifers in amounts that promote a high rate of gain has been shown to markedly decrease age at puberty [8, 13-15], including heifers with *Bos indicus* influence [81]. However, no studies have examined whether the source of dietary energy (concentrate or forage), and thus the type of ruminal volatile fatty acid production pattern, is an important factor in this process.

In the current study, we set out to examine these relationships during a period of development in the heifer that is critical for early onset of puberty, and the associated endocrine patterns. In the current study, dietary treatment had no effect on age at

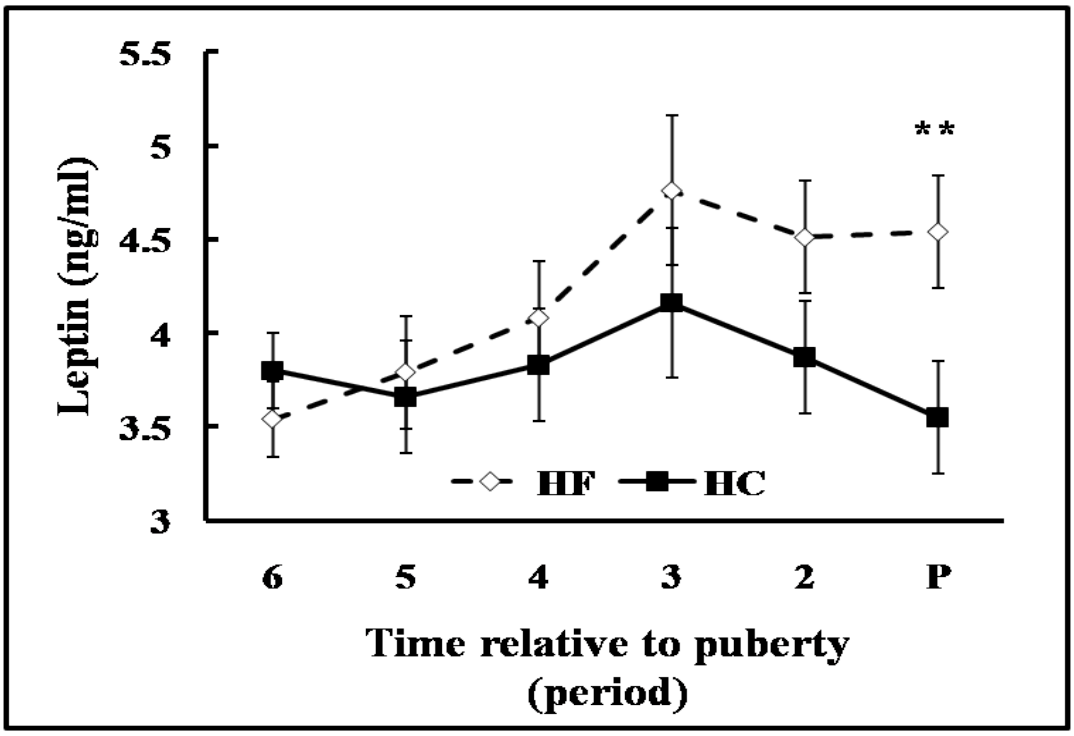

FIG. 8. Mean (±SEM) circulating concentrations of leptin during the common growth period preceding puberty (P) in heifers fed high-forage (HF) and high-concentrate (HC) diets. Concentrations of leptin in HF heifers were greater than HC heifers at puberty (**$P < 0.03$).

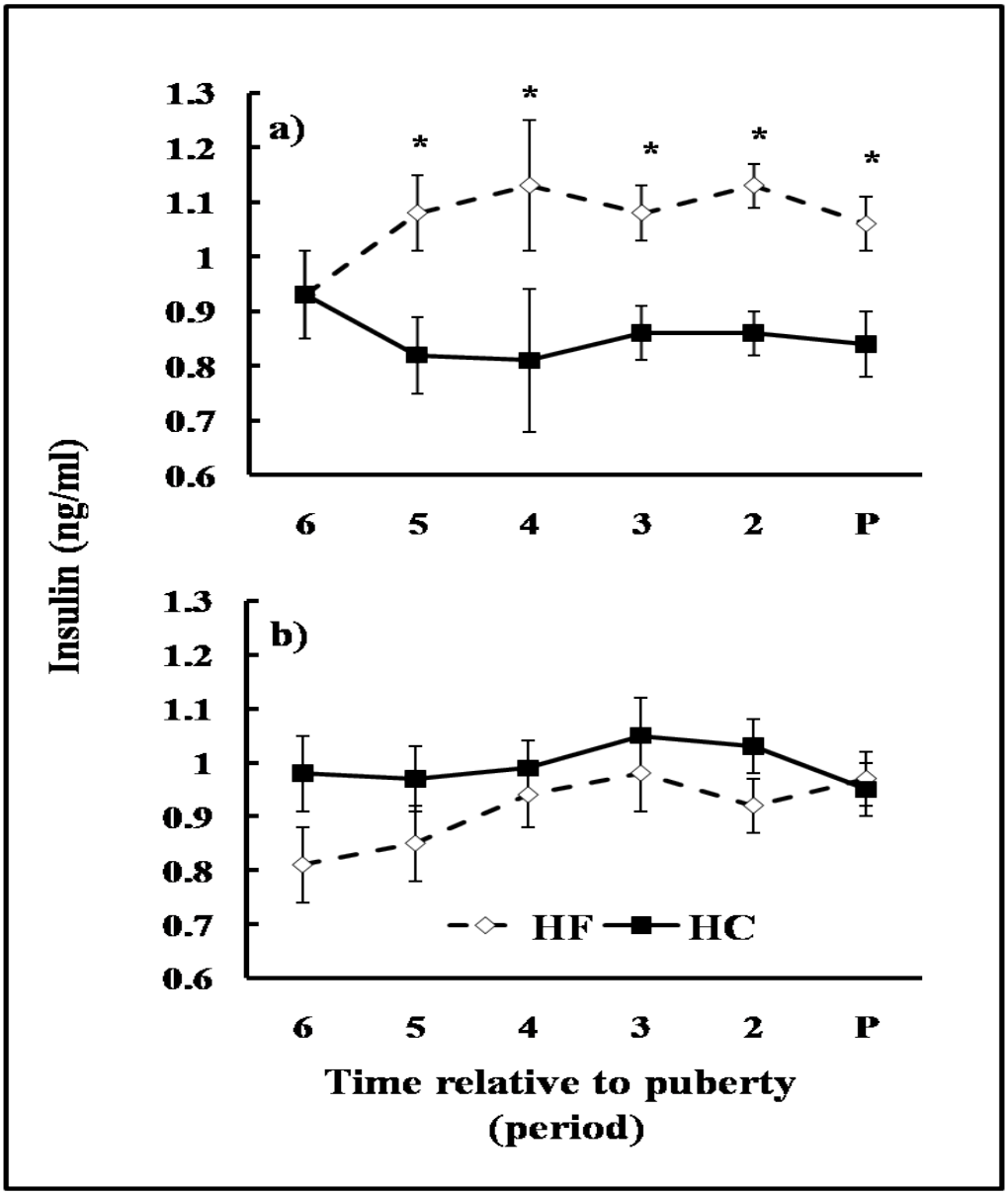

FIG. 9. Mean (± SEM) circulating concentrations of insulin during the common growth period preceding puberty (P) in heifers fed high-concentrate (HC) and high-forage (HF) diets. In replicate 1 (a), insulin was greater in HF than HC heifers at puberty, and during the 4 preceding periods (*$P < 0.02$). Neither diet nor period was a significant source of variation in replicate 2 (b) heifers.

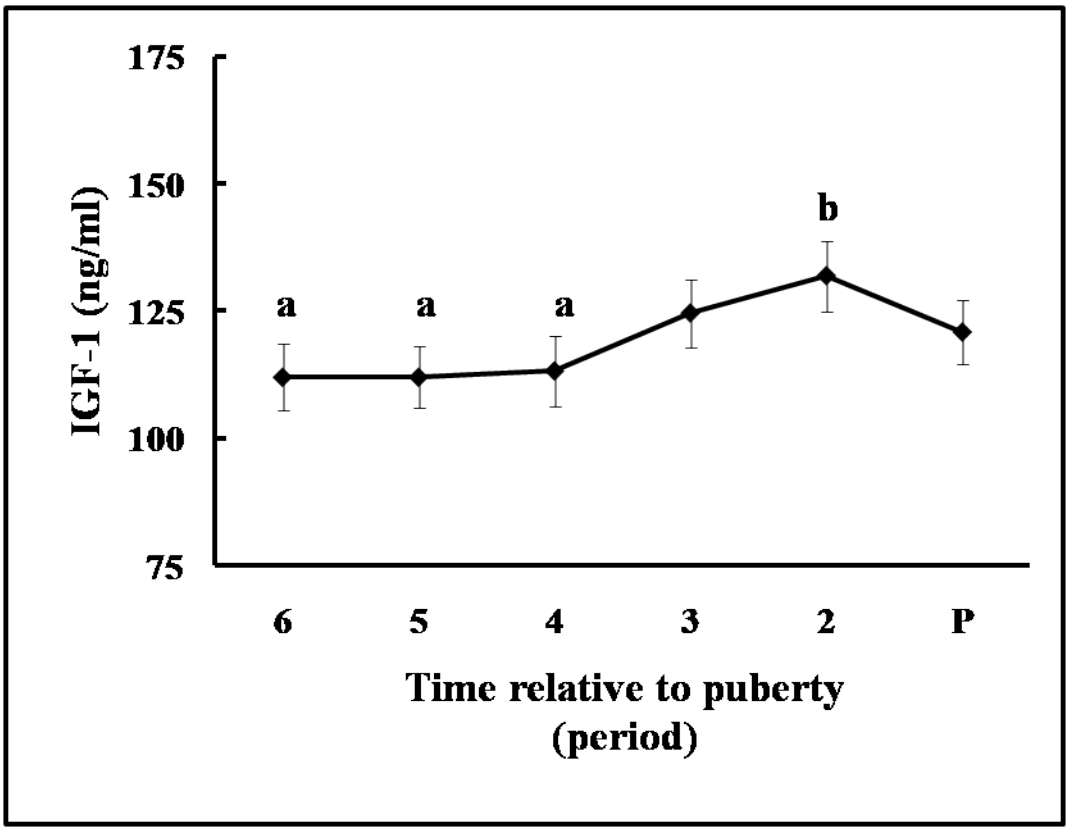

FIG. 10. Mean (± SEM) concentrations of IGF-1 in all heifers during the common growth period preceding puberty (P). Mean concentrations of IGF-1 differed by period (a,b $P < 0.0004$) however did not differ by diet or gain (Fig. 10).

puberty; however, heifers in both HG groups reached puberty at younger ages (54 wk) than heifers in the LG groups (60 wk). Nonetheless, very few heifers exhibited precocious puberty (≤ 43 wk). Several studies by Gasser and co-workers [13-15], in addition to previous observations in our laboratory [81], demonstrated that early weaned heifers fed high-concentrate diets until puberty become pubertal at earlier ages than heifers fed control diets. Unlike the studies by Gasser and others [13-15] and Garcia and others [81], where a high incidence of precocious puberty was observed, heifers in the current study were not fed experimental diets continuously until puberty was confirmed. In the current study, heifers were weaned at approximately 3.5 mo of age and following a 2 wk acclimatization period, were fed experimental diets for 14 wk, after which they were switched to a common growth diet to stimulate a moderate average daily gain. The timing and duration of the dietary treatment period is similar to an additional study by Gasser and others [8] in which heifers were weaned at approximately 3 months of age and fed high-concentrate or control diets for 70 days, after which half from each group were switched to the opposite diet until puberty. A greater percentage (67%) of heifers fed the high-concentrate diets continuously after weaning demonstrated precocious puberty than those switched to the control diet (47%) and those fed a control diet and switched to a high-concentrate diet (47%) [8]. Although a high incidence of precocious puberty was not observed in the current study, it must not be overlooked that heifers gaining body weight more rapidly during the juvenile period reached puberty at earlier ages that those gaining at a slower rate.

It is possible that individual genotype and phenotype is also an important consideration affecting age at puberty. *Bos indicus* x *Bos taurus* crossbred cattle are typically later maturing (14-16 mo of age) than straight *Bos taurus* cattle [19] which usually reach puberty between 11 and 14 mo of age. However, there is great variability in this phenomenon in both straight *Bos taurus* and *Bos indicus* x *Bos taurus* cattle. Although the heifers used in the current study were quite similar to those used previously in this laboratory, it is possible that there was a greater number of heifers with a later-maturing phenotype (larger frame score; heavier mature weight) compared to those involved in the earlier report [81]. Based on results reported in the current study, and results from the latter of Gasser's studies [8], we speculate that heifers exhibit heightened sensitivity to dietary intake from 3-7 months of age, and that the period of time following 7 months of age is equally critical for accelerating pubertal development. Additionally, perhaps had the dietary treatment period been extended beyond 7 months of age, a greater number of animals would have reached precocious puberty. Since no difference in age at puberty was observed between heifers fed high-concentrate and high-forage diets, it would appear that only rate of body weight gain is a significant factor in accelerating pubertal development. Nonetheless, since neither HG group exhibited high rates of precocious puberty, additional work would be required in order to extend this conclusion to the occurrence of precocious puberty.

The pattern of circulating leptin in the HC/HG heifers during the 14-wk dietary treatment period differed markedly from all other groups. Beginning at approximately 24 wk of age, after 8 wk of feeding, heifers in the HC/HG group exhibited a marked

increase in concentrations of circulating leptin that was sustained for the remainder of the 14-wk post-weaning period. Concentrations of leptin in all other treatment groups remained relatively unchanged during the same period. Importantly, once heifers in the HG-HC treatment group were switched to the common growth diet, concentrations of leptin decreased to values similar to those observed in all other treatment groups. Leptin signaling is essential for normal reproductive function to occur [65], and although leptin alone has not been demonstrated to accelerate the maturation of the hypothalamic-hypophyseal complex in heifers [70, 73], it is believed to have a permissive effect on the initiation of puberty. Garcia et al. and others reported that normally-developing heifers exhibit a progressive increase in circulating leptin as puberty approached [69, 81]. In the current study, a gradual increase in serum leptin in heifers in 3 of 4 dietary treatment groups (HF/HG, HF/LG, HC/LG) was observed during the 14-wk dietary treatment period. However, in the HC/HG group, the abrupt increase noted in serum leptin between 24 and 30 wk of age did not wane until heifers were switched to the common growth diet at the end of the 14-wk dietary treatment. This distinguishing feature of the HC/HG group could represent an important signal for neurons within the hypothalamus during a critical window of development and is likely indicative of metabolic responses to the combination of high-energy feeding and high-rate of gain. In particular, perhaps sustained leptin signaling after 7 months of age, in addition to the 3-7 month juvenile

period, is necessary for acceleration of pubertal development. Interestingly, HC heifers demonstrated a progressive decrease in concentrations of leptin as puberty approached, and HF heifers exhibited greater concentrations of leptin at puberty.

Circulating concentrations of insulin also was affected by rate of gain during the 14-wk dietary treatment period, although not to the same extent as leptin. Similar to leptin, HF heifers exhibited greater concentrations of circulating insulin than HC heifers during the common growth period, although only in replicate 1. It is of no surprise that leptin and insulin displayed similar patterns during the common growth period since both hormones tend to change in the same direction in response to dietary alterations. However, the elevation of both hormones in HF heifers was unexpected, particularly since only heifers in one replicate demonstrated a difference in serum insulin.

In summary, the endocrine responses observed in the current work are similar to those normally associated with pubertal development in heifers [25, 69, 81]. In general, it appears that ADG in the current experiment was the most important variable affecting age at puberty. However, the pattern of leptin secretion that was unique to HC/HG heifers is an interesting and potentially relevant finding that may contribute to our understanding of hormonal cues involved in acceleration of the pubertal process. The dramatic increase observed for this variable in the HC/HG group suggests the possibility that dietary energy source does play an important role in regulating hormonal cues

during development. Thus, had we extended the duration or altered the timing of the experimental feeding period postweaning, we may have observed a high frequency of precocious puberty similar to that observed previously [8, 13-15, 81]. In the experiment described in the previous chapter, we observed a marked reduction in expression of NPY, AgRP, and GHR genes in the ARC of heifers fed the HC/HG diet compared to HF/LG. Those changes are consistent with a reduction in inhibitory tone within the reproductive neuroendocrine system. Thus, although heifers in that experiment were slaughtered well before puberty, observations from two studies, including differential gene expression in the ARC (Experiment 1) and abrupt increases in circulating leptin (current experiment), indicate that HC/HG diets can create unique metabolic and hormonal effects that distinguish them from both HF/LG and HF/HG diets. Thus, further studies are warranted to integrate this information into a cohesive strategy for managing the pubertal process in heifers.

CHAPTER V

SUMMARY AND CONCLUSIONS

Studies reported herein demonstrated that weaning heifers at approximately 3 months of age and feeding two levels of dietary concentrate for 14 wk effects differential changes in gene expression within the ARC of the hypothalamus. The three genes of interest, *NPY*, *AGRP*, and *GHR*, are of particular importance since they are thought to be involved in processes relaying metabolic signals to the central reproductive axis through neuronal pathways emanating from the within the ARC. Our findings support previous studies demonstrating the involvement of *NPY* and *AGRP* in mediating pubertal development, as well reproductive cyclicity in mature animals. In this context, there is strong evidence that both genes respond to metabolic signaling (leptin and insulin) by concurrently stimulating feeding behavior, suppressing the effects of *POMC*, and exerting inhibitory effects on the reproductive neuroendocrine axis. Although the relationship between pubertal development and the decreased expression of *GHR* in the high-concentrate high-gain heifers is not as clear, previous reports indicate that *GHR* is localized in the majority of all NPY/AGRP neurons, and it appears that GH mediates the effects of NPY via the GHR. Finally, our results suggest that IGF-1, insulin and leptin are likely involved in regulating expression of these genes, particularly since high-concentrate high-gain heifers exhibited increased concentrations of all three hormones, and decreased expression of all three genes of interest.

In the second experiment, although only a few heifers attained precocious puberty, it is clear that increased ADG during the juvenile period decreased the age at puberty. The level of dietary concentrate did not appear to affect age at puberty; however, since only few animals attained precocious puberty, it is difficult to draw definitive conclusions regarding the role of this factor in precocious puberty. It is possible that extending the dietary treatment period in the types of cattle used in our experiments would help clarify this issue. The most striking observation in the second experiment appears to be the elevation in concentrations of leptin in HC/HG heifers during the 14-wk dietary treatment period. Of equal importance is that after heifers were switched to the common growth diet, leptin decreased to concentrations similar to heifers in all other treatments during the 14-wk dietary treatment period. It is possible that sustained increases in leptin signaling during the juvenile period that extend past 7 months of age is necessary for accelerated maturation of the reproductive neuroendocrine axis. In conclusion, we have demonstrated the ability to modify expression of a multitude of genes within the ARC by controlling intake, and feeding high- and low-concentrate diets during the juvenile period. Furthermore, we have pinpointed three genes (*NPY*, *AGRP*, and *GHR*) previously demonstrated to be linked to metabolism and reproduction that were differentially expressed between dietary treatments. As these genes are regulated by leptin and insulin, and it is possible that elevated leptin during the juvenile period, as demonstrated at slaughter in HC heifers (experiment 1) and in temporal concentrations in HC/HG heifers (experiment 2) may

modulate the expression of genes important for the metabolic control of puberty, such as *NPY*, *AGRP*, and *GHR*.

Furthermore, it appears that, although heifers exhibit heightened sensitivity to dietary intake from 3-7 months of age, the period of time following 7 months of age may also contribute to the expression of precocious puberty in *Bos indicus* x *Bos taurus* heifers. Since precocious puberty was not a prominent feature in Experiment 2, it is not possible to discern whether occurrence of the phenomenon is dependent upon a high-concentrate diet. Thus, further studies are warranted.

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APPENDIX A

PROCEDURES

Insulin-like Growth Factor-I Radioimmunoassay

1. Iodination:

   Reference: Iodination grade r-hIGF-1 (R&D Systems Inc., Minneapolis, MN; Cat # 291-G1)

   Reaction: 3 µg of hormone, 0.5 mCI of ^{125}I, 3.5 µg Chloramine T, 60 sec, 22 µg Na-Metabisulfate

2. Antibody: Polyclonal anti-human IGF-1 (rabbit anti-human IGF-1; **LOT #** NHPP) *Dilution: 1:50,000*

3. Standards: r-hIGF-1 (R&D Systems Inc., Minneapolis, MN; Cat # 291-G1) *Range: 0.156 – 10 ng/ml*

4. Reference preparation: CSS from cows with IGF-1 added in (see lab notebook/notes)

5. RIA Procedure

   a) Label assay sheets and polypropylene tubes (IGF-1 sticks to glass)

      4 NSB, 9 TC, 3 "0", standards in triplicate, references in triplicate, and unknown samples in triplicate

   b) Day 1: Extraction

      i) Pipette 50µl of unknown sample or references and 950 µl 0.2 M Glycylglycine Hydrochloride Solution into polypropylene extraction tubes
      ii) Vortex and place in room temperature water bath for 24 hr

   c) Day 2: Assay Procedure

      i) Remove extraction tubes from water bath, vortex, add 1 ml IGF-1 assay buffer, vortex and place in water bath for additional 6 hr
      ii) Following incubation, remove tubes from water bath, vortex briefly and pipette the following:

NSB: 400µl IGF-1 assay buffer

0 STD: 300µl IGF-1 assay buffer

STDs: 100µl STD + 200µl IGF-1 assay buffer

References: 100µl extracted reference + 200µl IGF-1 assay buffer

Unknowns: 100µl extracted sample + 200µl IGF-1 assay buffer

Vortex tubes and incubate overnight at 4^{o} C (minimum time)

d) Day 3: Assay Procedure

i) Pipette 100µl anti-IGF-1 primary antibody (diluted in IGF-1 assay buffer) into all except NSB and TC tubes and incubate for 1 hr at 4^{o} C

ii) Pipette 100µl ^{125}I IGF-1 tracer (~20,000 cpm/tube; diluted in IGF-1 assay buffer) into all assay tubes, vortex, and incubate at 4^{o} C for 16-24 hr

iii) Prepare precipitated secondary antibody/NRS

Make 1:8 dilution of 2^{nd} antibody (Who 30) in IGF-1 assay buffer

Make 1:150 dilution of NRS in IGF-1 assay buffer

Mix 2^{nd} antibody and NRS solutions 1:1 and incubate overnight at 4^{o} C

e) Day 4: Secondary Antibody

i) Finish 2^{nd} antibody preparation

ii) Briefly stir 2^{nd} antibody/NRS solution and centrifuge for 30 min at 2900 rpm at 4^{o} C

iii) Decant supernatant and bring back to original volume with new IGF-1 assay buffer

iv) Pour buffer from each tube with pellet into a glass homogenizer and homogenize pellet into solution. Combine into one beaker after homogenizing

v) Pipette 200µl of 2nd antibody/NRS solution into each assay tube, vortex and incubate at 4^{o} C for 4 hr

vi) Add 200µl 15% polyethylene glycol + 1 ml 0.01 M PBS into all tubes except TC tubes

vii) Centrifuge tubes at 4^{o} C for 45 min at 3600 rpm, decant supernatant (do not leave tubes upside down for more than 30 sec or pellet may slip) and count pellet on gamma counter

INSULIN RADIOIMMUNOASSAY

Using Siemens Coat-A-Count Insulin RIA Kit

References: 1) Accorsi PA, Govoni N, Pezzi C, Seren E, Taminini C. Reprod Dom Animal Leptin, GH, PRL, Insulin and Metabolic Parameters Throughout the Dry Period. 2005 40; 217-223

2) Siemens Healthcare Diagnostics Coat-A-Count Insulin Kit, Los Angeles, CA

I. Assay Set Up

A) Label assay sheet and number six (6) polypropylene test tubes (12 x 75 mm) as follows: Tube #'s

a) 4 Total Count (TC) 1-3

b) 2 Non-specific Binding (NSB) 4-5

B) Label lime green antibody coated tubes (supplied by the kit) for the:

C) standards, references, and unknown samples in duplicate as follows:

Tube #'s

c) 3, 0 STD tubes 6-8

d) Triplicate tubes for each of the following 9-35

STD's: 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0 and 2.5 ng/ml

e) Duplicate tubes for the Low, Mid, and High references 36-41

f) Duplicate tubes for the serum blank (CSS) 42-43

g) Duplicate tubes for each serum/plasma unknown sample 44- onward

II. Assay Protocol (2 day assay)

A) Pipette 200 µl of PBS-1% BSA into NSB and 0 standard tube

B) Pipette 200 µl of each of the standards in triplicate

C) Pipette 200 µl of each unknown sample, references and css in duplicate

D) Pipette 1.0 ml of insulin tracer into every tube using the Eppendorf repeater pipettor with the insulin tip from the isotope room. Cover with aluminum foil and vortex briefly.

NOTE: The ^{125}I-insulin has to be at **room temperature for at least 30 minutes before** dispensing into assay tubes. **No more than 10 minutes** should elapse during the dispensing of the tracer, to ensure equal incubation time for all the tubes. Therefore, run only **350 maximum** in an assay to ensure a 10 minute dispense time for the tracer.

E) Incubate for 18-24 hours at room temperature in the isotope room.

F) After completion of incubation, transfer all tubes **except** total count tubes to Styrofoam racks and decant thoroughly into waste receptacle in the ISO lab hood. Strike or thump tubes sharply on adsorbent paper

to shake off all residual droplets. The bound insulin will stick to the insides of the antibody coated tube and will not be dislodged by the thumping. Be sure to remove all visible moisture from the tubes since this increases counting precision.

G) Load tubes into gamma counter with insulin clip and press start.

Cresyl Violet Staining for bovine Hypothalamic Tissue Sections Fixed in 4% Paraformaldehyde

I. Reagents and Materials

1. 250 ml 4% Paraformaldehyde

   10 g Paraformaldehyde
   125 ml .2 M PB
   125 ml ddH20
   (Add paraformaldehyde with 125 ml .2 M PB and 50 ml ddH20, heat to approximately 65□C; add remaining ddH20 slowly until solution is dissolved)
2. Cresyl Violet solution (filter before each use)

3. Glacial Acetic Acid

4. 70 %, 95 % and 100% Ethanol

5. Acidified 70% Ethanol

   5 ml acetic acid
   245 ml 70% Ethanol
6. 10X Acetate Buffer

   2.5 g Sodium Acetate (MW 136.08)

   Dissolve in approximately 450 ml ddH20
   Adjust pH to 3.5 with glacial acetic acid (approximately 20 ml)
   Adjust final volume to 500 ml

II. Procedure

1. Air dry slides and place in 4% PAF for 15 minutes

2. Dip twice in ddH20

3. Place slides in 1X Acetate Buffer for 5 minutes

4. Place slides in Cresyl Violet solution for 20-30 minutes

5. Dip twice in Acidified 70% Ethanol

   *Acidified 70% Ethanol should not be used for more than 2 racks

6. Dehydrate sections through Ethanol as follows

   a) 70% EtOH- Dip three times
   b) 95% EtOH- Dip several time for 1 minute
   c) 100% EtOH for 5 minutes

7. Place slides in Citrisolv 3 times for 5 minutes each

8. Cover slip using DPX

RNA Isolation: RNAqueous®-Micro (Ambion) Protocol for Isolation of Small Quantities of RNA

Fresh Frozen Sections on Slides

Reagents and Materials

RNAqueous®-Micro Kit
1 Micro filter Cartridge Assembly/Sample
1 Micro Elution Tube/Sample
Wash Solution 1 (4□C; verify that 100% EtOH was added to the bottle)
Wash Solution 2/3 (4□C; verify that 100% EtOH was added to the bottle
Lysis Solution (4□C)
DNase (-20□C)
10X DNase I Buffer (-20□C)
DNase Inactivation Reagent (-20□C)
Elution Solution (4□C)

Additional Materials

1.5 ml Micro Centrifuge Tube/Sample
5, 0.65 ml Micro Centrifuge Tubes/Sample
100% Ethanol

I. Preparation

a) Label a Micro Filter Cartridge Assembly and Micro Elution Tube for each sample
b) Aliquot 185 µl of Wash Solution 1 into .64 ml Micro Centrifuge Tube and warm to RT
c) Aliquot 370 µl of Wash Solution 2/3 into .65 ml Micro Centrifuge Tube and warm to RT
d) Heath aliquot (50µl per sample) of Elution Solution to 95□C on heat block
e) Heat water back to 42□C

II. Tissue Harvest

a) Fill 1 ml syringe to 400µl (0.4 ml) with Lysis Solution
b) Scrape appropriate tissue section with 25 gauge 5/8 needle

c) Transfer scraped tissue and small amount of Lysis Solution to 1.7 ml Micro Centrifuge Tube
d) Repeat procedures b and c until desired amount of tissue is harvested
e) Adjust volume in Micro Centrifuge tube to 400µl with remaining Lysis Solution
f) Incubate sample for 30 min at 42□C; vortex briefly after 15 minutes of incubation
g) Centrifuge for 30 sec to collect fluid at bottom of tube
Remove 10 DNase I Buffer from -20ºC and thaw on ice

III. Preparation of Micro Filter Cartridge Assembly and Lysate Mixture

a) Add 30 µl of Lysis Solution to center of filter and allow to soak for ≥5 minutes
b) Add 3 µl of LCM Additive to Lysate Mixture and mix well by briefly vortexing
c) Briefly centrifuge Lysate Mixture to collect the fluid at the bottom of the tube
d) Add 200 µl of 100% Ethanol to Lysate Mixture tube*

IV. Lysate Mixture Filtration

a) Centrifuge Micro Filter Cartridge Assembly for 30 sec to remove Lysis Solution
b) Load 150 µl Lysate/Ethanol mixture into prepared Micro Filter Cartridge assembly and close cap tightly**
c) Centrifuge Lysate Mixture for 1 min at 10,000 x g
d) Repeat steps b-d until all Lysate/ethanol mixture is passed through the filter

V. Filter Wash

a) Add 180 µl of Wash Solution 1 to the filter and close cap tightly
b) Centrifuge for 1 min at 10,000 x g
c) Add 180 µl of Wash Solution 2/3 to the filter and close cap tightly
d) Centrifuge for 30 sec at 10,000 x g
e) Repeat steps c and d with a second 180 µl aliquot of Wash Solution
f) Remove Micro Filter Cartridge from the Collection Tube and discard flow-though liquid

g) Replace Filter, close cap and centrifuge assembly for 1 min to remove residual fluid and to dry filter

VI. RNA Recovery

a) Transfer Micro filter Cartridge from Collection Tube to Micro Elution Tube
b) Add 10 µl of preheated (95□C) Elution Solution to center of filter
c) Close cap and allow solution to sit for 5 min at RT
d) Centrifuge assembly at 10,000 x g for 1 min to elute RNA from filter
e) Repeat steps b-d with a second 10 µl aliquot of preheated Elution Solution collecting the elute in the same Elution Tube
f) Pipette entire sample into a fresh DNase-free .65 ml Micro Centrifuge Tube

VII. DNase I Treatment and DNase Inactivation

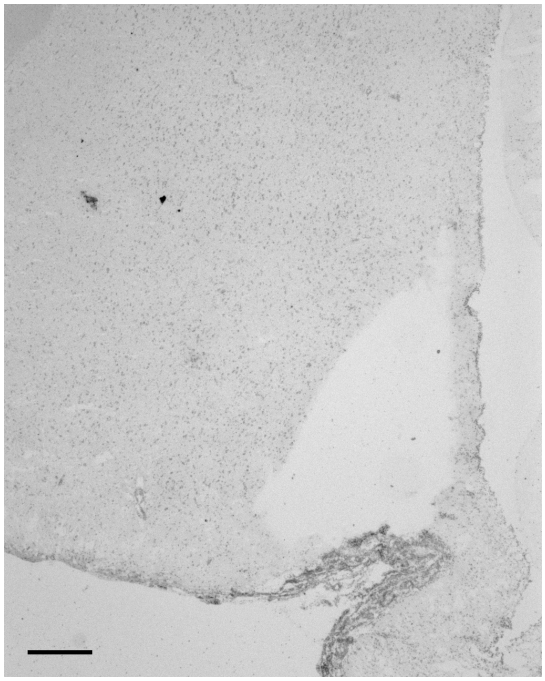
a) Add 2 µl of 10X DNase Buffer and 1 µl of DNase to the sample and mix gently
b) Incubate DNase reaction for 20 min 37□ C (Remove DNase Inactivation Reagent from -20□C and allow to thaw at RT)
c) Vortex DNase Inactivation Reagent vigorously to re-suspend slurry
d) Add 2.2 µl of DNase Inactivation Reagent to reaction and incubate at RT for 2 min
e) Vortex and incubate at RT for additional 1 min
f) Centrifuge the reaction for 1.5 min at 10,000 x g
g) Transfer 20 µl of RNA to a fresh DNase-free .65 Micro Centrifuge Tube and store at -80□C

*Volume of 100% EtOH added is dependent on if you are recovering Large RNA Species, or Large and Small RNA Species. See RNAqueous®-Micro manual for additional information.

**Caps on Collection Tubes and Elution Tubes tend to be stiff and can pop open. Bend back and forth to loosen hinge and make sure cap is on tight.

APPENDIX B

IMAGES



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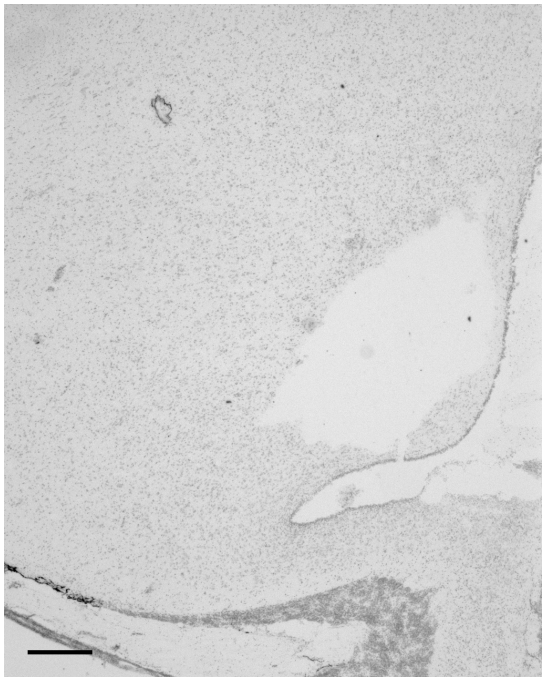

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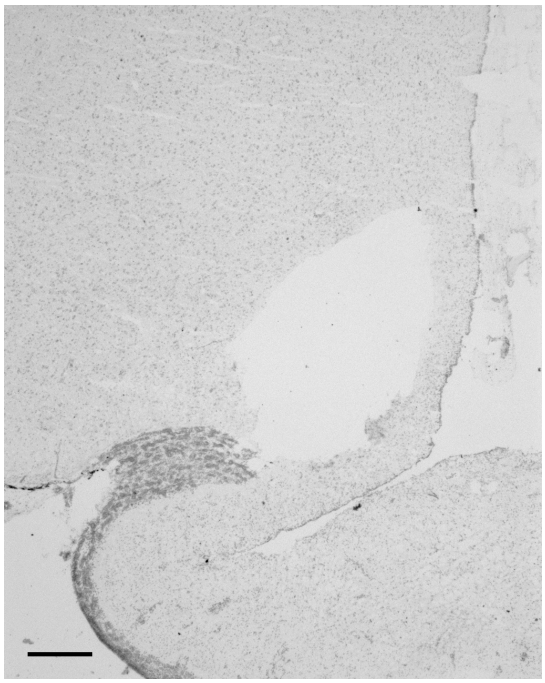

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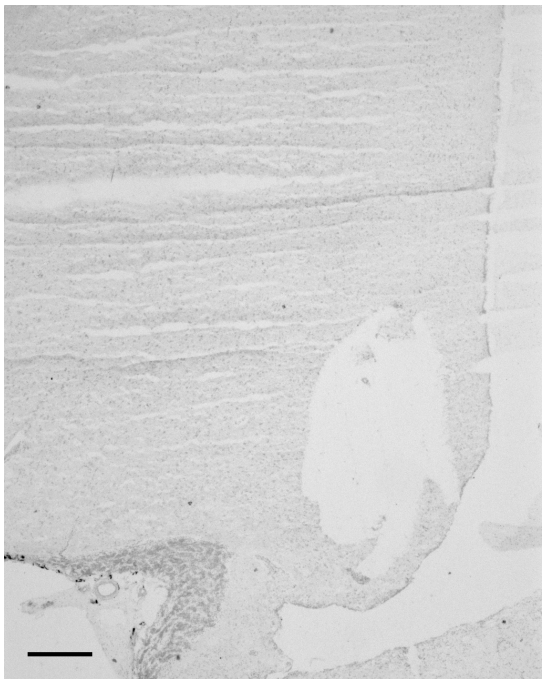

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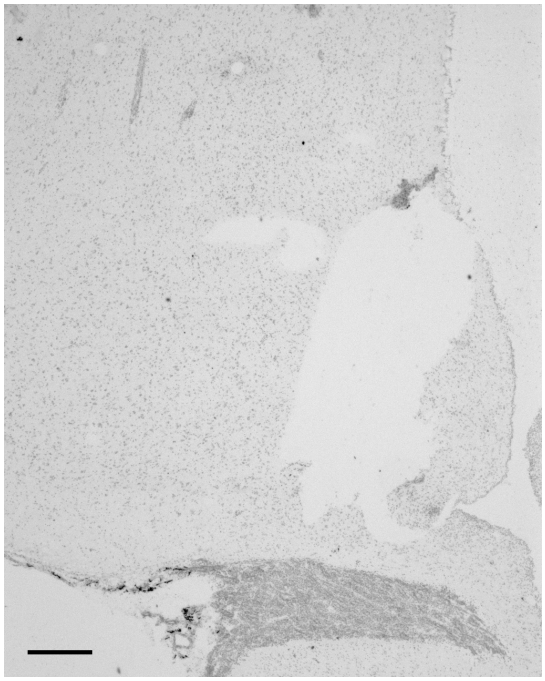

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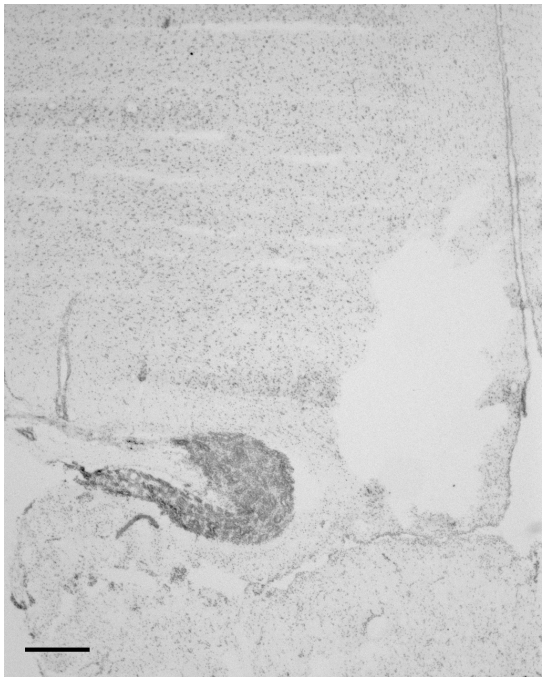

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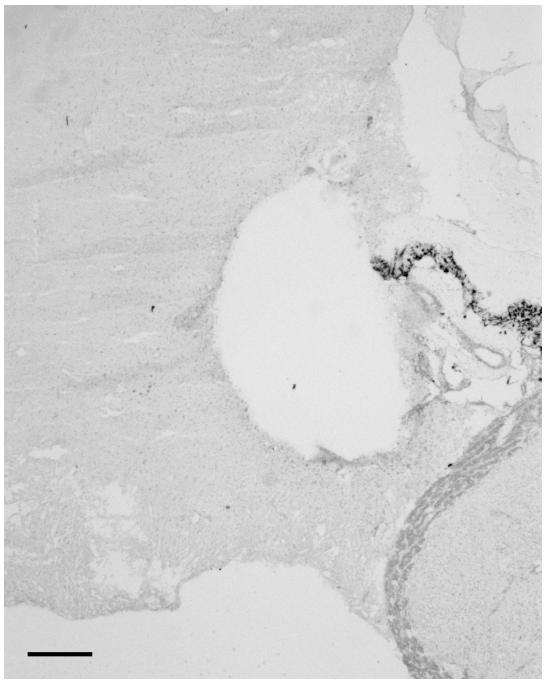

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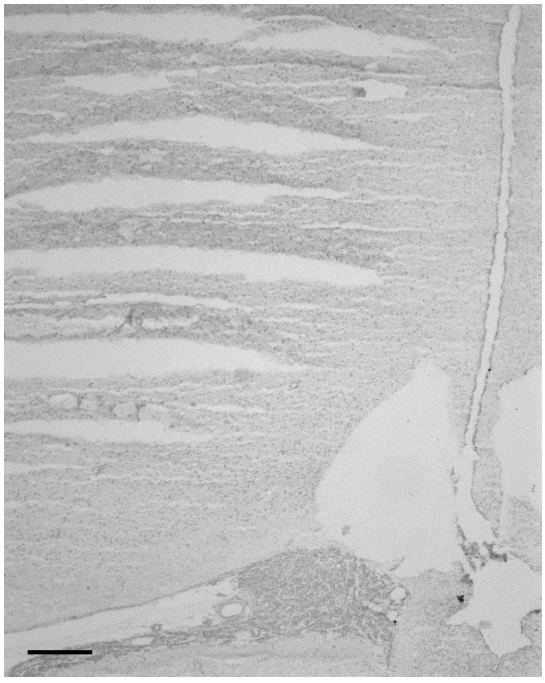

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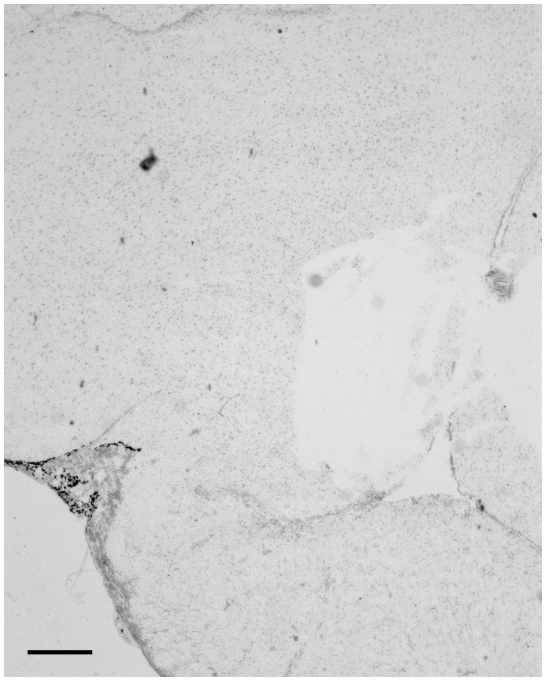

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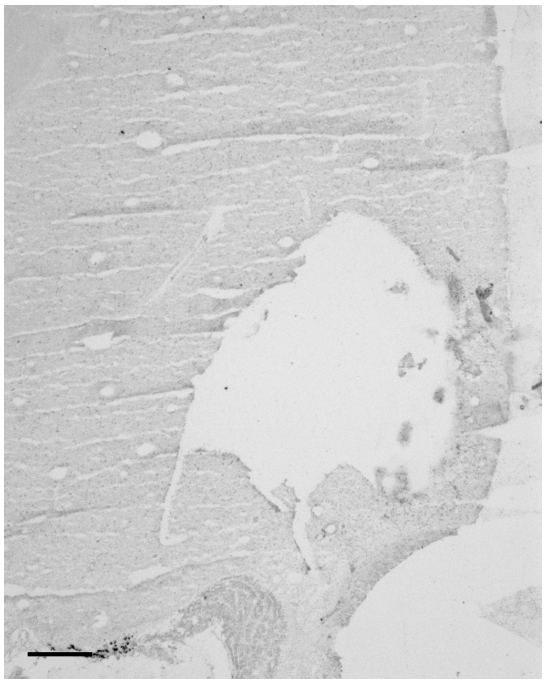

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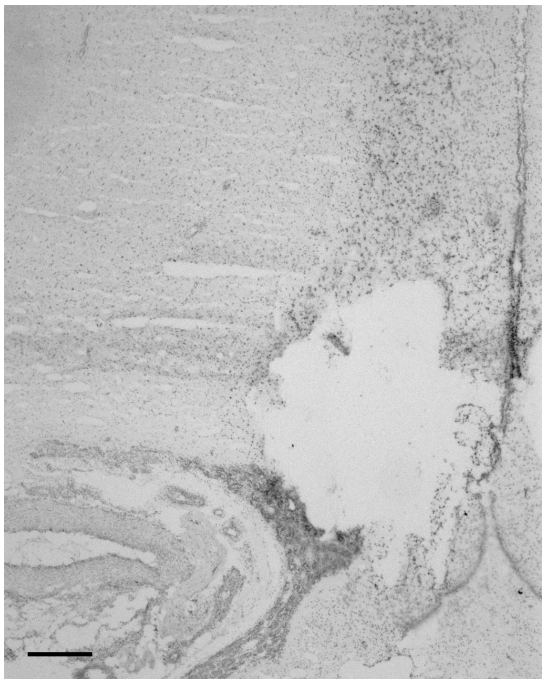

Heifer 7592

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