NUTRITIONAL PROGRAMMING OF PUBERTY IN BEEF HEIFERS

A Dissertation

by

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ABSTRACT

Nutritional and metabolic statuses during postnatal development control the timing of pubertal maturation in mammals. Studies reported herein had two overall aims. In Aim I, objectives were to characterize functional and morphological modifications that occur in the neuroendocrine system of prepubertal heifers in response to increased body weight (BW) gain during calfhood. We first tested the hypothesis that hypothalamic release of neuropeptide Y (NPY), an important inhibitory peptide involved in the integration of metabolism and reproduction, would be decreased in heifers that gained BW at higher rates. Confirming our hypothesis, heifers that gained 1 kg/day had lower concentrations of NPY in the third ventricle cerebrospinal fluid than heifers that gained 0.5 kg/day. These data corroborate previous results from our group that demonstrated that similar nutritional treatments result in decreased hypothalamic *NPY* expression and neuronal projections to gonadotropin-releasing hormone (GnRH) cells. We then tested the hypothesis that proopiomelanocortin (POMC) cells, a critical excitatory metabolic-sensing pathway in the hypothalamus, could be involved in the nutritional control of puberty. Heifers that gained BW at higher rates had greater *POMC* expression in the arcuate nucleus and an increased number of kisspeptin neurons innervated by fibers containing melanocyte-stimulating hormone alpha (αMSH), a product of the *POMC* gene. Very few GnRH neurons were observed in close apposition to αMSH fibers and these projections were not dependent on metabolic status. Results suggest that melanocortin signaling through arcuate kisspeptin neurons may be an

important mechanism involved in the nutritionally-programed acceleration of puberty in heifers. In Aim II we hypothesized that metabolic programming of processes underlying puberty could be shifted temporally during calfhood through the use of a stair-step, compensatory growth model. Results indicated that nutritional restriction between 6.5 to 9 mo of age did not affect the proportion of heifers pubertal by 12 mo of age when compared to continuous accelerated BW gain. Furthermore, we observed that increasing nutrient availability between 6 and 9 mo of age may potentially time pubertal onset consistently at around 12 mo of age while avoiding precocious puberty. Therefore, similar managerial strategies may allow optimal timing of sexual maturation in replacement beef heifers.

DEDICATION

This dissertation is dedicated to my parents and my wife,

for their love, endless support and encouragement.

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NOMENCLATURE

IIIV	Third ventricle
ACTH	Adrenocorticotropic hormone
ADG	Average daily gain
AgRP	Agouti-related peptide
ARC	Arcuate nucleus
AVPV	Anteroventral periventricular nucleus
BCS	Body condition score
BW	Body weight
cARC	Caudal arcuate nucleus
CART	Cocaine and amphetamine regulated transcript
CSF	Cerebrospinal fluid
DMI	Dry matter intake
END	Endorphin
GH	Growth hormone
GnRH	Gonadotropin-releasing hormone
GPR54	G-protein coupled receptor 54
ICV	Intracerebroventricular
Ig	Immunoglobulin
IGF1	Insulin-like growth factor 1
LH	Luteinizing hormone

mARC	Middle arcuate nucleus
MBH	Mediobasal hypothalamus
MC-R	Melanocortin receptor
MSH	Melanocyte-stimulating hormone
NPY	Neuropeptide Y
PMV	Ventral premammillary nucleus
POA	Preoptic area
POMC	Proopiomelanocortin
rARC	Rostral arcuate nucleus
RIA	Radioimmunoassay
SAS	Statistical analysis software
STAT3	Signal transducer and activator of transcription 3
T3	Triiodothyronine
T4	Thyroxine

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CHAPTER I

INTRODUCTION

Puberty is a key developmental process, during which complex events must occur to culminate in the maturation of the hypothalamic-pituitary-gonadal axis and, consequently, in first ovulation (Parent et al., 2003). Sexual maturation and initiation of estrous cycles in heifers well in advance of the initial breeding season is critical for early conception and pregnancy (Byerley et al., 1987, Bagley, 1993), and results in increased lifetime productivity (Lesmeister et al., 1973). Genetics and nutrition play key roles in attainment of puberty (Moran et al., 1989, Patterson et al., 1992) and improvement in post weaning metabolic status is an important factor regulating age at puberty and age at first calving (Patterson et al., 1992).

As heifers approach puberty, there is a decline in the sensitivity to estradiol negative feedback, resulting in increased pulsatile release of luteinizing hormone (LH). This increased release of gonadotropins stimulates the development of the dominant follicle, leading to increased concentrations of circulating estradiol, and ultimately causing the preovulatory surge of LH and first ovulation (Schillo et al., 1982, Day et al., 1987). Although numerous studies have demonstrated that nutrition and metabolism play major roles in controlling pubertal onset, the neuroendocrine pathways regulating this process have not been completely elucidated. However, it is clear that hormones such as leptin, insulin, and other metabolic cues collaborate to control neuroendocrine

reproductive maturation by targeting various hypothalamic circuitries (Gamba and Pralong, 2006, Pralong, 2010).

Among the central neuropeptides that have been described as potential mediators regulating pubertal onset is kisspeptin, the product of the *Kiss1* gene. Kisspeptin appears to act as an essential gatekeeper of sexual maturation and fertility, mediating important reproductive processes, including maturation of the hypothalamic-pituitary-gonadal axis, steroidal feedback regulation of gonadotropin secretion, and the preovulatory surge of LH, among others (Gianetti and Seminara, 2008, Pineda et al., 2010). Studies have demonstrated that deletions and inactivating mutations of the kisspeptin receptor (*GPR54*) gene were related to a failure to attain puberty and hypogonadotropic hypogonadism (de Roux et al., 2003, Seminara et al., 2003). Moreover, kisspeptin has been described as a potent stimulator of gonadotropin secretion in a large diversity of species (Roa et al., 2008, Felip et al., 2009, Roa et al., 2009, Roseweir and Millar, 2009).

Other neuropeptides that have been proposed as mediators of the effects of metabolic status on timing of puberty include neuropeptide Y (NPY) and the melanocortins. Central administration of NPY has been shown to inhibit gonadotropin releasing hormone (GnRH)/LH output in ewes (Barker-Gibb et al., 1995). In addition, daily injections of leptin have been shown to reduce hypothalamic *NPY* mRNA abundance in adult mice (Ahima et al., 1996). Insulin also has been shown to inhibit hypothalamic NPY gene expression in rodents, when centrally administered (Schwartz et al., 1992). Moreover, proopiomelanocortin (*POMC*) gene expression was reduced in lean, ovariectomized ewes but could be restored after leptin treatment (Backholer et al.,

2010a). Similarly, leptin positively regulates *POMC* mRNA expression in rodent species (Elias et al., 1999). Recent studies have demonstrated that POMC cells communicate with kisspeptin neurons in the arcuate nucleus (ARC) in ewes, forming complex reciprocal connections, and altering expression of genes in the target cells (Backholer et al., 2010b). Therefore, it has been proposed that this network of kisspeptin-, NPY-, and POMC-containing cells could coordinate the central nervous system control of reproduction.

An expansion of our understanding of the foregoing processes in female mammals in general, and the integration and application into management of food-producing species, are essential for improving reproductive efficiency. For example, recent studies have demonstrated the ability to greatly reduce age at first ovulation in beef heifers by early-weaning calves at 3 to 4 mo of age and feeding a high energy diet for as little as 70 days (Garcia et al., 2003, Gasser et al., 2006a-d). However, while assuring the onset of puberty well before targeted breeding, this approach requires increased managerial vigilance to avoid accidental pregnancies in skeletally-immature heifers. Furthermore, the costs associated with the intensive nutritional management required in this approach is one of the main factors limiting its wide adoption. Therefore, strategies are needed that facilitate nutritional imprinting of the reproductive neuroendocrine system during early calfhood, while minimizing feeding costs, optimizing the consistent establishment of estrous cycles by 11 to 12 mo of age, and avoiding precocious puberty (puberty at < 300 days of age).

The objectives of work reported in this dissertation were to determine if 1) increased rate of BW gain during early calfhood, resulting in an accelerated onset of puberty, is associated with decreasing concentrations of NPY in the cerebrospinal fluid (CSF) collected from the third ventricle (IIIV); 2) increased rate of BW gain during early calfhood results in early functional changes in arcuate *POMC* gene expression, and hypothalamic melanocortin secretion and neuronal projections; and 3) a stair-step compensatory growth nutritional model can produce consistent acceleration of pubertal onset, with a high proportion of heifers achieving puberty around 12 mo of age while avoiding precocious puberty.

CHAPTER II

REVIEW OF LITERATURE

2.1 Puberty in heifers

Age at first ovulation varies widely in cattle, depending on genetic and environmental factors (Moran et al., 1989), and important events occur during the last several weeks preceding onset of pubertal in heifers. Moreover, studies have demonstrated that there is a progressive increase in fertility as heifers experience multiple estrous cycles before the first breeding (Byerley et al., 1987, Patterson et al., 1992, Bagley, 1993); therefore, attaining puberty well in advance of the first breeding season is fundamental for obtaining optimal conception rates. Furthermore, heifers that calve early during the calving season and rebreed effectively each year thereafter have increased productivity throughout their lifetime (Lesmeister et al., 1973).

During the last 2 to 3 mo prior to first ovulation, there is a gradual decline in sensitivity of the hypothalamus to estradiol negative feedback, an increase in GnRH/LH pulse frequency, leading to growth of larger antral follicles, and enhanced estradiol secretion (Day et al., 1987, Evans et al., 1994, Rawlings et al., 2003). Ultimately, there is a peak in estradiol secretion by the dominant follicle that stimulates a preovulatory surge of LH which induces ovulation (Kinder et al., 1987). The first ovulation in heifers is usually followed by a luteal phase of short duration, which eliminates the chances of pregnancy (Day et al., 1987, Kinder et al., 1987).

Mechanisms regulating the decrease in the hypothalamic response to estradiol negative feedback during the peripubertal period are not completely determined. Recent studies indicate that changes in sensitivity to estradiol negative feedback influence the GnRH neuron indirectly, with specific neuropeptides such as kisspeptin, NPY, and POMC acting as mediators of this process (Gianetti and Seminara, 2008, Backholer et al., 2010b). Moreover, the activity of these neuropeptides is likely regulated by nutritional and metabolic factors which play a key role in signalizing the individual's energy status to the neuroendocrine reproductive axis (Schwartz et al., 1992, Gamba and Pralong, 2006, Pralong, 2010).

2.2 Influence of nutrition on pubertal onset

Nutrition, both pre- and postweaning, has a large impact on the pubertal maturation process in heifers. Studies have demonstrated that pubertal onset can be significantly hastened when heifers are fed diets that promote a relatively high rate of gain after weaning (Garcia et al., 2003, Gasser et al., 2006a-d). Moreover, a reduction in age at puberty was also observed in heifers that exhibited increased preweaning BW gain (Arije and Wiltbank, 1971, Greer et al., 1983). In contrast, when dietary intake is restricted, the timing of first ovulation is delayed (Foster and Olster, 1985, Bergfeld et al., 1994).

In particular, dietary energy intake has been demonstrated to have a pronounced potential to influence the timing of pubertal onset. In a series of studies conducted at The Ohio State University (Gasser et al., 2006a-d), crossbred Simmental and Angus heifers

were weaned around 3 mo of age and fed either a high-concentrate (60% corn) or a control diet (30% corn) until detection of puberty. The authors observed that heifers fed a high-concentrate diet had an anticipated reduction of estradiol negative feedback on LH release, increased frequency of LH pulses, increased follicular development, and a greater incidence of precocious puberty when compared to heifers fed a control diet. Moreover, the same group of researchers reported that early-weaned heifers that were fed a high-concentrate diet from 126 to 196 days of age presented with a similar proportion of heifers exhibiting precocious puberty as heifers fed the same diet from 126 days of age until puberty was detected. Also, heifers fed a control diet from 126 to 196 days of age, and subsequently switched to a high-concentrate diet, presented a low incidence of precocious puberty. According to these observations, rate and timing of BW gain during the early calfhood period are key factors regulating pubertal onset. Therefore, it has been proposed that metabolic programming of the brain for early puberty in heifers is established mainly before 6.5 mo of age.

2.3 Metabolic factors and neuropeptides

Over the years, several factors have been proposed to mediate metabolic and nutritional signaling to the central nervous system. These include glucose (He et al., 1999, Oltmanns et al., 2001), fatty acids (Schneider and Wade, 1989), and growth hormone (GH)/Insulin-like growth factor 1 (IGF1; Hiney et al., 1996, Longo et al., 1998). More recently, several studies have demonstrated an important role for insulin and leptin in modulating the function of GnRH neurons (reviewed by Gamba and

Pralong, 2006). This is evidenced by the observation that mice that have undergone a neuron-specific excision of the insulin receptor (Bruning et al., 2000) or mice lacking a functional leptin gene (Chehab et al., 1996), displayed hypogonadism secondary to impaired release of GnRH.

2.3.1 Insulin

Insulin serves an important role in the control of nutrient substrate metabolism by insulin-sensitive tissues, including the liver, skeletal muscle, and adipose tissue. In the presence of insulin, substrates resulting from food digestion are taken up and metabolized, and energy surplus is stored as fat (reviewed by Gamba and Pralong, 2006). In addition, insulin has been also proposed to be involved in the signaling of satiety to the brain (Woods et al., 1979, Bruning et al., 2000). Hypothalamic neurons that have an important role in controlling energy homeostasis and food intake such as NPY and POMC express insulin receptors (Schwartz et al., 1992, Pardini et al., 2006). Furthermore, intracerebroventricular (ICV) infusion of insulin was shown to inhibit food intake in normal rats and reverse the hyperphagic syndrome of insulin-deficient rats (McGowan et al., 1990).

It has been demonstrated that insulin stimulates GnRH secretion by primary hypothalamic neuronal cell cultures and increases the expression of GnRH (*GNRH1)* mRNA in a GnRH neuronal cell line produced by conditional immortalization of adult rodent GnRH cells (Gnv-3; Gamba and Pralong, 2006). Furthermore, when administered centrally to sheep via ICV cannula, insulin stimulates pulsatile secretion of LH (Miller et

al., 1995). Recent studies have focused on elucidating the pathways by which insulin regulates the neuroendocrine reproductive axis; however, the precise mechanisms that underlie this process and the interactions between neurons involved are only beginning to be revealed.

Hypothalamic neurons such as NPY/Agouti-related peptide (AgRP) and POMC/cocaine- and amphetamine-regulated transcript (CART) were shown to express insulin receptors (Pardini et al., 2006). Furthermore, central administration of insulin has been shown to inhibit NPY expression in the ARC (Schwartz et al., 1992). Because NPY has been shown to inhibit GnRH (Gazal et al., 1998) and gonadotropin secretion (Catzeflis et al., 1993, Gazal et al., 1998), insulin has the potential to indirectly stimulate GnRH release by diminishing NPY release. In addition, transgenic mice lacking insulin receptors in kisspeptin neurons had decreased circulating concentrations of LH with delayed initiation of puberty, suggesting that this might be an important pathway involved in the metabolic regulation of sexual maturation (Qiu et al., 2013).

Gonadotropin releasing hormone-expressing cell lines (GN11), generated by directed oncogenesis of GnRH neurons, were shown to express insulin receptor mRNA, protein, and specific insulin binding sites, indicating that insulin may directly regulate GnRH neuronal activity (Kim et al., 2005). Moreover, *in vitro* studies have demonstrated that primary hypothalamic neuronal cell cultures (Burcelin et al., 2003) as well as GnV-3 cells (GnRH-expressing cell line generated by conditional immortalization of adult primary rat hypothalamic neurons; Salvi et al., 2006) show an increase in *GNRH1* gene expression and greater GnRH secretion after insulin stimulation. These observations

support the premise that insulin regulation of the neuroendocrine reproductive axis results, at least in part, from a direct effect upon GnRH neurons.

2.3.2 Leptin

Leptin, an adipose-derived hormone positively correlated with body fat mass (Ahren et al., 1997), plays a key function in signaling energy store status to the central reproductive axis (Zieba et al., 2005). Moreover, one of leptin's most important roles is to act as a peripheral satiety cue to the hypothalamus (Schwartz et al., 1997). Studies have revealed that leptin deficiency in rodents is associated with hyperphagic obesity and hypogonadotropic hypogonadism (Zhang et al., 1994), and both can be reversed by leptin treatment (Campfield et al., 1995, Chehab et al., 1996). In addition, it has been shown that leptin administration hastens onset of puberty in normal mice (Chehab et al., 1997, Mounzih et al., 1997).

Feed restriction in prepubertal heifers markedly reduces leptin (*Ob*) mRNA abundance in adipose tissue, as well as circulating concentration of leptin and LH pulse frequency (Amstalden et al., 2000). In addition, it has been shown that leptin administration inhibits a fasting-mediated reduction in the pulsatile secretion of LH in prepubertal heifers (Maciel et al., 2004a). In studies performed in mature cows, central infusion of recombinant ovine leptin increased plasma LH concentrations in fasted but not in control-fed cows (Amstalden et al., 2002), suggesting that administration of leptin can increase LH secretion only when concentrations of leptin are below a "critical" threshold (Williams et al., 2002). These results support the notion that leptin plays a

passive or permissive, rather than causal, role in timing the progression of sexual maturation.

Despite the universal recognition of the critical effects of leptin in the regulation of pubertal onset, some disagreement has persisted regarding the specific pathways and mechanisms by which leptin controls the function of the gonadotropic axis. Numerous studies have provided evidence that leptin's actions on the secretion of gonadotropin are mediated at the hypothalamic level; however, leptin directly stimulates the secretion of LH from rat and cattle adenohypophyseal explants (De Biasi et al., 2001, Amstalden et al., 2003), and rat and pig adenohypophyseal cells (Ogura et al., 2001, Barb et al., 2004), indicating that leptin can modulate gonadotropin secretion at both sites.

Additional studies that measured GnRH release directly in CSF collected from the IIIV of cows described an increase in mean GnRH concentration and size of individual pulses after leptin administration (Zieba et al., 2005). Despite the abundant expression of leptin receptors within the hypothalamus (Zamorano et al., 1997), the majority of GnRH neurons do not express leptin receptors (Finn et al., 1998), indicating that leptin may regulate GnRH secretion primarily via interneuronal signaling mechanisms. Morphological studies have demonstrated that populations of NPY neurons in the ARC co-express leptin receptor (Baskin et al., 1998), and NPY appears to mediate most of leptin's neuroendocrine actions on the GnRH/LH system (Kalra et al., 1999, Ahima et al., 2000). Furthermore, POMC neurons in the ARC were also shown to be regulated by leptin (Thornton et al., 1997, Cone, 2005). In addition to acting as food

intake suppressors (Cowley et al., 2001), melanocortins stimulate the secretion of LH in rats (Watanobe et al., 1999a).

Recent studies have shown that leptin may play an important role in the regulation of *Kiss1* expression within the hypothalamus. *Kiss1* mRNA levels in the ARC are decreased in *ob/ob* mice that lack a functional leptin gene, but increase following leptin treatment (Smith et al., 2006a). In addition, diabetic-rats with severe hypoleptinemia demonstrate reduced abundance of *Kiss1* mRNA in the hypothalamus, which is normalized after ICV infusion of leptin, but not of insulin (Castellano et al., 2009). Leptin also increased *Kiss1* expression in a mouse hypothalamic cell line (Luque et al., 2007). Moreover, *Kiss1* transcript levels in the ARC and POA were lower in hypogonadotropic lean ewes than animals of normal BW, and central administration of leptin partially restored *Kiss1* mRNA abundance in lean animals (Backholer et al., 2010b). Therefore, it is reasonable to propose that the hypothalamic *Kiss1/GPR54* system represents a parallel pathway through which leptin regulates GnRH neuronal function.

Nonetheless, a recent study demonstrated that mice lacking leptin receptor specifically in kisspeptin neurons had normal pubertal development and fertility (Donato et al., 2011). In contrast, authors found that deletion of the leptin receptor from the ventral premammillary nucleus (PMV) prevented puberty and fertility. Corroborating these findings, Quennell et al. (2011) demonstrated that leptin induction of STAT3 phosphorylation, a major intracellular signaling mechanism activated by leptin, does not occur in kisspeptin neurons. Altogether, these recent findings suggest that a substantial

part of the effects of leptin on hypothalamic *Kiss1* expression may be transmitted indirectly through intermediate pathways.

The PMV contains a large concentration of neurons expressing the leptin receptor (Elias and Purohit, 2013). A large proportion of these cells coexpresses the neurotransmitters glutamate and nitric oxide, and directly innervates GnRH and kisspeptin neurons (Donato et al., 2011, Louis et al., 2011). Bilateral lesions of the PMV result in reduced LH secretion and *GNRH1* mRNA abundance, and reduced activation of GnRH neurons (Donato et al., 2009). Administration of leptin to fasted PMV-lesioned rats failed to increase concentrations of LH, indicating that the PMV is a critical site for the stimulatory actions of leptin on fasting-induced suppression of the reproductive neuroendocrine system (Elias and Purohit, 2013). In addition, Donato et al. (2011) used a conditional knockout technique, in which the leptin receptor is endogenously re-expressed in PMV neurons of a leptin receptor null-reactivable mouse model, to demonstrate that pubertal development and fertility can be rescued by re-expression of the leptin receptor selectively in PMV neurons. Authors suggest that PMV neurons are possibly stimulated by leptin and through the release of glutamate, a classical excitatory neurotransmitter, may directly activate their terminal targets (e.g., GnRH and kisspeptin cells).

2.3.3 Kisspeptin

Kisspeptin belongs to a large Arg-Phe-amide (RFamide) family of neuropeptides derived from the *Kiss1* gene that operate though the G-protein coupled receptor GPR54

(Kotani et al., 2001). Originally, *Kiss1* was isolated as a suppressor gene of tumor metastasis in a human malignant melanoma cell line (Lee et al., 1996). However, in 2003, an important role for kisspeptin in pubertal onset was first demonstrated. Studies in both humans (de Roux et al., 2003) and mice (Seminara et al., 2003) demonstrated that mutations within or deletion of the kisspeptin receptor (GPR54) prevented normal sexual maturation, resulting in infertility. Later, *Kiss1* knockout mice were described to show a similar phenotype to the *GPR54* knockout mice, demonstrating that kisspeptin acting through GPR54 is critical for normal pubertal development (d'Anglemont de Tassigny et al., 2007).

The mechanisms and signals whereby hypothalamic expression of *Kiss1/GPR54* is controlled have not been entirely elucidated. *Kiss1* expression is under the control of estrogens and androgens, with differential effects depending on the hypothalamic site (Tena-Sempere, 2010). Expression in the ARC is repressed by sex steroids in rodents (Smith et al., 2005), sheep (Smith et al., 2007), and monkeys (Shibata et al., 2007). However, sex steroid regulation of *Kiss1* mRNA expression at the anteroventral periventricular nucleus (AVPV) of the hypothalamus in rodents has opposite effects, with both androgens and estrogens having stimulatory effects (Smith et al., 2005). Additionally, as mentioned before, there are several lines of evidence that leptin also plays an important role in the regulation of the hypothalamic kisspeptin system.

The exogenous administration of kisspeptin has been shown to stimulate the gonadotropic axis at, or prior to, pubertal onset in several species. In prepubertal female rats, central administration of kisspeptin increases plasma concentrations of LH and

advances vaginal opening by approximately 4 days compared to controls (Navarro et al., 2004). When peripherally administered to prepubertal Holstein heifers, kisspeptin increased plasma concentrations of LH, inducing peak responses around 30 minutes after a single injection (Kadokawa et al., 2008). Moreover, ICV administration of kisspeptin in sheep leads to a potent secretion of GnRH into the CSF, with a parallel rise in serum LH (Messager et al., 2005). The effects of kisspeptin on secretion of LH can be completely blocked by the administration of a GnRH antagonist, indicating that kisspeptin's site of action lies within the hypothalamus (Shahab et al., 2005).

In adult rodents, the majority of GnRH neurons (~80%) have been found to express *GPR54* mRNA (Irwig et al., 2004, Han et al., 2005). Moreover, a large population of GnRH neurons (~40%) is likely to be responsive to kisspeptin from the time of birth and the number of detectable GnRH neurons expressing *GPR54* increases to adult-like levels well before onset of pubertal in the mouse (Han et al., 2005, Herbison et al., 2010). Electrophysiological investigations have shown that kisspeptin exerts powerful and prolonged stimulatory actions upon the electrical excitability of most adult GnRH neurons, which results from the ability of GPR54 to affect several ion channels in the GnRH neuron (Clarkson et al., 2010).

Mapping of kisspeptin cells in rodents (Mikkelsen and Simonneaux, 2009) and sheep (Franceschini et al., 2006) has documented the presence of two major neuronal populations found in the ARC and AVPV. Recent investigation in rodents demonstrated that there is a dramatic induction of *Kiss1* mRNA and protein levels observed in the AVPV throughout postnatal development (Clarkson et al., 2010). This up-regulation of

Kiss1 abundance within the AVPV has been investigated in female mice and found to be entirely dependent upon estradiol (Clarkson et al., 2010). Briefly, expression of *Kiss1* in the AVPV was markedly reduced in mice that were ovariectomized before pubertal onset and was completely restored by estradiol administration. Additionally, a complete lack of *Kiss1* expression in the AVPV of the mature aromatase-knockout female mouse demonstrates that estradiol is critical to drive the postnatal increase in *Kiss1* abundance in the AVPV during sexual maturation (Clarkson et al., 2010). Therefore, the authors suggest that AVPV kisspeptin neurons might represent an estradiol-dependent GnRH neuron amplification mechanism, which is brought into play in late postnatal stages to complete the process of pubertal development.

On the other hand, the kisspeptin neuronal population located in the ARC appears to be differentially regulated by steroid hormones. In the female sheep, ovariectomy during the juvenile period results in an increased number of kisspeptin-immunoreactive cells in the ARC of prepubertal ewes (Nestor et al., 2012). Moreover, authors observed that kisspeptin close contacts on GnRH neurons also increase after ovariectomy in prepubertal lambs and are greater in postpubertal compared to prepubertal ovary-intact ewes. Thus, Nestor et al. (2012) proposed that a peripubertal increase in kisspeptin production within the ARC would occur in response to a decrease in the steroid-negative feedback, resulting in increased GnRH/LH secretion that characterizes pubertal maturation in the ewe.

2.3.4 Neuropeptide Y

Neuropeptide Y is a potent orexigenic peptide that is elevated under feed-deprived situations, and has been described to suppress the secretion of LH in ruminants (McShane et al., 1992, Gazal et al., 1998). Populations of NPY neurons in the ARC have been demonstrated to express functional leptin and insulin receptors (Schwartz et al., 1992, Barker-Gibb et al., 1995, Backholer et al., 2010a), and have been implicated in several physiological functions, especially playing a role as controllers of metabolism and feeding behavior (Welch et al., 1994). Central administration of NPY produces a potent feeding response even in satiated animals, ultimately leading to obesity if infusion is continued over long periods of time (Gamba and Pralong, 2006). Moreover, abundance of *NPY* mRNA in hypothalamic neurons of the ARC has been demonstrated to be increased during unfavorable metabolic conditions (Brady et al., 1990, Schwartz et al., 1996).

Neuropeptide Y activates at least five different receptor subtypes, and among them, Y1 and Y5 have been associated in the regulation of food intake (Gerald et al., 1996) and in the regulation of the neuroendocrine reproductive axis (Raposinho et al., 1999). The Y5 receptor subtype is expressed by GnRH cells (Campbell et al., 2001) and administration of a specific Y5 antagonist blocks the NPY-mediated inhibition of LH secretion (Raposinho et al., 1999). Furthermore, porcine NPY (Y1/Y2/Y5 agonist) directly inhibited the firing of GnRH neurons in female mice, and through the use of further agonists it has been demonstrated that Y1and Y5 are the main receptor subtypes responsible for suppressing GnRH neuron activity (Roa and Herbison, 2012).

In addition, a high endogenous NPY-ergic tone constrains normal sexual maturation in juvenile rodents (Gamba and Pralong, 2006). These results imply that inhibition of the neuroendocrine reproductive axis that occurs during unfavorable metabolic conditions might be mediated by an increase in hypothalamic NPY release (Brady et al., 1990, Raposinho et al., 1999). This hypothesis is bolstered by the fact that central infusion of NPY in prepubertal rats is followed by a delay of pubertal maturation similar to that seen in food-restricted animals (Pralong et al., 2000). Likewise, it has been demonstrated recently that prepubertal heifers that were weaned around 3 mo of age and fed a high-concentrate diet for 91 days, to promote rapid weight gain and facilitate early onset of puberty, presented with a decrease in *NPY* gene expression (3.5 fold) in the hypothalamic ARC when compared to heifers fed a low-concentrate diet (Allen et al., 2012). Using a similar nutritional regimen, Alves et al. (2011) observed that the proportion of GnRH cell bodies in close proximity to NPY immunoreactive fibers in the preoptic area and hypothalamus was reduced in heifers gaining BW at higher rates. Thus, these findings suggest that functional and morphological modifications involving the hypothalamic NPY circuitry during early postnatal development may be involved in the metabolic programming of accelerated puberty in females.

2.3.5 Melanocortins

Among the neuropeptide systems contained in the ARC, the melanocortin system has been described as an important pathway by which peripheral signals communicate with the brain. Several important melanocortin receptor ligands originate from the

precursor polypeptide, POMC, which comprise adrenocorticotropic hormone (ACTH) and the different forms of melanocyte-stimulating hormone (MSH). POMC is produced in the periphery and in the central nervous system, predominantly within the ARC of the hypothalamus. These neurons project to several hypothalamic regions and directly to sympathetic efferent projections in the spinal cord (reviewed by Seeley et al., 2004).

The melanocortin-4 receptor (MC4R) is broadly expressed in the brain, including numerous regions of the hypothalamus, but also in the nucleus accumbens and the dorsal motor nucleus of the vagus (Kishi et al., 2003). This receptor has an important role in the regulation of food intake, energy balance, and glucose homeostasis (Cone, 1999). Acute administration of the non-selective MC3R/4R agonist αMSH and βMSH is very effective in reducing food intake (Kask et al., 2000). The endogenous MC4R antagonist, AgRP, and the synthetic selective MC4R antagonist, HS024, are also very effective in stimulating food intake after acute injections (Kask et al., 1998, Rossi et al., 1998). The effects of AgRP are not as potent as for NPY, one of the most potent stimulators of food intake. However, the food intake effects of AgRP are not limited to a few hours. Actually, a single administration of AgRP can continue to increase food intake in rats for a period of up to six days (Hagan et al., 2000).

Food restriction and weight loss are reported to result in increased *AgRP* and decreased *POMC* gene expression in the ARC of mice (Schwartz et al., 1997, Mizuno et al., 1998) and ovariectomized ewes (Backholer et al., 2010a). In contrast, positive energy balance results in increased *POMC* gene expression in the ARC of mice (Hagan et al., 1999). In addition, prepubertal heifers fed a high-concentrate diet that promotes a

rapid weight gain and hastens pubertal onset exhibited a 5-fold reduction in *AgRP* gene expression in the ARC when compared to heifers fed a low-concentrate diet (Allen et al., 2012). Both insulin and leptin have been proposed to signal energy balance status to the melanocortin system. Insulin and leptin receptors are expressed in POMC neurons and central administration of both, insulin and leptin, can stimulate *POMC* gene expression in fasting animals (Mizuno et al., 1998, Benoit et al., 2002). Moreover, leptin-deficient mice had enhanced abundance of *AgRP* and reduced expression of *POMC* that can be regularized by exogenous leptin treatment (Schwartz et al., 1997, Mizuno et al., 1998).

Considering the important role of the melanocortin system in signaling energy balance to the brain, some studies indicate the possibility that this system could contribute to the metabolic control of the reproductive axis. Central administration of SHU9119 (a nonselective MC3R/4R antagonist) or HS014 (a selective MC4R antagonist) decreased the magnitude of the LH surge in normally-fed steroid-primed ovariectomized female rats (Cowley et al., 2001). Moreover, they also blocked the leptin-stimulated LH surge in starved rats (Watanobe et al., 1999b). These results indicate that, as with food intake, effects of leptin on the reproductive axis could be mediated, at least in part, through the MC4R. One important aspect to consider is that POMC, a complex prohormone, can be enzymatically cleaved into various neuroactive peptides (e.g., β-endorphin and αMSH) with contrary effects on reproductive and metabolic functions. The neuropeptide αMSH reduces feed consumption and stimulates lordosis behavior in female rats (Woods et al., 1998, Ahima et al., 2000, Cone et al.,

2001), while β-endorphin stimulates feed intake and inhibits LH secretion (Leadem and Kalra, 1985, Bray, 1993).

Recently, NPY and POMC cells have been shown to project to kisspeptin cells in the ARC of ovariectomized and intact ewes, indicating that melanocortin cells may play an important role in synchronizing effects of peripheral signaling agents on the reproductive system (Backholer et al., 2010b). In support of these histochemical data is the observation that kisspeptin neurons communicate with NPY and POMC cells, increasing *NPY* and reducing *POMC* mRNA expression in the ARC (Backholer et al., 2010b). Thus, authors suggested that the network created between these three cell types could coordinate the brain control of reproductive functions and metabolic homeostasis. In addition, POMC neurons from the ARC project to the preoptic area and make apparent direct synaptic contact with GnRH neurons in mice (Leranth et al., 1988, Cravo et al., 2011). Therefore, a direct regulation of GnRH neurons by POMC cells cannot be discarded. In fact, Roa and Herbison (2012) observed that the majority (~70%) of GnRH neurons was potently excited by αMSH in female mice, and this resulted from the direct postsynaptic activation of MC3R and MC4R.

2.4 Stair-step compensatory gain nutritional model

Considering the importance of nutritional and metabolic statuses controlling pubertal maturation in heifers, approaches that enable targeting of pubertal onset to a desirable age range, while optimizing other aspects of growth and development, are needed. To achieve this goal, a better comprehension of the neural plasticity associated

with temporal and metabolic programming of the brain is fundamental. The stair-step compensatory gain nutritional model (Park et al., 1987) presents a valuable tool to address this issue. This model makes use of alternating dietary energy-restriction and refeeding phases to potentiate compensatory growth, increase feed conversion and optimize mammary gland development, resulting in increased energy and protein utilization and enhanced lactational performance during subsequent lactation cycles (Park et al., 1998).

It has been demonstrated that several changes in metabolic and endocrine statuses occur during compensatory growth in cattle (Blum et al., 1985, Yambayamba et al., 1996, Hornick et al., 2000). During feed restriction, there is a reduction in basal metabolism, largely caused by a decrease in the volume and metabolic activity of the viscera (Yambayamba et al., 1996). This condition results in a reduction in anabolic hormones and enhanced concentrations of catabolic hormones. Plasma concentrations of insulin, IGF1, 3.5.3'-triiodothyronine (T3) and thyroxine (T4) decrease, while circulating concentrations of cortisol and GH increase (Blum et al., 1985, Hornick et al., 2000). The increase in plasma concentrations of GH is associated with GH resistance, which results from a lower nutrient influx that has been demonstrated to reduce the release of somatostatin by the hypothalamus and thus reduce inhibitory effects on the synthesis and release of GH (Thomas et al., 1990). Moreover, decreased plasma concentrations of insulin, T3, and T4 result in reductions in synthesis of GH receptor and in plasma concentrations of GH-binding proteins. This reduces binding of GH with target tissue receptors (Maes et al., 1983).

During refeeding, an acceleration of BW gain occurs as a result of several processes. First, a moderately low basal metabolism is observed because the live weight of the heifers is less than in age-matched animals, and sparing mechanisms are preserved beyond the restricted feeding period for several weeks (Hornick et al., 2000, Hausman et al., 2004). The lower metabolic rate is probably a consequence of the reduced visceral weight during the initial period of compensatory gain (Drouillard et al., 1991), which may indicate that a relatively higher proportion of energy and protein are available to cover growth requirements. Furthermore, enhanced feed intake and improved nutrient digestibility have also been reported (Hornick et al., 2000). These events are probably the result of changes in endocrine status during the refeeding phase. Plasma concentrations of insulin are normalized within a day after the beginning of the refeeding period (Blum et al., 1985, Hornick et al., 2000). High insulin concentrations might stimulate the uptake of metabolites, such as glucose by the hypothalamus, resulting in decreased secretion of GH (Buonomo and Baile, 1990). Plasma concentrations of leptin are lower in animals under feed restriction and after refeeding a 40% increase was observed when body condition score increased by 1 point on a 1 to 9 scale (Leon et al., 2004). These data are congruent with the reported high correlation between the amount of abdominal and peripheral fat and plasma leptin (Ahren et al., 1997, Amstalden et al., 2000).

Because the stair-step compensatory nutritional regimen significantly alters the metabolic and endocrine status of prepubertal heifers during specific periods of the juvenile development, it may have important effects on the programming of the

reproductive neuroendocrine system. Thus, compensatory growth at specific periods of development may not only improve feed intake and nutrient digestibility as mentioned above, but may also be used as a potential strategy to optimally program the onset of puberty in heifers.

2.5 Expected impact of this research

Developing managerial strategies that optimize the timing of sexual maturation of beef heifers to allow maximum lifetime productivity remains a major goal of the beef industry. Studies that address functional and morphological changes within the hypothalamus that underlie this process, may contribute to a better understanding of the mechanisms involved in the nutritional acceleration of puberty in bovine females. The outcome of this research is expected to be used for designing nutritional strategies that aim to exploit postnatal neuroendocrine plasticity to optimally time pubertal onset consistently at around 12 mo of age, while avoiding precocious puberty in beef heifers. This expansion in the knowledge base of the metabolic control of sexual maturation will be beneficial to advance the fundamental understanding of bovine reproductive neuroendocrinology and may positively impact replacement-heifer production systems.

CHAPTER III

NUTRITIONAL ACCELERATION OF PUBERTY IN HEIFERS IS ACCOMPANIED BY A DECLINE IN HYPOTHALAMIC NEUROPEPTIDE Y RELEASE

3.1 Synopsis

Feeding a high-concentrate diet to heifers during the juvenile period, resulting in increased BW gain and adiposity, leads to early maturation of the reproductive neuroendocrine system. Herein, we tested the hypothesis that hypothalamic release of NPY, an inhibitor of GnRH and LH secretion in ruminants, is decreased in heifers that gained BW at higher rates. Crossbred heifers, weaned at approximately 3.5 mo of age, were fed to gain either 0.5 (LG; n = 12) or 1.0 kg/day (HG; n = 13) for 30 wk. At approximately 7.5 mo of age, a subgroup of heifers (n = 6/group) was selected randomly to be surgically fitted with third ventricle (IIIV) guide cannulas. Concentrations of progesterone were determined in blood samples collected twice weekly starting at 7.5 mo of age to determine pubertal status in all heifers. At 8 (n = 6/group) and 9 mo of age (LG, n = 6; HG, n = 3), IIIV cerebrospinal fluid (CSF) and blood samples were collected intensively for 6 h. Jugular blood sampling continued for an additional 6 h. Mean BW and circulating concentrations of leptin were greater ($P < 0.05$) in HG when compared to LG heifers at week 6 and 8 of the experiment, respectively, and they remained greater thereafter. Pulsatile patterns of GnRH (CSF) and LH (plasma) secretion were highly correlated (0.87; $P < 0.01$). Collectively, all LH pulses detected in the initial 6h of the

intensive sampling period began coincident with or within two sampling points after the onset of a GnRH pulse. At 8 mo of age, frequency of LH pulses was greater ($P < 0.01$) in the HG (3 pulses/12 h) than in the LG (1 pulse/12 h) group. At 9 mo of age, HG heifers had lower ($P = 0.02$) concentrations of NPY in the IIIV CSF (0.6 ng/ml) than LG (1.1 ng/ml) heifers. In addition, the percentage of pubertal heifers by 9 mo of age was greater ($P = 0.03$) in the HG gain group when compared to LG. These observations support the hypothesis that accelerating BW gain by feeding a high-concentrate diet during the juvenile period decreases hypothalamic NPY release, leading to maturation of the reproductive neuroendocrine axis and hastening pubertal onset in heifers.

3.2 Introduction

The pubertal initiation of high-frequency episodic secretion of GnRH is largely dependent upon metabolic cues throughout prepubertal development. Growth restriction during the juvenile period has been demonstrated to delay puberty (Foster and Olster, 1985, Kile et al., 1991, I'Anson et al., 2000) and, in contrast, increased adiposity accelerates reproductive maturation in mammals (Lee et al., 2007, Tena-Sempere, 2007 Rosenfield et al., 2009). In cattle, feeding a high-concentrate diet to heifers during the juvenile period, resulting in increased BW gain and adiposity, leads to early maturation of the reproductive neuroendocrine system and earlier puberty (Gasser et al., 2006a,c). Although the mechanisms involved in this process have not been completely elucidated, leptin and leptin-sensitive cells in the central nervous system appear to play a critical role (Garcia et al., 2003, Maciel et al., 2004b, Zieba et al., 2005, Allen et al., 2012).

Even though leptin, a hormone secreted mainly by adipocytes and positively correlated with body fat mass (Ahren et al., 1997), does not affect secretion of luteinizing hormone (LH) in adequately fed ewes (Henry et al., 1999) and cows (Amstalden et al., 2002), it prevents fasting-induced reduction in LH pulsatility in prepubertal heifers (Maciel et al., 2004a), suggesting that leptin plays a permissive, rather than causal, role in timing the process of pubertal development. Because several studies have demonstrated that leptin does not act directly on GnRH neurons (Finn et al., 1998, Hakansson et al., 1998, Quennell et al., 2009), its influence on the metabolic control of reproductive maturation is likely communicated through intermediate pathways.

Neuropeptide Y (NPY) neurons in the arcuate nucleus (ARC) express the leptin receptor (Baskin et al., 1999), play an orexigenic role in energy homeostasis (Wang et al., 1997), and are responsive to changes in nutritional status (Kalra and Kalra, 2003). Furthermore, NPY has been proposed to mediate the inhibitory effects of undernutrition on reproductive function (Kalra and Crowley, 1984). Contrary to observations in rats in which NPY can have either stimulatory or inhibitory effects (depending on gonadal steroid milieu) on LH release (Sahu et al., 1987), NPY has predominantly inhibitory actions on the release of LH in ruminants irrespective of steroidal influences (Gazal et al., 1998, Estrada et al., 2003, Morrison et al., 2003). This effect of NPY is largely due to inhibition of GnRH release (Gazal et al., 1998) and may be mediated by a direct action on GnRH neurons (Klenke et al., 2010). In addition, central infusion of NPY in prepubertal rats is followed by a delay of pubertal maturation similar to that seen in food-restricted animals (Pralong et al., 2000). Thus, we reasoned that a decrease in

hypothalamic NPY release may be associated with pubertal maturation of the hypothalamic-adenohypophyseal-gonadal axis. To test this hypothesis, we examined whether feeding a high-concentrate diet during the juvenile period, a strategy known to accelerate BW gain, would result in lower concentrations of NPY in third-ventricle (IIIV) cerebrospinal fluid (CSF) concomitantly with increases in pulsatile GnRH/LH secretion and advanced onset of puberty in heifers.

3.3 Materials and methods

All animal-related procedures used in the present study were approved by the Institutional Agricultural Animal Care and Use Committee (IAACUC) of the Texas A&M University System (AUP# 2009-151).

3.3.1 Animals and nutritional model

Twenty-five crossbred heifers (½ Angus, ¼ Hereford, ¼ Brahman) were utilized in two replicates over a 2-yr period (1 replicate/yr). Within each year, heifers were weaned at approximately 3.5 mo of age (age at weaning = 106 ± 3 d), stratified by date of birth and assigned randomly to be fed individually until 11 mo of age either to achieve a relatively low rate of BW gain (LG, 0.5 kg/d; n = 6/replicate) or a high rate (HG, 1 kg/d; replicate 1, n = 6; replicate 2, n = 7). Diets were balanced using the level 2 solution of the Large Ruminant Nutrition System (LRNS; http://nutritionmodels.tamu.edu/lrns.htm), which is based on the Cornell Net Carbohydrate and Protein System as described by Fox et al. (2004). Targeted average

daily gain was attained by adjustments in the dry matter intake based on BW gain determined every 2 wk. Ingredients and diet chemical composition are presented in Table 3.1.

Table 3.1. Ingredients and chemical composition of diets fed to prepubertal beef heifers in the current study. Diet A was provided to heifers during the first 10 wk of the experiment and diet B was fed to heifers from week 11 until the end of the study (30 wk).

Item	Diet A[1]	Diet B[1]
Ingredients[2]		
Alfalfa hay, %	22.81	17.68
Cottonseed hulls, %	6.62	10.54
Rolled corn, %	40.18	51.05
Cane molasses, %	4.40	5.19
Cottonseed meal, %	6.51	7.13
Corn gluten feed, %	17.95	6.73
Urea, %	—	0.56
Producers 12:12 premix, %	0.56	0.28
Calcium carbonate, %	0.84	0.84
Chemical composition[2]		
Metabolizable energy, Mcal kg	2.59	2.62
Crude protein, %	15.00	14.8
Digestible intake protein, %	10.65	10.80

[1] Diets were balanced using the Large Ruminant Nutrition System (LRNS)
[2] Dry matter basis

Heifers were allocated individually to pens measuring 14.6 m x 3.1 m and fed an acclimation diet for 2 wk post-weaning. After the acclimation period, heifers were fed solely Diet A until 6.5 mo of age; afterwards, animals started to receive Diet B until the end of the experiment (11 mo of age). Blood samples from the coccygeal vasculature were collected twice a month for the duration of the experiment for determination of

serum concentrations of leptin. Starting at 7.5 mo of age, blood samples were collected twice weekly until the end of the experiment or until puberty was determined (at least three consecutive samples with concentrations of progesterone ≥ 1 ng/ml), whichever occurred first. Blood samples were placed on ice immediately after collection. Serum was obtained from blood samples by centrifugation (2,200 X g for 20 min at 4°C) and stored at -20°C until hormone concentration assessment.

3.3.2 Surgical cannulation of the IIIV and intensive sampling

Starting at 6 mo of age, heifers were placed frequently in stanchions and acclimated to intensive handling conditions to minimize the effects of stress on the variables measured. At 7.5 mo of age (age = 228 ± 3 d), heifers (n = 3/group/replicate) were randomly selected to be surgically fitted with IIIV cannulas as described in detail previously (Gazal et al., 1998). The location and function of the cannulas were verified by radiography and continuous flow of CSF. A period of at least 2 wk was allowed for heifers to recover from surgery.

At approximately 8 mo of age (age = 248 ± 5 d), heifers were fitted with jugular catheters on the previous day of the intensive sampling. At the same time, animals were treated prophylactically with antibiotics as reported previously (Gazal et al., 1998). On the sampling day, heifers were placed in stanchions and 20 cm of polyethylene tubing (0.58 mm i.d. X 0.96 mm o.d.; Intramedic Clay Adams Brand, Becton Dickinson, Sparks, MD) was inserted using aseptic technique through the guide cannula. Tubing was adjusted until CSF flowed easily using a blunt 22-gauge needle and tuberculin

syringe. The end of the tubing was connected to another 60-cm section of polyethylene tubing. The collection end of the tubing was secured approximately 40-50 cm away from the heifer's head to facilitate semi-remote sampling. Blood samples (6 ml), via extensions connected to the jugular catheter and remotely secured, were collected simultaneously with CSF samples (600 μl) at 15- (replicate 1: n = 3/group) or 10-min intervals (replicate 2: n = 3/group) for 6 h and intensive blood sampling continued for an additional 6 h. The basis of the slight difference in intensive sampling intervals between the 2 replicates was to putatively improve the sensitivity of detection for GnRH and LH pulses. Analyses of IIIV CSF and plasma samples from replicate 1 had demonstrated that frequencies of GnRH and LH pulses were relatively low. However, the sensitivity of detection of secretory episodes in replicate 2 was not improved as a result of this change. In all cases, void volumes of CSF and blood created by the indwelling tubing and their extensions were discarded before sample collection. Blood samples were dispensed in tubes containing 100 μl of heparin solution (10,000 IU/ml) and 5% EDTA, and placed on ice immediately. Plasma was separated by centrifugation (2,200 X g for 20 min at 4°C) and stored at -20°C until LH determination. Cerebrospinal fluid samples were placed on ice immediately and, within 30 min, stored at -20°C until NPY analysis. Intensive blood and CSF sampling procedures were repeated at approximately 9 mo of age (age = 276 ± 3 d) in heifers that had not achieved puberty yet (LG, n = 6; HG, n = 4). Because one HG heifer did not have a functional IIIV cannula at 9 mo of age, only jugular blood samples for LH determination were collected at this time point.

3.3.3 Radioimmunoassays

To confirm the pubertal status of heifers, circulating concentrations of progesterone were determined by a commercial RIA kit (Coat-A-Count, Siemens Healthcare, Malvern, PA) as reported previously (Fajersson et al., 1999). Sensitivity was 0.1 ng/ml with intra- and interassay CV of 5.5% and 13%, respectively. Circulating concentrations of leptin were determined in triplicates using a highly specific ovine leptin RIA validated for use in bovine serum (Delavaud et al., 2000), with intraassay CV of 6%. Plasma concentrations of LH were measured in duplicate 200-µl aliquots with a validated RIA (McVey, Jr. and Williams, 1991). The sensitivity of the assay averaged 0.1 ng/ml, and average intra- and interassay CV were 5% and 9.5%, respectively. Concentrations of GnRH were determined in duplicate 200-µl CSF samples as described by Ellinwood et al. (1985). Antiserum BDS-037 (Dr. Alain Caraty, INRA Centre de Tours, Nouzilly, France) was used as the source of first antibody at a working dilution of 1:50 000. The sensitivity of the assay was 0.5 pg/ml, and average intra- and interassay CV were 5% and 16%, respectively. Finally, concentrations of NPY were determined directly in duplicate CSF samples using a commercial bovine RIA kit (Peninsula Laboratories, Belmont, CA). The sensitivity of the assay averaged 0.25 ng/ml, with intra and interassay CV of 4.5% and 11.5%, respectively.

3.3.4 Pulse detection and statistical analysis

Body weight gain and concentrations of leptin, GnRH, LH and NPY were analyzed by general linear mixed models for repeated measures using the mixed

procedure (PROC MIXED) of the Statistical Analysis System (SAS 9.3; SAS Institute, Inc., Cary, NC). Sources of variation were treatment, replicate, time and their interactions. Time was used as the repeated variable, and heifer was used as the subject. Because several heifers in the HG group were detected as pubertal by 9 mo of age, age was not considered a source of variation in the overall statistical model, and the test for treatment effects on concentrations of GnRH, LH and NPY at 8 and 9 mo of age were performed independently. The frequency and amplitude of LH pulses were determined using a pulse-detection algorithm, Pulsefit 1.2 (Kushler and Brown, 1991). Temporal coincidences between GnRH and LH pulses within heifers were determined as defined by Gazal and colleagues (1998). Means for GnRH and LH pulse frequencies data were contrasted by using Student's *t*-test. Percentage of pubertal heifers was analyzed using the CATMOD procedure of SAS (SAS 9.3; SAS Institute, Inc.). Main effects were considered significant when $P \leq 0.05$.

3.4 Results

3.4.1 Body weight gain and circulating concentrations of leptin

Because no replicate effect was observed for BW gain and circulating concentrations of leptin, data were pooled by treatment. Mean (±SEM) BW at the beginning of the experiment did not differ between groups (140.9 ± 3.2 kg). Body weight increased linearly in both dietary groups (Fig. 3.1). As expected based on the experimental design, HG heifers had greater ($P < 0.05$) BW than LG heifers starting at week 6 of the experiment and continuing throughout the study ($P < 0.05$). Mean

circulating concentrations of leptin increased ($P < 0.05$) markedly beginning at week 8 of the experiment in HG heifers and remained greater ($P < 0.05$) than in LG heifers until the end of the experiment (Fig. 3.2).

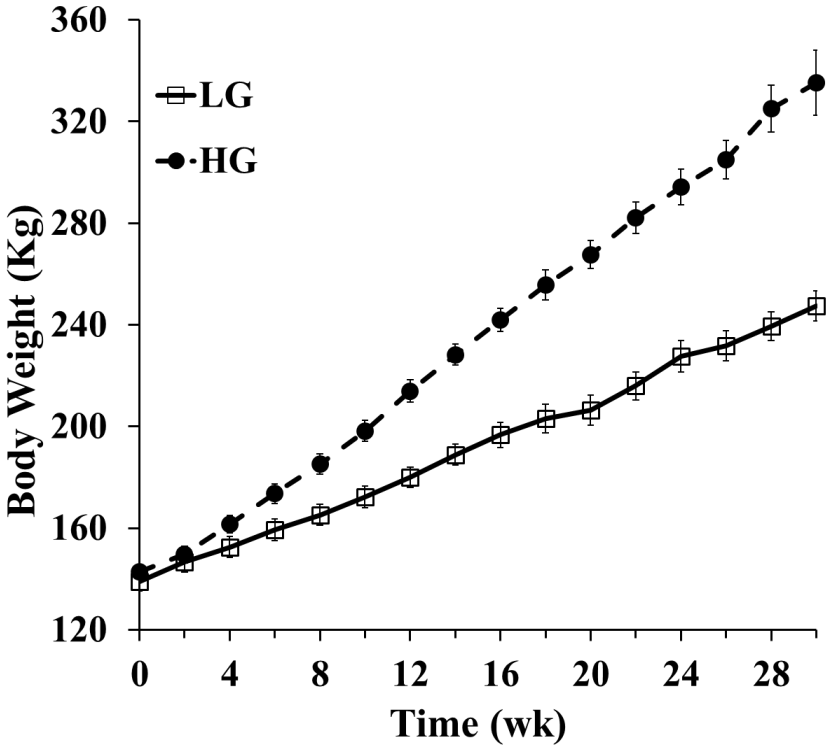

Fig. 3.1. Mean (±SEM) BW of heifers weaned at approximately 3.5 mo of age and fed for 30 wk either to achieve a low rate of BW gain (LG, n = 12) or a high rate of BW gain (HG, n = 13). Body weight of HG heifers was greater than LG heifers beginning at week 6 and continuing throughout the study ($P < 0.05$).

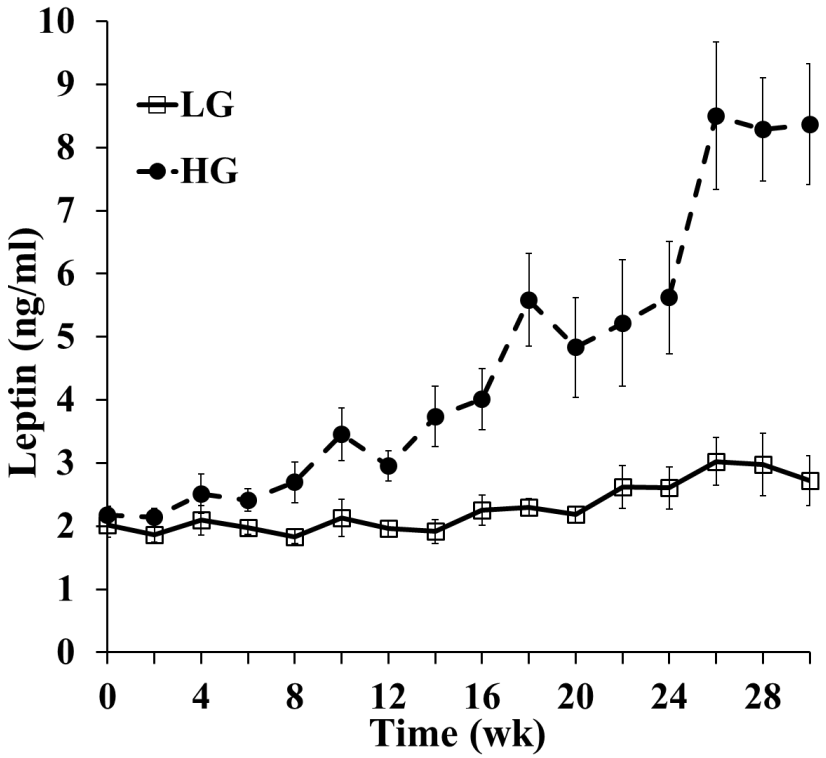

Fig. 3.2. Mean (±SEM) circulating concentrations of leptin of heifers weaned at approximately 3.5 mo of age and fed for 30 wk to achieve either a low rate of BW gain (LG, n = 12) or a high rate of BW gain (HG, n = 13). Concentrations of leptin were greater ($P < 0.05$) in HG heifers than in LG heifers beginning at week 8 of the experiment and remained greater until the end of the study ($P < 0.05$).

3.4.2 Pulsatile patterns of IIIV CSF GnRH and plasma LH release, and concentrations of NPY in IIIV CSF relative to mean peripheral leptin

No replicate effect was observed for mean concentrations of GnRH, LH, and NPY or pattern of secretion; therefore, data were pooled by treatment. Concentrations of NPY in the CSF collected from the IIIV of heifers at hourly intervals during the 6 h sampling period did not present a pattern indicative of pulsatile secretion; therefore, comparison between experimental groups was performed based on mean concentrations. At 8 mo of age, concentrations of NPY in the IIIV CSF tended to be lesser ($P = 0.17$) in HG (1.29 ± 0.25 ng/ml) than in LG heifers (1.8 ± 0.26 ng/ml; Fig. 3.3B). At 9 mo of age, concentrations of NPY in the IIIV CSF were significantly less ($P = 0.02$) in HG prepubertal heifers (0.56 ± 0.17ng/ml) than in LG (1.1 ± 0.07 ng/ml; Fig. 3.3B). At 8 and 9 mo of age, intensively sampled HG heifers presented greater ($P \leq 0.05$) concentrations of leptin than LG heifers (Fig. 3.3A).

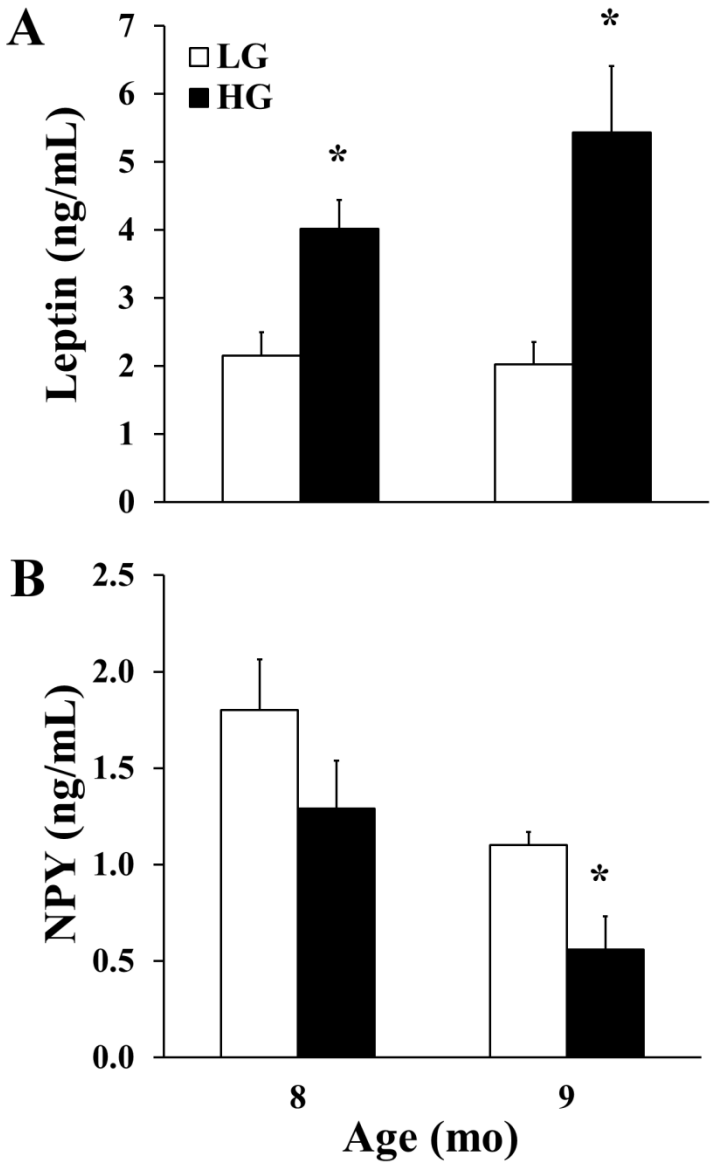

Fig. 3.3. Mean (±SEM) concentrations of NPY in the IIIV CSF (B) and leptin in peripheral blood (A) of LG and HG heifers at 8 (LG, n = 6; HG, n = 6) and 9 mo of age (LG, n = 6; HG, n = 3). Concentrations of leptin were greater in HG than in LG heifers at 8 and 9 mo of age (*$P \leq 0.05$). Mean concentration of NPY in the IIIV CSF was smaller in HG than in LG group at 9 mo of age (*$P = 0.02$).

Individual GnRH (CSF) and LH (plasma) secretion patterns in four representative prepubertal heifers from the LG and HG groups are shown in Figure 3.4.

GnRH was secreted in the IIIV CSF in a pulsatile pattern, consistent with previous reports in mature cows (Gazal et al., 1998). A parallel pattern of CSF GnRH and plasma LH secretion was observed. Over simultaneous CSF and blood sampling periods (totaling 126 h), 16 LH pulses were identified, and 13 (81.3%) occurred in exact temporal synchrony or within one sample after a GnRH pulse. All LH peaks (100%) occurred within 2 sampling points after onset of a GnRH pulse. Mean concentrations, amplitude, and frequency of GnRH pulses did not differ between experimental groups within the 6h sampling period. Nonetheless, at 8 mo of age, mean frequency of LH pulses monitored over 12 h was greater ($P < 0.01$) in the HG (2.8 ± 0.6) than in the LG group (1 ± 0) before puberty occurred in any heifer (Fig. 3.5). At 9 mo of age, there was no significant difference between LH pulse frequency in HG (3.8 ± 1.4) versus LG heifers (2 ± 0.8; Fig. 3.5). No differences in LH pulse amplitude or mean concentrations were observed between experimental groups at 8 and 9 mo of age.

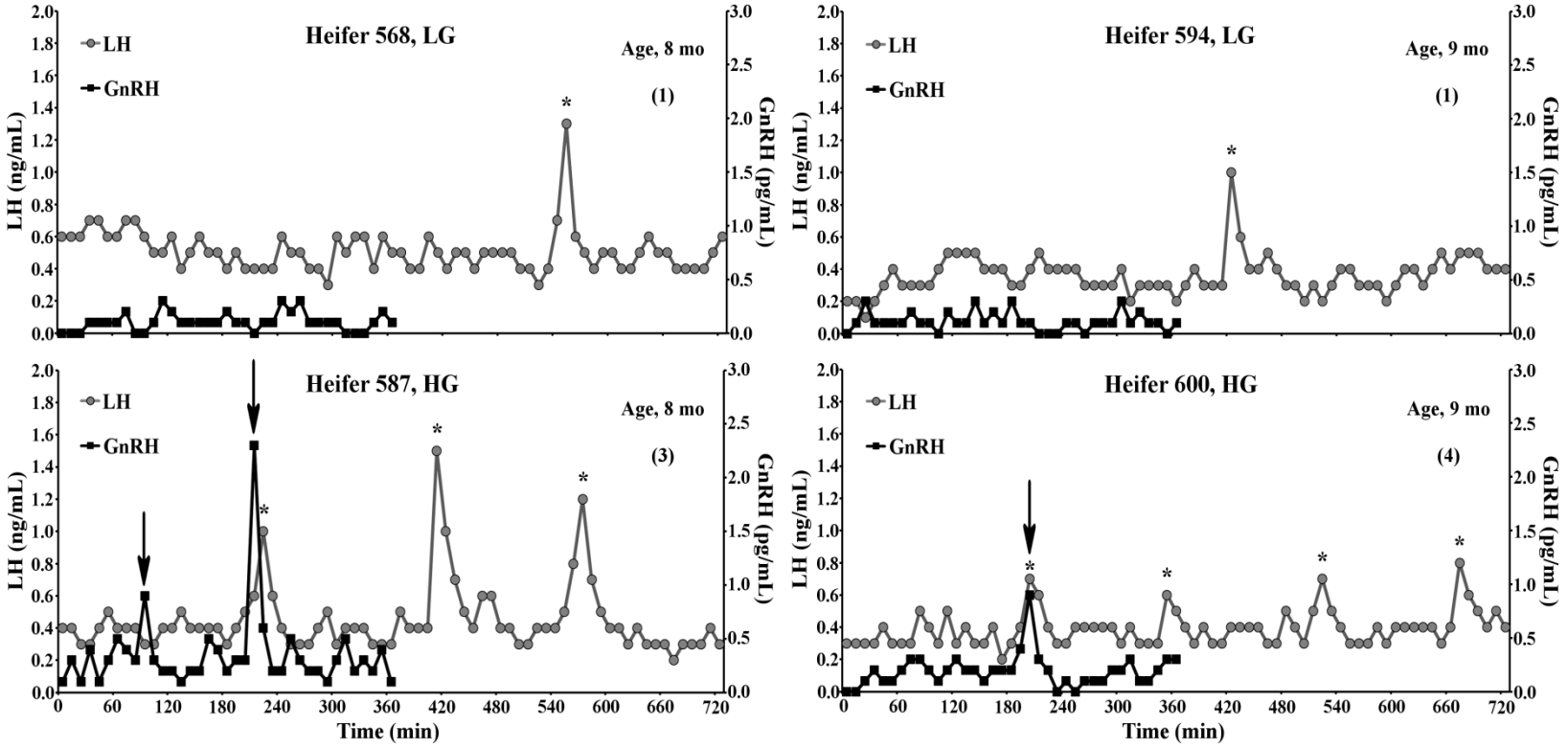

Fig. 3.4. Patterns of CSF GnRH (black lines) and plasma LH (grey lines) secretion in four representative prepubertal heifers from the LG (#568 and #594) and HG group (#587 and #600) at 8 and 9 mo of age. Gonadotropin releasing hormone and LH pulses detected by Pulsefit [32] are denoted by arrows and asterisks, respectively. Luteinizing hormone pulses detected within the 12-h sampling period are enumerated in parentheses.

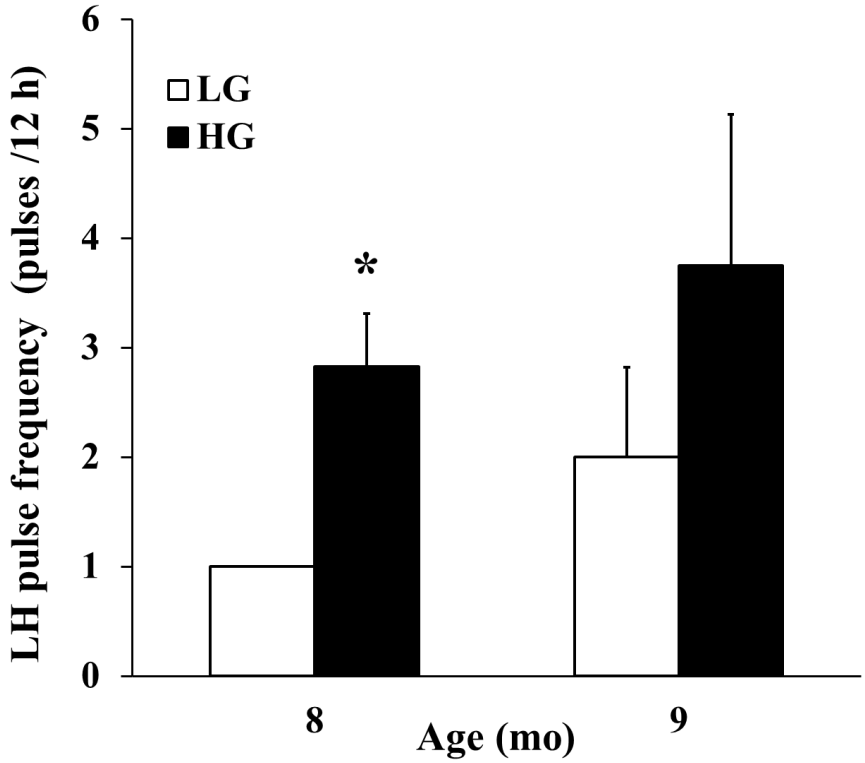

Fig. 3.5. Mean (± SEM) frequency of LH pulses of LG and HG heifers at 8 (LG, n = 6; HG, n = 6) and 9 mo of age (LG, n = 6; HG, n = 4). Luteinizing hormone pulse frequency was greater in HG heifers than LG heifers at 8 mo of age (*$P < 0.01$).

3.4.3 Pubertal onset

Percentage of pubertal heifers was greater ($P < 0.03$) in HG than in LG group starting at 9 mo of age (Fig. 3.6). A replicate effect was observed for the percentage of pubertal heifers in the HG group beginning at 9 mo of age, with a greater ($P < 0.05$) percentage of pubertal animals in the replicate 1 than in replicate 2 (Fig. 3.6). By 11 mo of age, 100% (6/6) of the HG heifers from replicate 1 had already achieved puberty, while only 14.29% (1/7) from replicate 2 were pubertal. None of the LG heifers (0/12) in both replicates attained puberty until the end of the experiment (11 mo of age).

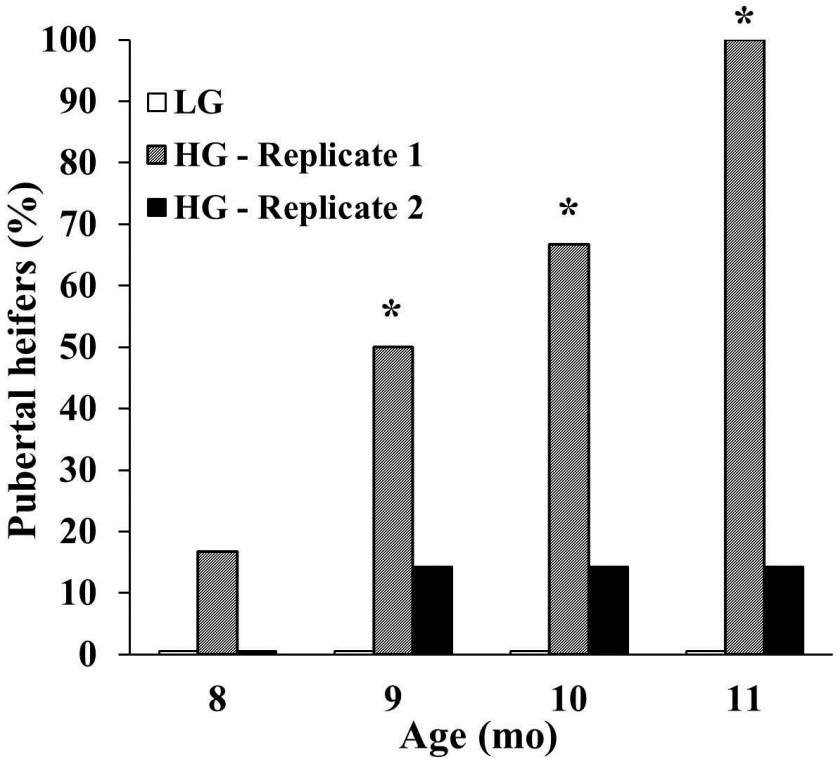

Fig. 3.6. Percentage of pubertal heifers in the LG (n = 12) and HG groups (replicate 1, n = 6; replicate 2, n = 7). Starting at 9 mo of age, percentage of pubertal heifers in the HG group was greater than in LG group ($P = 0.03$). A replicate effect was observed in the HG group beginning at 9 mo of age, with a greater percentage of pubertal animals in replicate 1 than in replicate 2 (*$P < 0.05$).

3.5 Discussion

Previous studies performed by our group have demonstrated that transcriptional and morphological alterations within the NPY hypothalamic pathway are involved in the sexual maturation of heifers (Alves et al., 2011, Allen et al., 2012). Results from the current study provide further evidence that the hypothalamic NPY system is a key metabolic-sensing circuitry involved in the nutritionally-mediated acceleration of puberty in heifers. Herein, we demonstrate that elevated BW gain, achieved by feeding a

high-concentrate diet to heifers during the juvenile period, leads to greater circulating concentrations of leptin and decreased hypothalamic release of NPY, characterized by lower concentrations of the peptide in the IIIV CSF. Furthermore, prepubertal heifers that gained BW at greater rates presented an increased LH pulsatile secretion and an advancement of pubertal onset. Since NPY has been demonstrated to have an inhibitory action on GnRH/LH release in ruminants (Gazal et al., 1998, Estrada et al., 2003, Morrison et al., 2003), we propose that the acceleration of sexual maturation seen in the present study is mediated, at least in part, by a diminished NPY inhibitory tone on GnRH episodic secretion.

The pubertal transition in females is characterized by an attenuation of estradiol negative feedback and increased pulsatile release of GnRH and LH that provide support for final maturation of ovarian follicles and enhance ovarian steroidogenesis (Gasser et al., 2006a). As anticipated, heifers fed a high-concentrate diet to promote accelerated BW gain during the juvenile period, a strategy known to advance onset of puberty, presented greater frequency of LH pulses during the peripubertal period. However, not surprisingly, mean concentrations of LH did not differ between groups. Similar results were reported in adult cows in which mean concentrations of LH were comparable during the early and mid-luteal periods even though the frequency of pulses was markedly higher during the early luteal period (Rawlings et al., 1980, Williams et al., 1983). The limited duration (6 h) of CSF sampling in the present study did not allow substantial comparison of frequency of GnRH pulses between dietary groups. However, the very high correlation observed between GnRH and LH pulses during the initial 6 h of

the sampling period together with the increased LH pulsatile release seen in HG heifers reinforce the notion that the pubertal "escape" from estradiol negative feedback is mainly regulated at the hypothalamic level.

Nutritional and metabolic signals are perceived largely by the hypothalamus (Cunningham et al., 1999, Schneider, 2004) and a major limiting factor for increased episodic secretion of LH during the prepubertal period is the lack of appropriate stimulation of the gonadotrophes by GnRH (reviewed by Amstalden et al., 2011). This evidence is supported by observations that ovarian function is stimulated in immature female monkeys treated with GnRH (Wildt et al., 1980), and in lambs treated with LH (Foster et al., 1984). Our group has demonstrated that leptin has a critical permissive role in controlling pubertal maturation in heifers (Maciel et al., 2004b, Zieba et al., 2005), and this regulation occurs mainly by leptin's effects on GnRH secretion (Maciel et al., 2004b). Nonetheless, several studies failed to demonstrate the presence of leptin receptors in GnRH neurons in rodents (Hakansson et al., 1998) and primates (Finn et al., 1998), suggesting that leptin's actions on GnRH release are likely mediated by intermediate pathways.

Neuropeptide Y, a potent stimulator of food intake (Clark et al., 1985) abundantly found in the hypothalamus (Allen et al., 1983), has been proposed as a major mediator of the inhibitory effects of undernutrition on reproductive function (Crown et al., 2007). In ruminants, central infusion of NPY suppresses GnRH and LH release (Gazal et al., 1998, Estrada et al., 2003, Morrison et al., 2003); in primates, it has been demonstrated that *NPY* mRNA and peptide concentrations are diminished during the

juvenile-pubertal transition (El Majdoubi et al., 2000). Thus, we reasoned that NPY may play an important role in the metabolic programing of puberty in heifers. Confirming our assumptions, prepubertal heifers in the HG group exhibited increased BW gain, greater concentrations of leptin during the juvenile period, and reduced concentrations of NPY in IIIV CSF at 9 mo of age compared to those in the LG group. Because there was only a tendency for there to be a reduced concentration of NPY in CSF at 8 mo of age in HG heifers, we presume that the nutritionally-mediated suppression of hypothalamic NPY release occurs shortly prior to the onset of puberty. In the current study, the majority of HG heifers (86%; 6/7) that achieved puberty during the study reached sexual maturation around or shortly after 9 mo of age.

Using similar nutritional models, our group has previously demonstrated that the expression of *NPY* in the ARC is decreased in prepubertal heifers that gained BW at a high rate (Allen et al., 2012). Furthermore, we also demonstrated that the proportion of GnRH neurons in close proximity to NPY fibers in the preoptic area and mediobasal hypothalamus are reduced in heifers with increased BW gain (Alves et al., 2011). Taken together, these findings suggest that increased hypothalamic NPY activity in juvenile heifers may play a role in restraining GnRH and LH pulsatile release necessary for final reproductive development and, as heifers approach a more positive state of body energy reserves, NPY inhibitory tone is suppressed culminating in reproductive competence.

Although no replicate effects were observed in BW gain and circulating concentrations of leptin, the proportion of HG heifers that reached puberty during this study differed between replicates. This variability in age at puberty in heifers of similar

genetic background and fed diets of analogous formulation to promote comparable BW gains has been previously observed by our group, and has been reported by others (Gasser et al., 2006a-c). Hence, it is possible that factors other than postnatal nutrition and genetic background may influence the maturation of the reproductive neuroendocrine system. For example, maternal nutrition during fetal development has been shown to cause adverse changes in brain functions that regulate nutrient intake, energy expenditure, and endocrine physiology. Some of these changes are characterized by alterations in expression of *NPY* and the number of NPY cells within the ARC (Warnes et al., 1998, Plagemann et al., 2000), and reduced ability of leptin to signal metabolic status postnatally (Guo and Jen, 1995, Levin and Dunn-Meynell, 2002, Ford et al., 2007). Therefore, further studies are required to determine if maternal nutrition may influence the responsiveness of the hypothalamic circuitry to metabolic cues in prepubertal heifers.

In summary, these results, in conjunction with previous data generated by our group, support the hypothesis that the hypothalamic NPY pathway is involved in the nutritional acceleration of puberty in heifers. Favorable metabolic status during early calfhood, mainly characterized by increased circulating concentrations of leptin, resulted in lower hypothalamic NPY release, greater pulsatile release of LH, and hastened onset of puberty. These results may contribute to the development of nutritional strategies that can exploit postnatal neuroendocrine plasticity to optimally time pubertal onset in replacement beef heifers.

CHAPTER IV

NUTRITIONAL PROGRAMMING OF ACCELERATED PUBERTY IN HEIFERS: INVOLVEMENT OF PROOPIOMELANOCORTIN NEURONS IN THE ARCUATE NUCLEUS

4.1 Synopsis

Integration of metabolic signals in the hypothalamus is believed to be essential for regulating the timing of pubertal development and subsequent fertility in mammalian females. Hypothalamic POMC neurons are considered a critical metabolic-sensing pathway controlling the reproductive neuroendocrine axis. Here, we report studies in prepubertal heifers to determine whether nutritional status during the juvenile period alters *POMC* expression in the ARC, αMSH (a product of the *POMC* gene) neuronal projections in the hypothalamus, and concentrations of αMSH in the IIIV CSF as an indicator of hypothalamic αMSH release. Proopiomelanocortin mRNA abundance was greater in the ARC of heifers that gained BW at a rate of 1 kg/day when compared to heifers that gained 0.5 kg/day between 4 and 8 months of age. Innervation of GnRH neurons by αMSH fibers was limited, and these projections did not appear to be regulated by nutritional status during the juvenile development. To the contrary, a large number of kisspeptin-immunoreactive soma and proximal dendrites in the ARC was observed in close proximity with αMSH-containing varicosities. Furthermore, heifers that gained BW at greater rates during the juvenile period exhibited an increase in the proportion of kisspeptin neurons innervated by αMSH fibers and an increased number of

αMSH close contacts per kisspeptin cell. Concentrations of αMSH in the CSF collected from the IIIV did not differ between groups. These results indicate that activation of the POMC-kisspeptin-GnRH pathway may be an important mechanism mediating the nutritional programming of accelerated puberty in heifers.

4.2 Introduction

In cattle, feeding a high-concentrate diet to heifers during the juvenile period, resulting in increased BW gain, adiposity and elevated circulating concentrations of leptin, leads to early maturation of the reproductive neuroendocrine system and earlier puberty (Day et al., 1984, Gasser et al., 2006, Cardoso et al., 2013). Importantly, a "critical window" for the nutritional programming of accelerated puberty seems to occur around 4 to 6.5 mo of age (early juvenile period), since a period of marked feed intake restriction after this time point does not appear to counter this affect by reducing the proportion of heifers reaching early puberty (Cardoso et al., 2013). The initiation of a characteristic high-frequency pattern of episodic GnRH release during pubertal transition is largely dependent upon metabolic cues received during early development. Although it is clear that the adipocyte-derived hormone, leptin, is critically involved in the maturation of the central reproductive system, the afferent neuronal pathways involved in this process remain unresolved.

POMC neurons in the ARC express the leptin receptor (Cheung et al., 1997, Elmquist et al., 1998) and are considered major central regulators of energy homeostasis (Cone, 1999, Xu et al., 2012). Deleterious mutations in the *POMC* gene or in the genes

encoding the melanocortin receptors, MC3R and MC4R, lead to hyperphagia and obesity, without compromising reproductive functions (Huszar et al., 1997, Yaswen et al., 1999, Chen et al., 2000). Nonetheless, transgenic overexpression of the MC3/4R endogenous antagonist, AgRP, leads to obesity and infertility (Ollmann et al., 1997). Furthermore, POMC neurons have been demonstrated to make direct synaptic contact with GnRH neurons in rats (Leranth et al., 1988, Cheung and Hammer, 1995) and αMSH, one of the products of *POMC*, activates GnRH neurons in female mice as result of direct postsynaptic activation of MC3R and MC4R (Roa and Herbison, 2012). At the functional level, central administration of SHU9119 (a nonselective MC3R/4R antagonist) and HS014 (a selective MC4R antagonist) decrease the magnitude of the LH surge in normally-fed, steroid-primed ovariectomized female rats (Watanobe et al., 1999). In addition, although leptin treatment prevents the fasting-induced inhibition of the LH surge in rats, MC3R/4R antagonists block these leptin effects (Watanobe et al., 1999). Moreover, the melanocortin receptor agonist, melanotan II, stimulates LH release in undernourished, ovariectomized, hypogonadotropic ewes (Backholer et al., 2010b).

Previous results from our laboratories have demonstrated that the expression of *POMC* in the ARC, detected by microarray analysis, was increased in juvenile heifers that gained BW at high rates between 4 and 6.5 mo of age when compared to heifers that gained BW at lower rates (Allen et al., 2012). In contrast, *AgRP* mRNA abundance in the ARC was decreased in heifers that gained BW at higher rates. Based on these results, we reasoned that functional and morphological modifications in melanocortin pathways may be involved in the nutritional control of pubertal development in bovine females.

Therefore, the studies described herein tested the hypotheses that accelerated BW gain during the juvenile period increases 1) *POMC* mRNA abundance in the ARC, 2) the number of αMSH-immunoreactive neurons in the ARC, 3) the αMSH innervation of GnRH and kisspeptin neurons, and 4) concentrations of α-MSH in the cerebrospinal fluid (CSF) collected from the third ventricle (IIIV) of prepubertal heifers.

4.3 Materials and methods

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

4.3.1 Animals and experimental procedures (Experiments 1 and 2)

Details of animal maintenance, nutritional regimens, and diet composition (same as reported in Chapter III, Table 3.1) have been described in detail elsewhere (Alves, 2011). Briefly, crossbred (¾ *Bos taurus*, ¼ *Bos indicus*) heifers were weaned at approximately 3.5 mo of age and assigned randomly to one of two dietary treatments: Low gain (LG) and High gain (HG). Heifers were fed individually and total feed intake was controlled to promote a daily BW gain of 0.5 Kg (LG) or 1 kg (HG). This dietary regimen has been previously reported to accelerate reproductive maturation in HG heifers, resulting in high incidence of heifers reaching puberty by 12 mo of age (Cardoso et al., 2012, Cardoso et al., 2013). Heifers were slaughtered using humane procedures at 8 mo of age and a block of brain tissue containing the septum, preoptic area (POA), and hypothalamus was obtained. To confirm the prepubertal status of heifers at the moment

of tissue collection, circulating concentrations of progesterone were determined in blood samples collected twice weekly starting at 7 mo of age and the ovaries were visually inspected at slaughter.

4.3.2 Experiment 1. Influence of body weight gain on POMC mRNA in the ARC of prepubertal heifers

4.3.2.1 In situ hybridization

Blocks of brain tissue obtained from heifers in the HG (n = 6) and LG (n = 5) groups were snap frozen in liquid nitrogen vapor and cut in series of 20-µm coronal sections using a cryostat. Tissue sections were thaw-mounted on SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA) and stored at -80°C until processing. A series of sections 200-µm apart that included the rostral to caudal extension of the ARC was used for detection of *POMC* mRNA by isotopic *in situ* hybridization.

A DNA template was generated by PCR amplification of hypothalamic ovine cDNA. Primers were designed using the ovine *POMC* sequence reported previously (GenBank accession #NM001009266). The PCR primers were extended at the 5' end with T3 and T7 RNA polymerase promoter sequences. Primers used in this reaction were: 5'**AATTAACCCTCACTAAAGGG**TGGCTCTTCTCCGAGGTCA3' (T3 promoter in bold) and 5'**TAATACGACTCACTATAGGG**AGAGGACAAGCGTTCTTACT3' (T7 promoter in bold). The 348 bp product obtained was sequenced and confirmed to contain a partial sequence of the ovine *POMC* cDNA. This sequence was then aligned to the *Bos taurus*

genome database using the BLAST tool and observed to be 97% identical to the bovine mRNA sequence. Sense and antisense ^{35}S-UTP-labeled RNA probes were generated by *in vitro* transcription using T3 and T7 RNA polymerase, respectively, as described previously (Redmond et al., 2011). Sections were processed and hybridized with radiolabeled *POMC* probe (10^6 cpm/150 μL) overnight at 55 °C as described previously (Redmond et al., 2011). After completing post-hybridization washes, sections were dried and exposed to photographic emulsion for 10 days in the dark at 4 °C. Slides were then developed using Kodak D-19 Developer and fixer (Eastman Kodak, Rochester, NY), and sections were counterstained with Cresyl violet and covered with glass slip using distyrene plasticizer and xylene (DPX, Electron Microscopy Sciences, Hatfield, PA).

Slides were analyzed using a bright- and dark-field microscope (Nikon eclipse 80i – Nikon Inc., Tokyo, Japan). The high density and proximity of *POMC* cells in the ARC limited the ability to count individual cells for analysis. Therefore, the abundance of *POMC* mRNA was determined by analyzing the density of silver grains in images captured through the rostral (rARC; 3 sections), middle (mARC; 5 sections) and caudal (cARC; 3 sections) regions of the ARC using a 10X objective. For determination of density of silver grains per cell, bright-field images of *POMC*-expressing cells were captured using a 40X objective throughout the rARC (5 cells), mARC (30 cells), and cARC (10 cells) of each heifer. Images were analyzed using the NIS Elements software (Nikon Inc.) after normalization by the background density of silver grains which was determined from regions adjacent to the area of interest using procedures described previously (Redmond et al., 2011).

4.3.3 Experiment 2. αMSH immunoreactivity and afferent projections to GnRH and kisspeptin neurons in prepubertal heifers

Blocks of tissue containing the septum, POA, and hypothalamus obtained from heifers in the HG (n = 5) and LG (n = 6) groups were cut in approximately 3 mm coronal blocks and placed in a solution of 0.1M phosphate buffer containing 4% paraformaldehyde for 48 h as described earlier (Alves et al., 2011). Tissue blocks were then immersed in a PB solution containing 30% sucrose for 5 to 7 days. Tissue blocks were then cut in 50-µm sections using a microtome, placed in cryoprotectant solution, and stored at -20°C until processing.

4.3.3.1 Immunohistochemistry

A series of sections 200-µm apart was cut sagittaly in two halves. One series of hemisections was processed for double-label detection of αMSH and GnRH by immunofluorescence. The contralateral series of hemisections was used for double-label immunofluorescent detection of αMSH and kisspeptin. Procedures were carried out on free-floating sections at room temperature under gentle agitation following procedures similar to those described previously (Amstalden et al., 2010). For the αMSH/GnRH double-label immunofluorescence, sections were incubated overnight at room temperature with mouse monoclonal anti-GnRH (1:10,000; Covance, Princeton, NJ; cat#SMI-41R), followed by incubation with Alexa 488 conjugated goat anti-mouse IgG (1:200; Molecular Probes, Eugene, OR; cat#A-11001). Detection of αMSH was performed by incubation with guinea-pig anti-human αMSH (1:40,000; Biosensis,

Adelaide, Australia; cat#GP-030-50) overnight at room temperature, followed by incubation with biotinylated Goat anti-Guinea Pig IgG (1:400; Vector Labs, Burlingame, CA; cat#BA7000) for 1 hr. Alexa 555 conjugated-streptavidin (1:250, Invitrogen, Carlsbad, CA; cat#S-21381) was used to detect αMSH immunoreactivity.

For the αMSH/kisspeptin double-label immunofluorescence, sections were submitted to heat-induced epitope retrieval by incubation in 10mM Sodium Citrate buffer (pH 6) at 90°C for 18 min to enhance detection of kisspeptin, as previously described (Alves, 2011). Sections were incubated overnight at room temperature with rabbit anti-kisspeptin10 (1:250,000; graciously provided by Dr. Alain Caraty, INRA Centre de Tours, Nouzilly, France; cat#INRA564), followed by biotinylated goat anti-rabbit IgG (1:400; Vector Labs; cat#BA-1000) and streptavidin-conjugated horseradish peroxidase (1:600; Vectastain Elite ABC, Vector Labs; cat#PK-6100). Alexa 555 conjugated-streptavidin (1:250; Invitrogen; cat#S-21381) was used to label kisspeptin immunoreactivity. Detection of αMSH was performed using guinea-pig anti-human αMSH (1:20,000; Biosensis; cat#GP-030-50) followed by incubation with Alexa 488 conjugated goat anti-guinea-pig IgG (1:200; Molecular probes; cat#A-11073). After completion of the immunostaining, sections were mounted in glass slides using Gelvatol medium.

Controls for the dual-label immunofluorescence procedure included omission of primary antibodies (anti-GnRH, anti-αMSH, and anti-kisspeptin10) and pre-absorption of primary antibodies with GnRH (100 μM; Sigma-Aldrich, St Louis, MO), αMSH (100 μM; Phoenix Pharmaceuticals Inc., Burlingame, CA), and ovine kisspeptin (1 μg/mL;

American Peptide Company Inc., Sunnyvale, CA). These procedures eliminated the fluorescent signal for each corresponding antigen.

Sections were analyzed using an epifluorescent microscope (Nikon eclipse 80i, Nikon Inc.) and all the quantification analyses were performed by an observer blind to the assignment of tissue sections to experimental groups. Close contacts were defined as direct apposition between αMSH-containing varicosities and GnRH- and kisspeptin-immunoreactive soma and proximal dendrites observed in the same focal plane using a 40X objective. In order to determine the number of αMSH immunoreactive cells and the proportion of arcuate kisspeptin cells innervated by αMSH fibers, 3 comparable sections of the rARC and cARC, and 5 comparable sections of the mARC, were selected from each animal. All GnRH neurons visualized in sections containing the POA and MBH were utilized to determine the proportion of GnRH cells in close apposition to αMSH-containing varicosities. Neurons were considered highly innervated by αMSH fibers when the number of contacts was equal or greater than the upper quartile of the total distribution of contacts per cell. For GnRH neurons, cells observed with 4 or more close contacts were considered highly innervated. For kisspeptin neurons, cells observed with 7 or more contacts were considered highly innervated.

4.3.4 Statistical analysis

The JMP software (SAS Inst. Inc., Cary, NC) was used for statistical analyses. The proportions of GnRH and kisspeptin neurons in close proximity to αMSH containing fibers were normalized using the arcsine square root transformation method.

Density of silver grains per region and per cell, number of immunoreactive αMSH cells in the ARC, mean number of αMSH close contacts to GnRH and kisspeptin neurons, and normalized proportions of GnRH and kisspeptin cells in close proximity to αMSH varicosities were compared between LG and HG groups using the Student's t-test. Each hypothalamic region was analyzed independently.

4.3.5 Experiment 3. Concentrations of αMSH in the CSF collected from the IIIV of prepubertal heifers

4.3.5.1 Animals and experimental procedures

The details of animal maintenance, nutritional regimens, diet composition, and experimental procedures are described in Chapter III (please, see Animals and Experimental Procedures). Briefly, 25 crossbred (¾ *Bos taurus*, ¼ *Bos indicus*) heifers were utilized in two replicates over a 2 yr period (1 replicate/yr). Within each year, heifers were weaned at approximately 3.5 mo of age (age at weaning = 106 ± 3 d), stratified by date of birth, and assigned randomly to be fed individually until 11 mo of age either to achieve a relatively low rate of BW gain (LG, 0.5 kg/d; n = 6/replicate) or a high rate of BW gain (HG, 1 kg/d; replicate 1, n = 6; replicate 2, n = 7). Targeted average daily gain was attained by adjustments in the dry matter intake based on BW gain determined every 2 wk. To assess pubertal status, circulating concentrations of progesterone were determined in blood samples collected twice weekly starting at 7.5 mo of age.

4.3.5.2 Surgical cannulation of the IIIV and intensive sampling

Starting at 6 mo of age, heifers were placed frequently in stanchions and acclimated to intensive handling conditions to minimize the effects of stress. At 7.5 mo of age (228 ± 3 d), a subgroup of heifers (n = 3/group/replicate) was randomly selected to be surgically fitted with a IIIV cannula as previously described in detail (Gazal et al., 1998). The location and function of the cannulas were verified by radiography and continuous flow of CSF. A period of at least 2 wk was allowed for heifers to recover from surgery.

At 8 mo of age (248 ± 5 d), heifers were placed in stanchions and blood samples (6 mL) were collected, via extensions connected to the jugular catheter and remotely secured, simultaneously with CSF samples (600 μL) at 15-min intervals (replicate 1: n = 3/group) or 10-min intervals (replicate 2: n = 3/group) for 6 h. Intensive blood sampling continued for an additional 6 h. Cerebrospinal fluid samples were placed on ice immediately and stored at -20°C within 30 min of collection until determination of αMSH concentrations. Intensive blood and CSF sampling procedures were repeated at 9 mo of age in heifers that had not been detected pubertal (LG, n = 6; HG, n = 3).

4.3.5.3 Radioimmunoassay

Concentrations of αMSH in the CSF were determined using a commercial RIA kit (Phoenix Pharmaceuticals, Inc.). Hourly CSF samples collected from the IIIV during the 6 h sampling period at 8 and 9 mo of age were used for this experiment. To enhance the detection of αMSH in samples, 1.2 mL of CSF was dried down in a Speed-Vac

concentrator (Savant, Farmingdale, NY) and pellets were completely solubilized in 240 μL of assay buffer. Subsequently, 100 μL of the reconstituted samples were assayed in duplicates. Recovery of added mass and parallelism between serial dilutions of samples with the standard curve were performed to validate the use of the commercial RIA kit in bovine CSF samples. The sensitivity of the assay averaged 6.0 pg/tube with intra and interassay CV of 2.5% and 9.2%, respectively.

4.3.5.4 Statistical analysis

Mean concentrations of αMSH were analyzed by general linear mixed models for repeated measures using the mixed procedure (PROC MIXED) of SAS (SAS Inst. Inc.). Sources of variation were treatment, replicate, time, and their interactions. Time was used as the repeated variable and heifer was used as the subject.

4.4 Results

4.4.1 Experiment 1. Hypothalamic POMC mRNA expression in prepubertal heifers

Hybridization of tissue sections with antisense probe to *POMC* mRNA labelled an abundant number of cells in the ARC of prepubertal heifers (Fig. 4.1). The mean density of silver grains in the rARC and mARC was greater ($P < 0.05$) in HG than LG

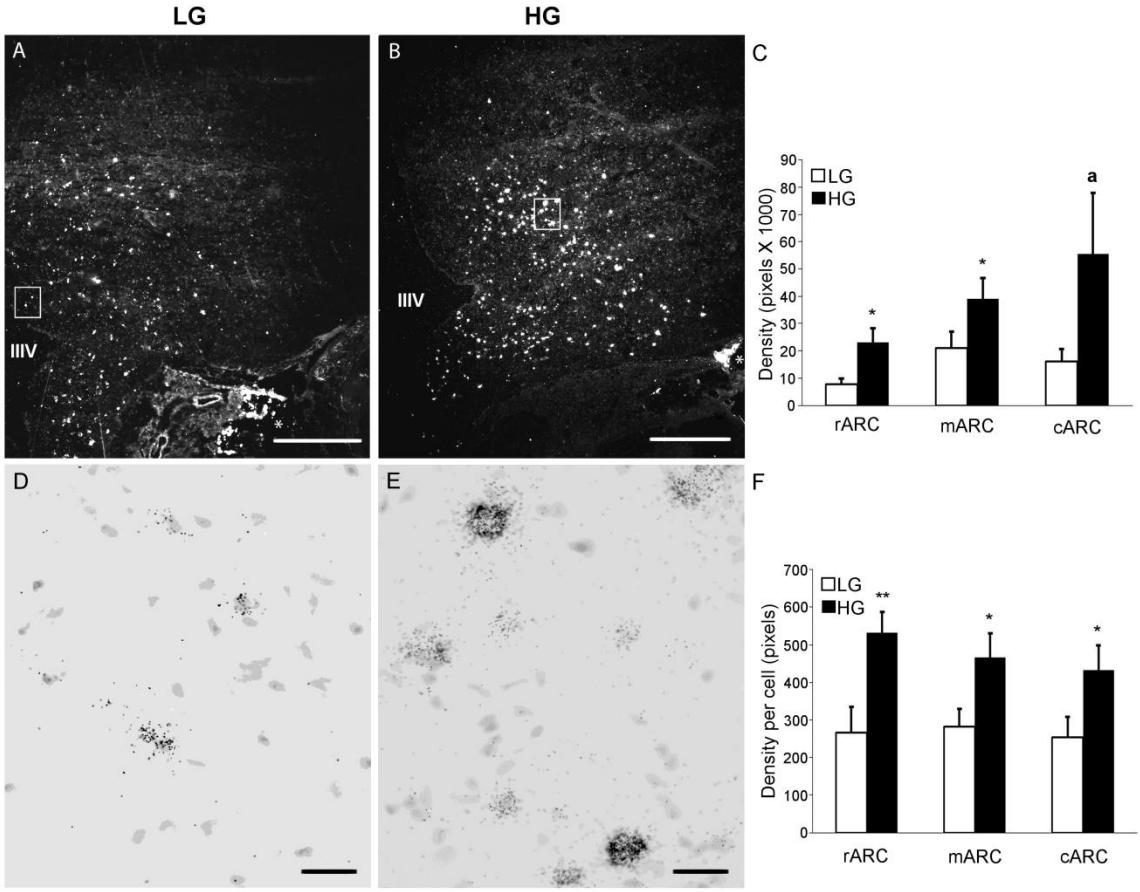

Fig. 4.1. *POMC* mRNA in the arcuate nucleus (ARC) of prepubertal heifers. (A, B) Low-magnification, dark-field image depicting accumulation of silver grains in the ARC in sections processed for *in situ* hybridization from representative low-gain (LG; A) and high-gain (HG; B) heifers. (C) Mean (± SEM) density of silver grains in the rostral (rARC), middle (mARC), and caudal (cARC) ARC. (D, E) High-magnification, bright-field images depicting silver grain accumulation over Cresyl violet-stained cells shown in A and B (boxed areas). (F) Mean (± SEM) density of silver grains per cell in the rARC, mARC, and cARC. Scale bars: (A, B) 500 µm; (D, E) 20 µm. IIIV, third ventricle. Asterisks in images A and B indicate artifacts at the meninges. $^{a}P = 0.07$; $*P < 0.05$; $**P < 0.01$.

heifers (Fig. 4.1C). In the cARC, a trend ($P = 0.07$) was observed for higher *POMC* mRNA signal density in HG than LG heifers (Fig. 4.1C). Furthermore, HG heifers demonstrated greater ($P < 0.05$) mean density of silver grains per cell than LG heifers in all 3 subregions of the ARC (Fig. 4.1F).

4.4.2 Experiment 2. αMSH immunoreactivity and afferent projections to GnRH and kisspeptin neurons in prepubertal heifers

4.4.2.1 αMSH immunoreactivity

Within the hypothalamus, the majority of αMSH-immunoreactive cells were detected in the ARC, with a smaller number of cell bodies observed in the subventricular extension of the ARC (Fig. 4.2). Most of the αMSH-immunoreactive neuronal projections were localized within the ARC, ventromedial hypothalamus (VMH), and median eminence, although the density of fibers in the median eminence was limited. In the mARC, the mean number of αMSH-immunoreactive cells was greater ($P < 0.05$) in HG than in LG heifers (Fig.4.2C). No significant differences were observed between groups for the number of αMSH cells in the rARC and cARC (Fig.4.2C).

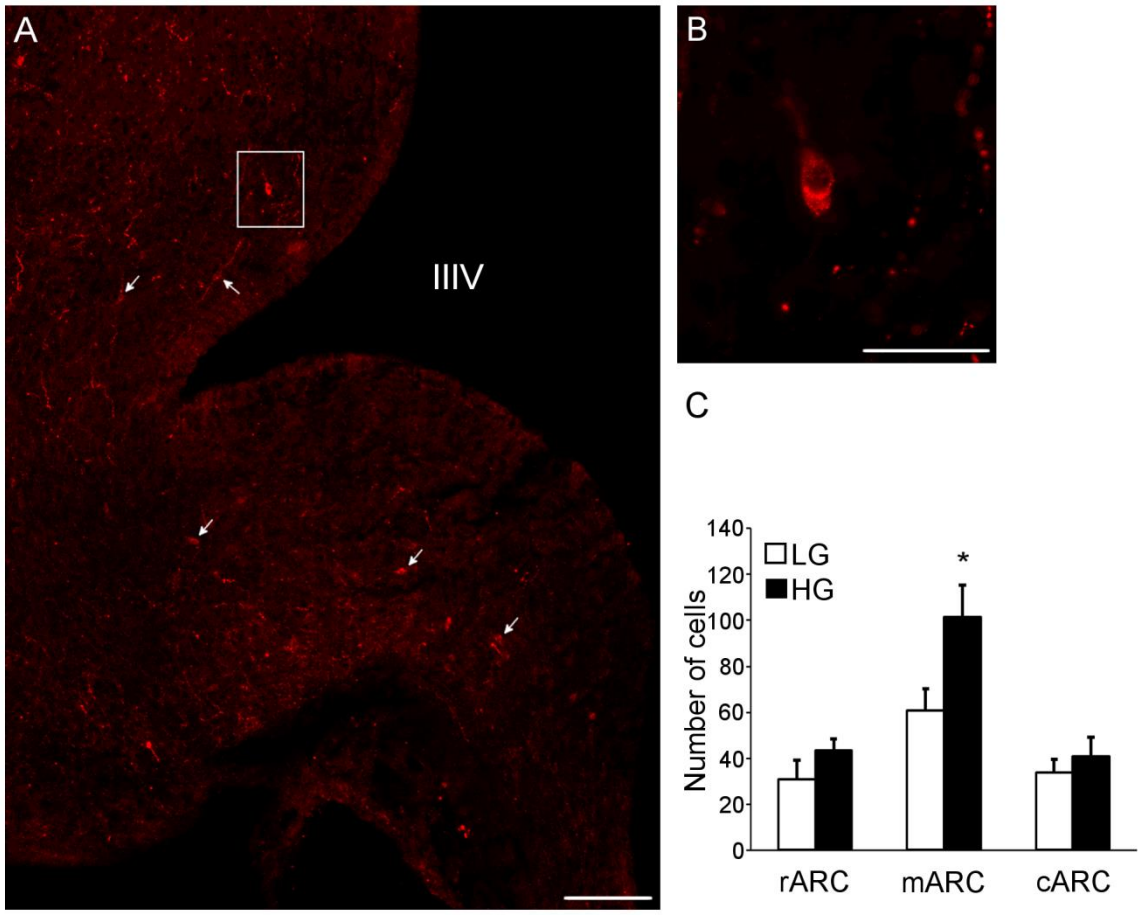

Fig. 4.2. αMSH immunoreactivity in the arcuate nucleus (ARC) of prepubertal heifers. (A) Low-magnification image demonstrating the distribution of αMSH-immunoreactive cell bodies (arrows) and fibers in the ARC and stalk of the median eminence. (B) High-magnification image depicting an αMSH-immunoreactive neuron shown in A (boxed area). (C) Mean (± SEM) number of αMSH-immunoreactive cells in the rostral (rARC), middle (mARC), and caudal (cARC) ARC. Scale bars: (A) 500 μm; (B) 50 μm. IIIV, third ventricle; LG, low-gain; HG, high-gain. *$P < 0.05$.

4.4.2.2 αMSH afferent projections to GnRH neurons

Similarly to previous findings in adult cows (Dees and McArthur, 1981), immunoreactive GnRH neurons were observed scattered in a rostral to caudal distribution from the septum to medial portions of the basal hypothalamus, with the majority of cell bodies localized in the POA at the level of the organum vasculosum of lamina terminalis (OVLT). Fibers containing αMSH were observed in close apposition (Fig. 4.3A) with approximately 20% of the GnRH neurons throughout the rostral-caudal distribution. No differences between dietary groups were observed in the mean number of GnRH cell bodies (Fig. 4.3B), percentage of GnRH neurons in close proximity with αMSH fibers (Fig. 4.3C), or mean number of αMSH-containing varicosities in close apposition to GnRH cell bodies and proximal dendrites (Fig. 4.3D). Only approximately 6% of the GnRH neurons analyzed were determined to be highly innervated (4 or more contacts) by αMSH fibers and no difference was observed between experimental groups.

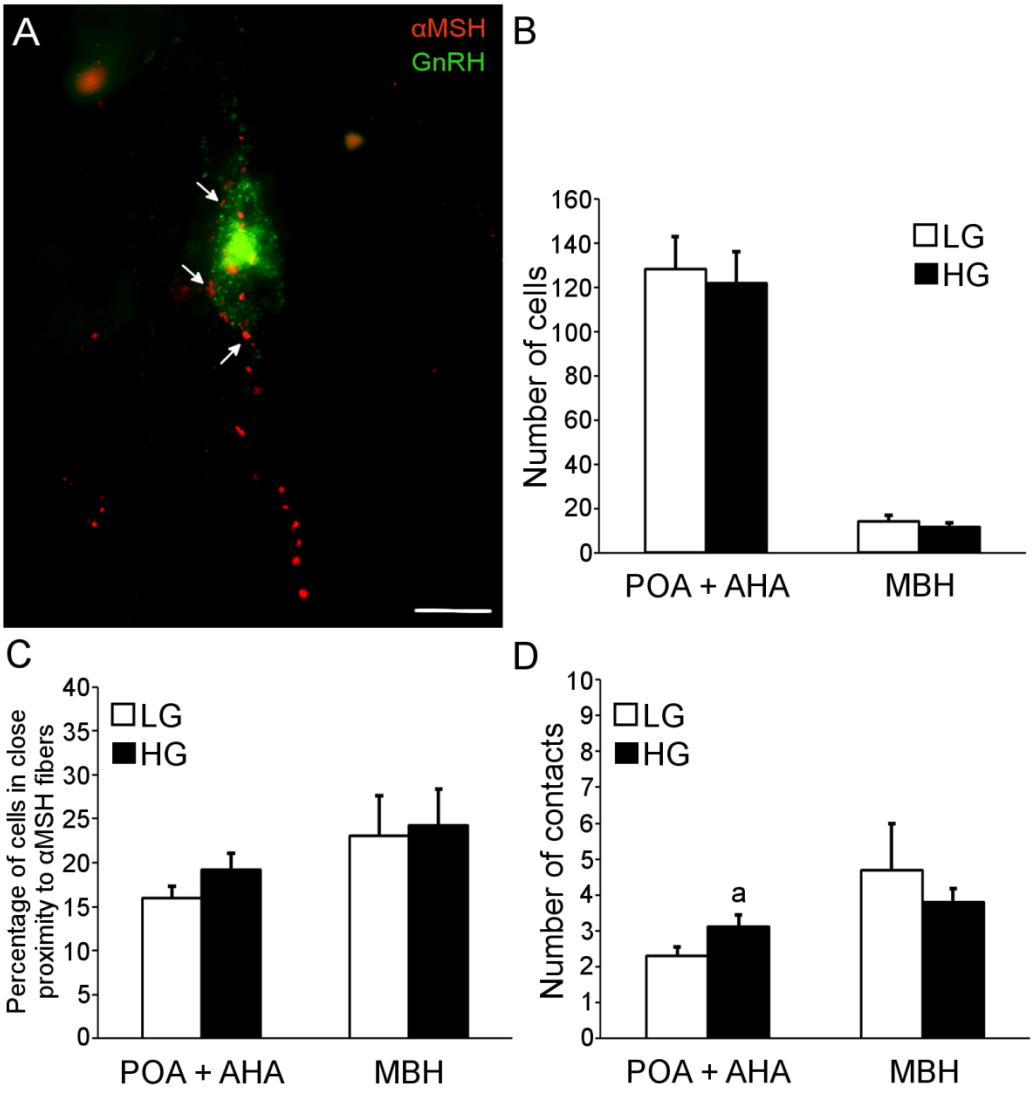

Fig. 4.3. Hypothalamic αMSH fiber projections to GnRH cell bodies and proximal dendrites in prepubertal heifers. (A) High-magnification image depicting a GnRH-immunoreactive neuron (green) in close apposition (arrows) to αMSH-containing varicosities (red). (B) Mean (± SEM) number of GnRH-immunoreactive cells in the preoptic area (POA) + anterior hypothalamic area (AHA) and mediobasal hypothalamus (MBH). (C) Mean (± SEM) percentage of GnRH-immunoreactive neurons in close proximity with αMSH-containing fibers in the POA+AHA and MBH. (D) Mean (± SEM) number of αMSH-containing varicosities in close contact with GnRH-immunoreactive soma and proximal dendrites in the POA+AHA and MBH. Scale bar: (A) 20 μm. LG, low-gain; HG, high-gain. [a]*P* = 0.09.

4.4.2.3 αMSH afferent projections to kisspeptin neurons

An abundance of kisspeptin-immunoreactive cell bodies was detected in the ARC of prepubertal heifers, and no differences in the number of neurons were observed between dietary groups (rARC, 18.60 ± 2.94; mARC, 39.10 ± 4.93; cARC, 12.73 ± 1.59). A large proportion of kisspeptin cells were visualized in close apposition with αMSH-containing varicosities (Fig 4.4A). In the rARC and mARC, the proportion of kisspeptin cells innervated by αMSH fibers was greater ($P < 0.05$) in HG than LG heifers (Fig 4.4B), but no difference was observed in the cARC. Likewise, among the innervated kisspeptin neurons, the proportion considered highly innervated (7 or more contacts) by αMSH fibers was also greater ($P < 0.001$) in the mARC of HG heifers when compared to LG (Fig. 4.4D). In addition, the number of contacts per innervated kisspeptin cell in the mARC was greater ($P < 0.001$) in the HG group (Fig 4.4C).

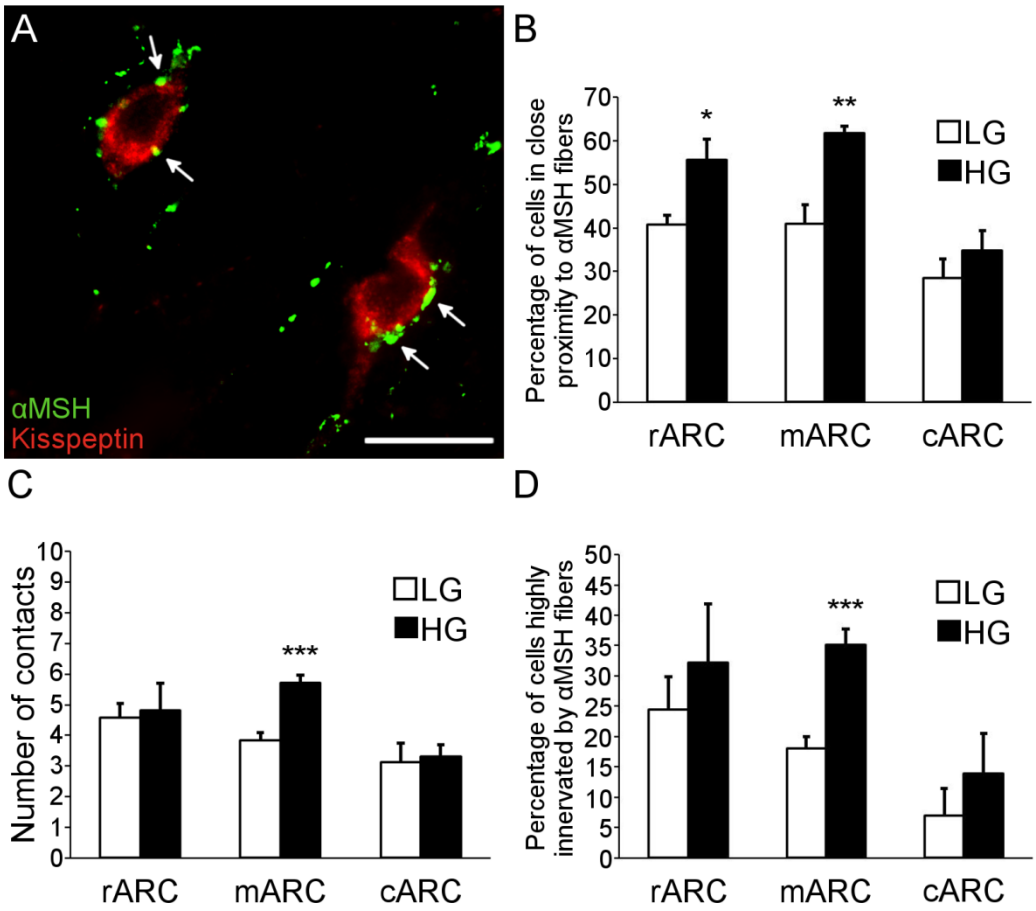

Fig. 4.4. αMSH fiber projections to kisspeptin cell bodies and proximal dendrites in the arcuate nucleus (ARC) of prepubertal heifers. (A) High-magnification image depicting kisspeptin-immunoreactive neurons (red) in close apposition (arrows) with αMSH-containing varicosities (green). (B) Mean (± SEM) percentage of kisspeptin-immunoreactive neurons in close proximity to αMSH-containing fibers in the rostral (rARC), medial (mARC), and caudal (cARC) ARC. (C) Mean (± SEM) number of αMSH-containing varicosities in close apposition with kisspeptin-immunoreactive soma and proximal dendrites in the ARC. (D) Mean (± SEM) percentage of kisspeptin-immunoreactive neurons highly innervated (7 or more contacts) by αMSH-containing fibers in the ARC. Scale bar: (A) 50 μm. LG, low-gain; HG, high-gain. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

4.4.3 Experiment 3. Concentrations of αMSH in the CSF collected from the IIIV of prepubertal heifers

No replicate effect was observed for mean concentrations of αMSH; therefore, data were pooled by treatment. Mean concentrations of αMSH from one HG heifer at 8 and 9 mo of age were observed to be two standard deviations greater than the mean for the HG group. Thus, data from this heifer were eliminated from analysis. Concentrations of αMSH determined hourly for a period of 6 h in the CSF collected from the IIIV of prepubertal heifers ranged from undetectable to 63 pg/mL and did not present an episodic pattern of secretion; therefore, comparison between experimental groups was performed based on mean concentrations. CSF concentrations of αMSH did not differ between experimental groups at 8 and 9 mo of age, when only 2 HG heifers remained prepubertal (Fig. 4.5).

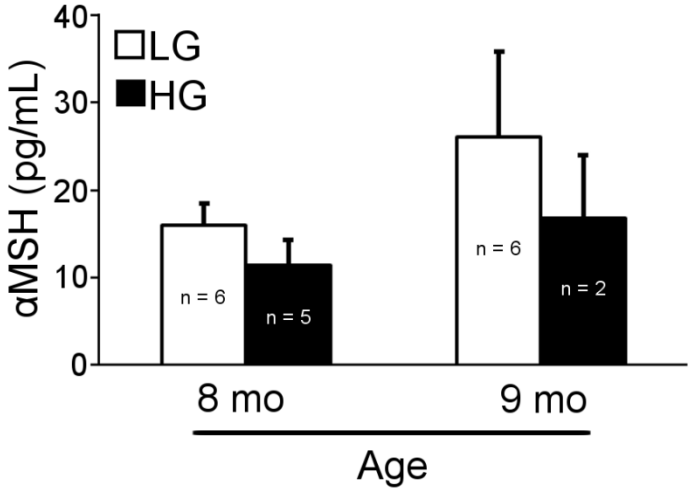

Fig. 4.5. Mean **(**± SEM) concentrations of αMSH in the cerebrospinal fluid collected from the third ventricle of Low-gain (LG) and High-gain (HG) prepubertal heifers at 8 and 9 mo of age.

4.5 Discussion

The results reported herein provide strong evidence for the involvement of hypothalamic POMC neurons in the nutritional control of pubertal development in heifers. Furthermore, these findings indicate that an indirect pathway encompassing kisspeptin neurons in the ARC may mediate the metabolic regulation of GnRH neurons by αMSH. A direct regulation of GnRH cells appears less likely because only a few of these neurons are observed in close apposition to αMSH-immunoreactive varicosities. The present results corroborate an earlier study (Backholer et al., 2010b) which demonstrated that POMC cells project to kisspeptin neurons in the ARC of ewes, and indicate that this pathway might be important for mediating melanocortin stimulatory effects on the reproductive axis.

The increase in *POMC* mRNA abundance in the ARC of prepubertal heifers that were fed a high-concentrate diet to gain BW at accelerated rates during the juvenile period demonstrates the responsiveness of the POMC gene to nutritional status. Previous data from our laboratories indicated that the nutritional regimen used in the current study leads to increased BW gain and greater circulating concentrations of leptin during the juvenile development (Allen et al., 2012, Cardoso et al., 2013). As mentioned previously, POMC neurons express leptin receptor (Cheung et al., 1997, Elmquist et al., 1998), and fasting, which leads to reduced circulating leptin, results in a reduction of *POMC* mRNA abundance in the ARC of mice (Schwartz et al., 1997). Moreover, transgenic mice lacking leptin and leptin receptor exhibit a reduction in *POMC* mRNA expression in the ARC. Likewise, intracerebroventricular infusion of leptin results in

upregulation of *POMC* mRNA in the ARC of lean, ovariectomized ewes (Backholer et al., 2010a). Therefore, the increased arcuate *POMC* expression observed in the current study likely occurs in response to increased leptin signaling in heifers fed to gain BW at an accelerated rate.

Proopiomelanocortin is posttranslationally cleaved into several melanocortins, including adrenocorticotropic hormone (ACTH) and α-MSH, and the opioid peptide β-endorphin (β-END) (reviewed by Pritchard et al., 2002). Although the various POMC-derived peptides have distinct physiological functions, melanocortins, including αMSH, have been demonstrated to reduce food intake (Fan et al., 1997) and exert excitatory effects on GnRH neurons (Backholer et al., 2009, Roa and Herbison, 2012). In the current study, the increase in the number of αMSH-immunoreactive neurons in the mARC is in agreement with the greater *POMC* mRNA expression observed in the ARC of HG heifers. This observation indicates that in heifers developing under the influence of a relatively increased nutritional status, the posttranslational processing of POMC leads to increased αMSH synthesis. This is conducive to the effects of adiposity in facilitating the pubertal activation of the reproductive axis. However, only a small percentage of the GnRH neurons were observed in close proximity to αMSH fibers in this study. Therefore, a direct input of αMSH onto GnRH neurons does not appear to be a primary pathway by which melanocortins may be involved in mediating the nutritional acceleration of puberty in heifers.

In order to investigate an alternative pathway by which αMSH may exert stimulatory effects on the secretion of GnRH, we determined whether αMSH projections

toward kisspeptin neurons are regulated by nutrition during the prepubertal period. In support of our hypothesis, we observed that innervation of kisspeptin neurons by αMSH was increased in heifers gaining BW at high rates during juvenile development. Projections containing POMC have been observed in proximity to kisspeptin neurons in sheep (Backholer et al., 2010b). However, melanotan II, a MCR agonist, has been reported to suppress arcuate *Kiss1* expression in ewes during the luteal phase (Backholer et al., 2009). Even though a direct inhibitory effect of melanocortin treatment on *Kiss1* expression in the ARC is possible, a potential rise in estradiol concentrations resulting from the increase in LH secretion that follows melanotan II infusion may negatively regulate *Kiss1* mRNA expression in the ARC. This notion is supported by the observation that estradiol inhibits *Kiss1* mRNA in the ARC of ovariectomized ewes (Smith et al., 2007). Thus, although kisspeptin neurons in the ARC appear to be an important intermediate pathway mediating the metabolic control of GnRH release, the mechanisms involved in this pathway are complex and remain to be clarified.

Kisspeptin cells in the ARC express the leptin receptor (Smith et al., 2006b) and conditions of low circulating concentrations of leptin are associated with suppression of *Kiss1* expression in the hypothalamus of prepubertal rats (Castellano et al., 2005). In addition, kisspeptin treatment alleviates the undernutrition-induced delay of puberty (Castellano et al., 2005, Kalamatianos et al., 2008); thus, kisspeptin neurons may mediate the permissive effect of leptin in the pubertal progression. Nonetheless, a recent study demonstrated that leptin induction of STAT3 phosphorylation, a major intracellular signaling mechanism induced by leptin, is absent in kisspeptin neurons

(Quennell et al., 2011). Additionally, Donato et al. (2011) reported that mice with selective deletion of leptin receptors in kisspeptin neurons undergo normal pubertal development, sexual maturation, and fertility. Therefore, indirect neuronal pathways appear to mediate leptin's effects on *Kiss1* mRNA expression/kisspeptin production. Because most of the POMC cells in the ARC express the leptin receptor (Cheung et al., 1997, Iqbal et al., 2001), it is plausible that the POMC system may be an important conduit for leptin's actions on kisspeptin neuronal activity in prepubertal heifers. This hypothesis is supported by the current study which indicates that the majority of kisspeptin neurons in the ARC are in close proximity with αMSH fibers. Moreover, the observation that kisspeptin neurons express *MC4R* in mice (Cravo et al., 2011) provides further support for the relevance of the αMSH-kisspeptin-GnRH pathway.

Interestingly, the hypothalamic neuropeptide Y (NPY) system has also been demonstrated to be involved in the nutritional control of puberty. In prepubertal heifers, a high proportion of GnRH neurons are observed in direct apposition with NPY-immunoreactive fibers and the magnitude of these projections is controlled by nutrition during the juvenile development (Alves et al., 2011). Because NPY inhibits GnRH release in cows (Gazal et al., 1998) and central administration of NPY delays the onset of puberty in rats (Pierroz et al., 1995), attenuation of inhibitory NPY tone is believed to be an important event during pubertal transition. Therefore, it appears that functional and structural changes in key metabolic-sensing circuitries, including the NPY and POMC pathways, are involved in the pubertal maturation of GnRH secretory activity.

Concentrations of αMSH were detected in the CSF collected from the IIIV of prepubertal heifers; however, no effect of dietary treatment was observed. Episodes of GnRH secretion can be effectively determined in the IIIV CSF of cows and are highly correlated with peripheral secretory episodes of LH (Gazal et al., 1998). In addition, NPY can also be successfully detected in the CSF from the IIIV of prepubertal heifers (Cardoso et al., 2012) and, as anticipated, its concentrations were lower in heifers that gained BW at high rates during the juvenile period (Cardoso et al., 2012). Through the placement of cannulas in different areas of the IIIV, Caraty and Skinner (2008) demonstrated that the median eminence region is the major, if not only, source of GnRH entering the cerebroventricular system in ewes. Contrary to GnRH and NPY projections, the density of αMSH immunoreactive fibers in the median eminence is limited in prepubertal heifers; therefore, it is possible that concentrations of αMSH in the IIIV CSF do not effectively reflect the release of this peptide in the hypothalamus.

In summary, nutritional status during the juvenile period regulates *POMC* expression in the ARC of prepubertal heifers. Although few GnRH neurons are observed in close apposition with αMSH fibers, kisspeptin neurons in the ARC may provide a neuronal network through which melanocortins stimulate the reproductive neuroendocrine system. These observations indicate that nutrition and metabolic signals may program the time of onset of puberty in heifers by regulating the POMC-kisspeptin-GnRH neuronal pathways.

CHAPTER V

USE OF A STAIR-STEP COMPENSATORY GAIN NUTRITIONAL REGIMEN TO PROGRAM THE ONSET OF PUBERTY IN BEEF HEIFERS

5.1 Synopsis

It was hypothesized that metabolic programming of processes underlying puberty can be shifted temporally through the use of a stair-step, compensatory growth model such that puberty is optimally-timed to occur at 11 to 12 mo of age. Forty crossbred beef heifers were weaned at approximately 3.5 mo of age, and after a 2-wk acclimation period, were assigned randomly to 1 of 4 nutritional groups: 1) Low Control (LC), restricted feed intake of a forage-based diet to promote BW gain of 0.5 kg/d until 14 mo of age; 2) High Control (HC), controlled feed intake of a high-concentrate diet to promote BW gain of 1 kg/d until 14 mo of age; 3) Stair-Step 1 (SS-1), ad libitum feed intake of a high-concentrate diet until 6.5 mo of age followed by restricted access to a high-forage diet to promote BW gain of 0.35 kg/d until 9 mo of age, ad libitum feed intake of a high-concentrate diet until 11.5 mo of age, and restricted intake of a high-forage diet to promote BW gain of 0.35 kg/d until 14 mo of age; and 4) Stair-step 2 (SS-2), reverse sequence of SS-1, beginning with restricted access to a high-forage diet. Body weight (every 2 wk) and circulating concentrations of leptin (monthly) were determined throughout the experiment. Concentrations of progesterone in blood samples collected twice weekly beginning at 8 mo of age were used to determine pubertal status. Body weight gain followed a pattern similar to that proposed in our experimental design.

Circulating concentrations of leptin increased following distinct elevations in BW, but decreased abruptly after feed intake restriction. Survival analysis indicated that the percentage of pubertal heifers in the LC group was lower ($P < 0.05$) than all other groups throughout the experiment. Although heifers in SS-1 were nutritionally restricted between 6.5 and 9 mo of age, the proportion pubertal by 12 mo of age did not differ ($P = 0.36$) from that of the HC group, with 80% and 70% pubertal in SS-1 and HC, respectively. In contrast, the proportion of heifers pubertal by 12 mo of age in the SS-2 group (40%) was lower ($P < 0.05$) than both HC and SS-1. However, by 14 mo of age, 90% of heifers in the SS-2 had also attained puberty compared to only 40% of the LC group. In summary, these data provide evidence that changes in the nutritional and metabolic status during the early juvenile period can program the onset of puberty that occurs months later, allowing optimal timing of sexual maturation in replacement beef heifers.

5.2 Introduction

Developing managerial strategies that optimize the timing of sexual maturation of heifers to allow maximum lifetime productivity remains a major goal of the beef industry. Because multiple estrous cycles before first breeding are often required to achieve optimal fertility (Byerley et al., 1987), assuring that puberty is attained in advance of 14 mo of age is critical for heifers to calve as 2-year olds (Lesmeister et al., 1973).

Age at puberty in heifers is controlled largely by genetic and environmental factors, among which nutrition has a major influence. Nutrient restriction during postnatal development delays puberty (Day et al., 1984), and feeding high-concentrate diets during the juvenile period can markedly increase the frequency of precocious puberty (puberty ≤ 300 days; Gasser et al., 2006a). Although the mechanisms underlying nutritionally-mediated precocious puberty have not been completely elucidated, leptin and leptin-sensitive cells in the hypothalamus appear to play a critical role (Garcia et al., 2003; Maciel et al., 2004b). For example, recent work in our laboratories has reported that heifers fed a high-concentrate diet to promote elevated BW gain during early calfhood exhibit altered expression of leptin-responsive genes within the arcuate nucleus (Allen et al., 2012). This functional regulation may underlie the process of accelerated puberty.

While valuable as a model for studying neuroendocrine changes regulating sexual maturation, precocious puberty is not a desirable phenomenon in a production setting. Therefore, a better understanding of the window of opportunity to nutritionally program and optimally time puberty is needed. Using a modified version of a previously reported stair-step nutritional regimen in dairy and beef heifers (Park et al., 1987; Park et al., 1998), the current study examined the ability of a stair-step compensatory gain nutritional regimen to program the onset of puberty in beef heifers at approximately 11 to 12 mo of age.

5.3 Materials and methods

This experiment was conducted at the Texas A&M University Nutrition-Physiology Research Center (College Station, TX). All animal-related procedures used were approved by the Institutional Agricultural Animal Care and Use Committee of the Texas A&M University System (AUP # 2009-151).

5.3.1 Animal procedures and nutritional model

Forty spring-born, crossbred heifers (½ Angus, ¼ Hereford, ¼ Brahman), weaned at approximately 3.5 mo of age (age at weaning = 109 ± 2 d), were stratified by date of birth, and assigned randomly to 1 of 4 dietary treatments (n = 10/treatment) in 2 experimental replicates (n = 20/replicate): 1) Low Control (LC; n = 10), heifers were fed forage-based diets to promote an average daily gain (ADG) of 0.5 kg from week 0 (4 mo of age) to week 40 (14 mo of age) of the experiment; 2) High Control (HC; n = 10), animals were fed high-concentrate diets to promote an ADG of 1 kg from week 0 to week 40 of the study; 3) Stair-step 1 (SS-1; n = 10), ad libitum feed intake of a concentrate diet from week 0 to week 10 (6.5 mo of age; Period 1) followed by restricted dry matter access of a high-forage diet to promote an ADG of 0.35 kg until week 20 (9 mo of age; Period 2), ad libitum feed intake of a high-concentrate diet until week 30 (11.5 mo of age; Period 3), and restricted intake of a forage-based diet to promote an ADG of 0.35 kg until week 40 of the study (Period 4); and 4) Stair-step 2 (SS-2; n = 10), reverse sequence of SS-1 with the dietary treatment beginning with restricted access to a forage-based diet.

Diets were balanced using the Large Ruminant Nutrition System (LRNS; http://nutritionmodels.tamu.edu/lrns.htm), which is based on the Cornell Net Carbohydrate and Protein System as described by Fox et al. (2004). Targeted ADG was attained by adjustments in the dry matter intake (DMI) based on BW gain determined every 2 wk. Dietary ingredients and chemical composition are presented in Table 5.1.

Table 5.1. Ingredients and chemical composition of diets fed to prepubertal beef heifers between 4 and 14 mo of age. Diet A1 was provided to high control (HC) and stair-step 1 (SS-1) heifers during dietary Period 1; Diet A2-3 was fed to HC heifers during Periods 2 and 3, to stair-step 2 (SS-2) heifers during Period 2, and to SS-1 heifers during dietary Period 3; Diet A4 was fed to HC and SS-2 groups during Period 4 of the experiment; Diet B1 was fed to low control (LC) and SS-2 heifers during dietary Period 1; Diet B2-4 was fed to LC heifers during Periods 2, 3, and 4 of the study, to heifers in the SS-1 group during Periods 2 and 4, and to SS-2 heifers during dietary Period 3.

	Diets[1]				
Item	A1	A2-3	A4	B1	B2-4
Ingredients[2]					
Alfalfa hay, %	6.78	3.22	2.25	2.57	2.60
Cottonseed hulls, %	6.44	2.41	2.14	24.44	24.89
Rolled corn, %	42.39	48.28	53.06	19.80	28.45
Cane molasses, %	7.90	2.41	3.94	4.50	3.86
Corn gluten feed, %	8.44	4.83	5.25	2.40	2.58
Urea, %	0.76	0.72	0.94	0.65	0.94
Soybean meal, %	12.46	12.87	10.35	7.10	8.25
Bermudagrass hay, %	13.70	24.14	21.42	37.90	27.63
Producers 12:12 premix, %	0.38	0.32	0.19	0.43	0.54
Calcium carbonate, %	0.75	0.8	0.46	0.21	0.26
Chemical composition[2]					
Metabolizable energy, Mcal/kg	2.59	2.64	2.71	2.24	2.34
Crude protein, %	16.90	17.00	17.00	13.70	14.50
Digestible intake protein, %	12.34	12.75	12.58	9.60	10.59

[1] Diets were balanced using the Large Ruminant Nutrition System
[2] Dry matter basis

Heifers in each group were allocated to 8 pens (n = 5/pen) measuring 10 x 6 m and equipped with a Calan Gate individual feeding system (American Calan, Northwood, NH). After weaning, heifers were fed a pre-experimental acclimation diet for 2 wk. During the first week of the adaptation period, heifers in all treatment groups were fed a forage-based diet (B1) ad libitum. During the second week of acclimation, heifers assigned to HC and SS-1 groups were fed a diet consisting of 50% B1 and 50% of a concentrate-based diet (A1) up to a maximum of 3.6 kg/heifer daily. Heifers assigned to LC and SS-2 groups were fed B1 through the second week of the adaptation period. After the 2-wk acclimation period, heifers were fed 100% of their respective treatment diets for the remainder of the experiment. During the transition between experimental periods, heifers transitioning from a high-forage diet to a concentrate-based diet were fed 50% of each diet for 1 wk prior to complete adaptation. Transition from a high-concentrate diet to a forage-based diet was performed without acclimation. At the end of each dietary period, body condition score (BCS) was scored on a scale of 1 to 9 (NRC, 2000) by visual appraisal performed by the same experienced technician.

Blood samples from the coccygeal vasculature were collected monthly for the duration of the experiment for determination of serum concentrations of leptin. During the transition between dietary periods (week 10, 20, and 30 of the experiment), additional blood samples were collected for determination of serum concentrations of leptin from heifers in the SS-1 and SS-2 groups at the time of diet transition, and at 2 and 4 wk after transition. Starting at 8 mo of age, blood samples were collected twice weekly for assessment of circulating concentrations of progesterone. Onset of puberty was

defined as the first time point that serum concentrations of progesterone indicated luteal activity for the duration of a normal estrous cycle. The criterion used for identification of the first normal estrous cycle with samples collected twice weekly was the presence of at least three consecutive samples with concentrations of progesterone ≥ 1 ng/mL (Day et al., 1984). Before feeding, blood samples were drawn into evacuated blood collection tubes (Vacutainer, 10 mL; Becton Dickinson, Franklin Lakes, NJ), placed on ice immediately, and centrifuged (2,200 x *g* for 30 min at 4°C) for collection of serum. Blood serum was stored at -20°C until hormone assays.

5.3.2 Hormone assays

Circulating concentrations of progesterone were determined using a commercial radioimmunoassay (RIA) kit (Coat-A-Count, Siemens Healthcare, Malvern, PA) as reported previously (Fajersson et al., 1999). Sensitivity of the assay was 0.05 ng/mL with intra- and inter-assay CV of 5.9% and 5.1%, respectively. Circulating concentrations of leptin were determined in triplicate in a single RIA using a highly-specific oleptin RIA validated for use in bovine serum (Delavaud et al., 2000) and as reported previously (Amstalden et al., 2000). Sensitivity of the assay was 0.1 ng/mL and intra-assay CV averaged 6%.

5.3.3 Statistical analysis

Because heifers were fed individually via the Calan Gate system, data were analyzed using heifer as the experimental unit. Changes in BW and concentrations of

leptin were analyzed using the mixed procedures for repeated measures (PROC MIXED) of SAS (SAS Inst. Inc., Cary, NC) and Satterthwaite approximation to determine the denominator degrees of freedom for the tests of fixed effects. Sources of variation were treatment, experimental replicate, week, and their interactions. Week was used as the repeated variable and heifer was used as the subject. The least squares means procedure was used to compare means when a significant difference was detected in the MIXED analysis. Kaplan-Meier survival analysis (GraphPad Software, La Jolla, CA) was used to evaluate the effect of treatment on the onset of puberty during the experimental period. The analysis used regression of the cumulative proportion of pubertal heifers as a function of week of the experiment. When the treatment effect was significant, contrasts were used to compare groups using the GLIMMIX procedure of SAS.

5.4 Results

5.4.1 Body weight gain and circulating concentration of leptin

Because no replicate effect was observed for BW gain, BCS and circulating concentrations of leptin, data were pooled by treatment. Mean (± SEM) BW at the beginning of the experiment did not differ between groups (150 ± 2 kg). Body weight gain in all groups followed a pattern similar to that projected in the experimental design

(Fig. 5.1). Heifers in the HC group had greater ($P < 0.05$) mean BW than LC heifers starting at week 6 of the experiment and continuing throughout the study. As expected, at the end of the first dietary period (week 10), heifers in the SS-1 group had greater ($P < 0.01$) BW than heifers in the SS-2 group. However, after changes in the targeted ADG during the second 10-wk dietary period, mean BW of animals in the SS-1 and SS-2 groups did not differ significantly at the end of period 2 (week 20). Because heifers in SS-1 were transitioned to ad libitum consumption of a high-concentrate diet during period 3 (weeks 20 to 30), and heifers in SS-2 were subjected to restricted intake of a forage-based diet, BW for SS-1 heifers was greater ($P < 0.05$) than SS-2 group at the end of period 3 (week 30). Because the majority of the heifers in the SS-1 (8/10) and HC (7/10) groups were pubertal before the beginning of dietary period 4 (week 30 to 40), and were removed from the study, growth and metabolic variables for these 2 groups were not analyzed for the last nutritional period of the experiment. Mean ADG and BCS during the different dietary periods are presented in Table 5.2.

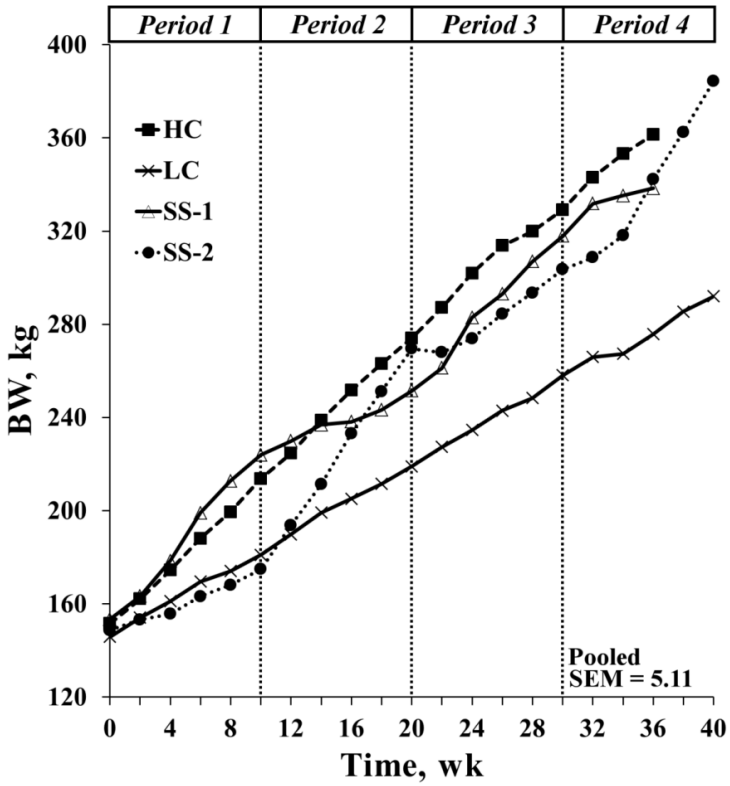

Fig. 5.1. Mean BW of heifers weaned at approximately 3.5 mo of age and subjected to 1 of 4 nutritional treatments. High control (HC; n = 10); Low control (LC; n = 10); Stair-step 1 (SS-1; n = 10); or Stair-step 2 (SS-2; n = 10). Body weight of HC heifers was greater than LC heifers beginning at week 6 ($P < 0.05$) and continuing throughout the study ($P < 0.01$). Because all heifers in the HC and SS-1 groups were pubertal by 36 wk of the experiment, data from these 2 groups are not presented beyond this time point.

Table 5.2. Average daily gain, BCS, and BW at puberty in early-weaned beef heifers receiving 1 of 4 nutritional treatments. High control (HC; n = 10); Low control (LC; n = 10); Stair-step 1 (SS-1; n = 10); or Stair-step 2 (SS-2; n = 10)[1]

	Nutritional regimen				
Item	LC	HC	SS-1	SS-2	SEM
Heifers, no.	10	10	10	10	
Mean ADG, kg					
weeks 0 to 10	0.50[b]	0.88[c]	1.01[d]	0.35[a]	0.04
weeks 10 to 20	0.57[b]	0.90[c]	0.43[a]	1.30[d]	0.03
weeks 20 to 30	0.57[a]	0.94[b]	1.07[c]	0.47[a]	0.03
weeks 30 to 40[2]	0.57[a]	—	—	1.01[b]	0.04
Overall	0.55[a]	0.90[d]	0.84[c]	0.76[b]	0.02
Mean BCS[3]					
week 10	5.05[b]	5.80[c]	6.25[d]	4.40[a]	0.11
week 20	4.95[a]	6.05[d]	5.40[b]	5.70[c]	0.08
week 30	5.10[a]	6.25[c]	6.22[c]	5.33[b]	0.08
week 40[2]	5.30[a]	—	—	6.00[b]	0.09
BW at puberty[4], kg	—	334.91[f]	302.55[e]	317.47[ef]	13.49

[a-d] Within a row, means without a common superscript differ ($P < 0.05$)

[e, f] Within a row, means without a common superscript tend to differ ($P = 0.09$)

[1] Least square means

[2] Because the majority of the heifers in the HC and SS-1 groups were pubertal before the beginning of Period 4, data from these 2 groups were not analyzed for the last period of the study

[3] BCS was determined by visual inspection

[4] Because only 40% (4/10) of LC heifers had reached puberty by the end of the study, data for BW at puberty were not analyzed for this experimental group

Mean (± SEM) circulating concentrations of leptin at the beginning of the experiment did not differ between groups (2.9 ± 0.3 ng/mL). Concentrations of leptin were greater ($P < 0.05$) in HC heifers when compared to LC heifers at week 8 and at week 16 of the experiment and remained greater ($P < 0.01$) than in LC heifers until the end of the experiment (Fig. 5.2). Animals in the SS-1 and SS-2 groups, as expected, consistently exhibited increased concentrations of leptin during the ad libitum consumption periods (SS-1, Periods 1 and 3; SS-2, Periods 2 and 4). Following transition to a period of feed restriction (SS-1, Periods 2 and 4; SS-2 Period 3), heifers in both groups demonstrated rapid declines in circulating concentrations of leptin (Fig. 5.2). Mean concentrations of leptin in SS-2 group remained persistently low during periods of nutrient restriction (Periods 1 and 3; Fig. 5.2); however, heifers in the SS-1 group demonstrated a rise ($P < 0.01$) in concentrations of leptin that occurred during the period of restriction of feed intake (Period 2; Fig. 5.2).

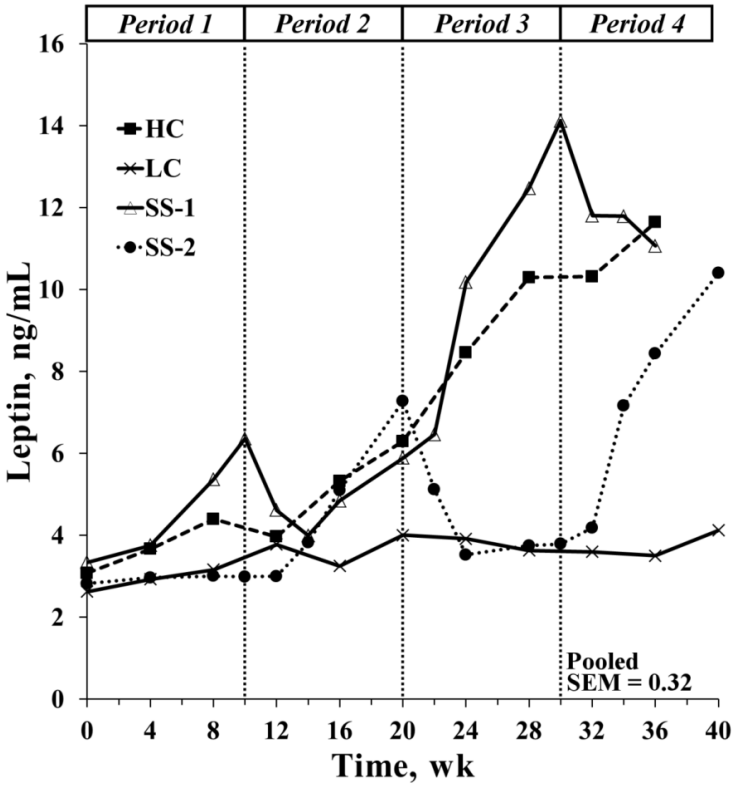

Fig. 5.2. Mean concentrations of leptin of heifers weaned at approximately 3.5 mo of age and subjected to 1 of 4 nutritional treatments. High control (HC; n = 10); Low control (LC; n = 10); Stair-step 1 (SS-1; n = 10); or Stair-step 2 (SS-2; n = 10). Concentrations of leptin were greater ($P < 0.05$) in HC heifers when compared to LC heifers at week 8 and at week 16 of the experiment and remained greater ($P < 0.01$) than in LC heifers thereafter. Because all heifers in the HC and SS-1 groups were pubertal by 36 wk of the experiment, data from these 2 groups are not presented beyond this time point.

5.4.2 Pubertal onset

No replicate effect was observed for age and BW at pubertal onset; therefore, data were pooled by treatment. Survival analysis indicated that heifers in the LC group attained puberty later ($P < 0.05$) than all other groups in the experiment. Furthermore, SS-2 heifers achieved puberty later ($P = 0.057$) than both HC and SS-1 groups, which did not differ ($P = 0.63$) in mean age of pubertal onset (Fig. 5.3). At 12 mo of age, the

proportion of pubertal heifers did not differ ($P = 0.36$) between SS-1 and HC groups, with 80% and 70% pubertal, respectively. In contrast, the proportion of heifers pubertal by 12 mo of age in SS-2 (40%) and LC (30%) was less ($P < 0.05$) than both HC and SS-1. However, by 14 mo of age, a greater ($P < 0.01$) proportion of heifers in the SS-2 had also attained puberty compared to LC heifers, with 90% and 40% pubertal in SS-2 and LC, respectively (Fig. 5.3).

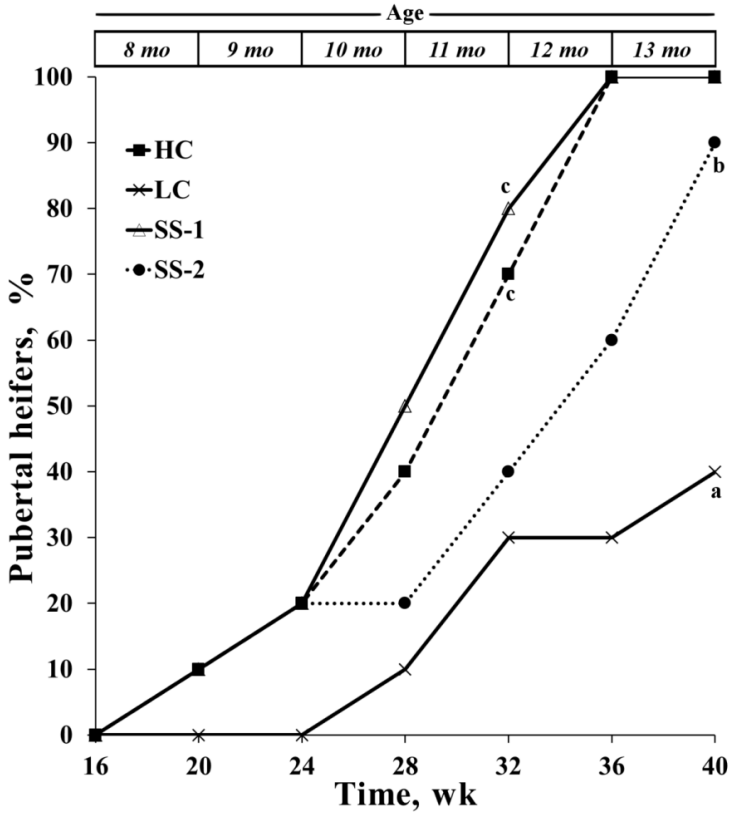

Fig. 5.3. Cumulative percentage of heifers that attained puberty after weaning at 3.5 mo of age and receiving 1 of 4 nutritional treatments. High control (HC; n = 10); Low control (LC; n = 10); Stair-step 1 (SS-1; n = 10); or Stair-step 2 (SS-2; n = 10).[a, b, c] Lines without a common superscript differ ($P \leq 0.057$).

Because only 40% (4/10) of LC heifers had reached puberty by the end of the experiment, comparisons of mean BW at onset of puberty were performed only between HC, SS-1, and SS-2 groups (Table 5.2). Heifers in the SS-1 group tended to be lighter ($P = 0.09$) at puberty than heifers in the HC group. Mean BW at puberty for SS-2 heifers did not differ from HC and SS-1 groups (Table 5.2).

5.5. Discussion

The first reports of a stair-step nutritional regimen involved a combination of energy restriction and re-feeding periods designed to induce compensatory growth and to improve lactation performance in dairy and beef heifers (Park et al., 1987; Park et al., 1998). These studies demonstrated that the stair-step approach results in greater BW gain, increased appetite and feed conversion efficiency, and improved mammary gland development (Park et al., 1987; Park et al., 1998; Ford and Park, 2001). The stair-step nutritional regimen reported by Park et al. (1987) also revealed that compensatory growth significantly influences metabolism and endocrine status of heifers. Based on these reports, we designed a similar nutritional approach that involved alternating dietary periods of feed intake restriction and ad libitum consumption that focused on programming the onset of puberty. Previous studies suggested that a critical window for nutritional programing of accelerated puberty appears to exist early in the juvenile development (Gasser et al., 2006a; Allen et al., 2012). Thus, we reasoned that a favorable nutritional and metabolic status between 4 and 6.5 mo of age would lead to functional changes in the neuroendocrine reproductive system that would persist after a

period of feed intake restriction between 6.5 and 9 mo of age. Moreover, upon re-feeding at 9 mo of age, a recurring positive metabolic status that is mainly characterized by a robust increase in concentrations of leptin would activate the previously programmed neuroendocrine pathways that lead to onset of puberty.

Results of the experiment reported herein confirm our hypothesis that timing of puberty can be shifted temporally by exposing heifers to a stair-step nutritional feeding regimen during the juvenile development period. Interestingly, even though heifers in the SS-1 were subjected to a significant restriction in feed consumption between 6.5 and 9 mo of age, the proportion of pubertal heifers by 12 mo of age did not differ between SS-1 and HC. Similarly, Gasser et al. (2006a) demonstrated that *Bos taurus* heifers weaned at approximately 4 mo of age and fed a high-concentrate diet that promoted a relatively high rate of BW gain between 126 and 196 d of age had accelerated puberty. However, puberty was not advanced at the same rate when heifers were fed a similar diet later during the juvenile development. Together these results indicate that during early calfhood development, plausibly between 4 and 6.5 mo of age, heifers are more sensitive to nutritional programming that can accelerate the timing of puberty. Importantly, even though there was a delay in sexual maturation of SS-2 heifers in the current study when compared to HC and SS-1 groups, the SS-2 group still demonstrated a hastening of pubertal onset when compared to LC. A majority (70%) of SS-2 heifers reached puberty between 12 to 13 mo of age, while gaining BW at high rates later in juvenile development compared to SS-1. Corroborating previous findings, heifers fed a high-concentrate diet to promote a continuous high rate of BW gain after weaning (HC)

exhibited an accelerated rate of pubertal maturation. This phenomenon is associated with an attenuation of estradiol negative feedback and increased pulsatile secretion of LH (Gasser et al., 2006b; Gasser et al., 2006c).

Although numerous studies have demonstrated that nutrition and metabolism play major roles in controlling pubertal development in mammals, the neuroendocrine pathways regulating this process have not been completely elucidated. In primates and rodents, GnRH neurons (which ultimately regulate secretion of LH and control puberty) do not appear to express leptin receptors (Finn et al., 1998; Quennell et al., 2009), suggesting that leptin's effects on GnRH release are likely mediated by intermediate pathways (Schneider, 2004; Amstalden et al., 2011). Information from other metabolic factors such as insulin, IGF1, and ghrelin, is also possibly integrated in a complex neural network that perceives and signals availability of metabolic fuels to the central reproductive system (Schneider, 2004; Amstalden et al., 2011). Hypothalamic NPY and POMC neurons are considered key pathways for nutritional control of reproduction (Crown et al., 2007; Amstalden et al., 2011). NPY has a predominant inhibitory action on the release of LH in ruminants (Gazal et al., 1998; Estrada et al., 2003; Morrison et al., 2003). Recent work conducted by our group has demonstrated that accelerated BW gain in association with high-concentrate diets during early calfhood results in decreased abundance of *NPY* mRNA in the arcuate nucleus and a reduced proportion of GnRH neurons in close proximity to NPY fibers when compared to lower rates of BW gain (Alves et al., 2011; Allen et al., 2012). In addition, we demonstrated that heifers fed in this manner have decreased concentrations of NPY in the cerebrospinal fluid collected

from the third ventricle when compared to heifers gaining BW at lower rates (Cardoso et al., 2012). The melanocortin system is also considered to play an important role in mediating the metabolic control of reproductive functions (Schneider, 2004). Melanocyte-stimulating hormone alpha, one of the products of the *POMC* gene in the hypothalamus, has been proposed to stimulate GnRH episodic release (Roa and Herbison, 2012). Using similar nutritional models, our group demonstrated that *POMC* mRNA abundance in the arcuate nucleus is increased in prepubertal heifers fed high-concentrate diets and gaining BW at high rates when compared to animals gaining BW at lower rates (Allen et al., 2012; Cardoso et al., 2013). Therefore, these results suggest that nutritional programming of early puberty in heifers might be mediated by transcriptional and morphological alterations within the hypothalamus that result in increased excitatory inputs and decreased inhibitory tone on GnRH neurons.

In the current study, the greater circulating concentrations of leptin in HC compared with LC heifers confirmed the elevated nutritional/metabolic status in that group. Leptin, a metabolic hormone secreted mainly by adipocytes, is positively correlated with body fat mass (Ahren et al., 1997) and has a critical role in the progression of sexual maturation in several species, including cattle (Zieba et al., 2005). Feed restriction in prepubertal heifers markedly reduces leptin mRNA abundance in adipose tissue as well as circulating concentration of leptin and the frequency of LH pulses (Amstalden et al., 2000). In addition, it has been demonstrated that leptin administration prevents a fasting-mediated reduction in frequency of LH pulses in prepubertal heifers (Maciel et al., 2004b). Leptin has also been demonstrated to regulate

the development of neuronal projections in the hypothalamus (Bouret et al., 2004). Herein, as expected, mean concentrations of leptin in SS-1 and SS-2 groups increased throughout the periods of ad libitum feed consumption, which occurred during early periods of juvenile development. Thus, this early elevation in circulating leptin may have programmed hypothalamic functions that facilitate the onset of puberty once adequate nutritional status is attained. Although concentrations of leptin decreased rapidly after the transition to dietary periods of intake restriction, the programming of neuroendocrine functions regulating reproductive maturation appears to have been established.

Interestingly, although concentrations of leptin remained low during periods of limited feed consumption in the SS-2 group, circulating concentrations of leptin in SS-1 rebounded during the period of feed restriction and remained elevated even though they were still under severe nutrient restriction. This indicates that accelerated BW gain during the early juvenile period through consumption of a high-concentrate diet, as seen in SS-1 heifers during the first dietary period, might program function of peripheral organs, including adipose tissue. The apparent increased capacity of adipose tissue in SS-1 heifers for leptin synthesis and secretion during the period of feed restriction between 6.5 and 9 mo of age supports this contention. Similarly, the ratio of leptin to fat mass in the human adolescent has been shown to be significantly greater in individuals with greater nutrient intake during the early postnatal period (Singhal et al., 2002). Moreover, Lopez et al. (2007) demonstrated that overfed neonatal rats growing up in small litters show marked hyperleptinemia during adulthood, indicating that the

programming of leptin synthesis and secretion in adipocytes through earlier exposure to heightened nutritional conditions might be one mechanism that links early nutrition with later obesity. Therefore, we believe that postnatal nutritional acceleration of puberty in heifers mediated by key alterations in the neuroendocrine system might also be facilitated by early programming of adipose tissue.

In summary, our results indicate that the age at onset of puberty can be nutritionally programed by exposing heifers to a stair-step nutritional regimen during juvenile development. In addition to previously characterized key alterations in the reproductive neuroendocrine system, postnatal nutritional acceleration of puberty in heifers might also be facilitated by early programming of leptin secretion in adipose tissue. Even though heifers are more sensitive to the nutritional acceleration of puberty during early calfhood (4 to 6.5 mo of age), managerial approaches that focus on increasing availability of nutrients around 6 to 9 mo of age (SS-2) can potentially time reproductive maturation consistently so that the majority of replacement beef heifers have reached puberty at 11 to 14 mo of age, while avoiding a high incidence of precocious puberty. Furthermore, if heifers are developed on a well-controlled nutritional regimen based on periods of nutrient restriction and realimentation, significant improvements in growth efficiency and life-long lactation performance could also be achieved (Park et al., 1987).

CHAPTER VI

CONCLUSIONS

Studies in different species, including rodents, ruminants, and primates have demonstrated that excessive BW gain and adiposity during juvenile development are associated with hastened pubertal onset in females. In the first overall aim, using prepubertal heifers, we characterized some important functional and morphological modifications that occur in two critical metabolic-sensing pathways, the orexigenic NPY and the anorexigenic POMC circuitries, which are involved in relaying metabolic signals to the central reproductive axis. Results reported herein, in conjunction with previous data generated by our group, contribute considerably to a better understanding of the mechanisms involved in the nutritional acceleration of puberty in bovine females. This knowledge may be used for designing nutritional strategies to optimally time pubertal onset in replacement beef heifers (as seen below in the second overall aim). Furthermore, increased interest in identifying the mechanisms linking metabolism and reproduction has been motivated by recent evidence associating childhood obesity with early onset of puberty in girls. Because these mechanisms appear to be similar in most mammals, results obtained in the current studies may also have important implications for human health.

Data collected under the second overall aim indicate that a stair-step, compensatory growth model can exploit postnatal neuroendocrine plasticity to time pubertal onset in beef heifers. Corroborating previous results, we observed that *Bos*

indicus influenced heifers are more sensitive to the nutritional acceleration of puberty during the early calfhood period (between 4 and 6.5 mo of age) compared to later stages of development. However, in order to avoid precocious puberty and time sexual maturation consistently between 11 to 14 mo of age it appears that nutritional strategies that focus on increasing nutrient availability around 6 to 9 mo of age may be a more appropriate strategy. Interestingly, we also observed that increased BW gain during early postnatal development may facilitate pubertal onset by acting on the adipose tissue. Although not investigated in detail in this study, results suggest that nutritional programing of adipocytes for increased leptin synthesis and secretion might be possible in heifers during early development. Thus, further studies that aim to characterize the cellular and molecular mechanisms involved in this process may have important value.

Finally, although similar diets, nutritional regimens, and animal genetic background were used in all the studies reported here, considerable variability was observed in the mean age of puberty between experiments/replicates. Therefore, it is possible that factors other than genetics and postnatal nutrition may influence the maturation of the reproductive axis in heifers. One plausible hypothesis is that maternal nutrition during fetal development could influence the responsiveness of the hypothalamic circuitry to metabolic cues in prepubertal heifers. Thus, further efforts to investigate this hypothesis are currently underway by our group.

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APPENDIX

ADDITIONAL METHODOLOGY

Progesterone radioimmunoassay

Single antibody RIA kit, Diagnostic Products Corporation, Los Angeles, CA

1. Iodination product: Iodination grade hP4
2. Antibody: Anti-human P4 coated tubes
3. Standards: Human serum with added P4 (0.1 – 20.0 ng/mL)
4. Reference: Human standard preparation added to bovine serum
5. RIA Procedure:

   Day 1: begin and complete assay

   1. Pipette in non-coated polypropylene tubes

      NSB – 100 µl of 0 Std

   2. Pipette in antibody coated tubes

      0 Std – 100 µl

      Stds – 100 µl

      Refs – 100 µl

      Unknowns – 100 µl

   3. Pipette 1 ml of ^{125}I –P4 provided in the kit to all tubes including two total count noncoated polypropylene tubes
   4. Vortex tubes briefly and incubate at room temperature for 3 h
   5. Pour off supernatant
   6. Count radioactivity of each tube using a gamma counter

Bovine LH radioimmunoassay

1. Iodinated Product: Iodinated grade oLH (AFP-8614B)
2. Antibody: Anti-ovine LH (AFP-192279 - Dr. Parlow - UCLA). Working dilution 1:1,000,000
3. Standards (stds): Iodination grade ovine LH (AFP-8614B)
4. References (refs): Ovine LH added to bovine serum
5. RIA Procedure:

   Day 1: Start assay

   1. NSB- 500 µl of 1% PBS-EW (egg white)
   2. 0 Std – 500 µl of 1% PBS-EW
   3. Stds – 200 µl std + 300 µl of 1% PBS-EW
   4. Refs – 200 µl ref + 300 µl of 1% PBS-EW
   5. Unknowns – 200 µl unknown sample + 300 µl of 1% PBS-EW
   6. Pipette 200 µl of PBS-EDTA + 1:400 NRS without primary antibody into NSB tubes only
   7. Pipette 200 µl of anti-oLH (diluted in PBS-EDTA + 1:400 NRS) into all tubes except NSB and TC tubes
   8. Pipette 100 µl ^{125}I-oLH (20,000 cpm/100 µl diluted in 1% PBS-EW) into all tubes
   9. Vortex tubes briefly and incubate for 24 h at 4°C

   Day 2: Add secondary antibody

1. Pipette 200 µl of goat anti-rabbit gamma globulin (SARGG: 2nd Ab; 1:50) diluted in PBS-EDTA without NRS into all tubes except TC tubes
2. Vortex tubes briefly and incubate for 48 h at 4°C

Day 4: Pour off assay

1. Add 3 ml of ice cold PBS (0.01 M: pH 7.0) to all test tubes except TC tubes
2. Centrifuge tubes for 1 h at 4°C at 3600 rpm
3. Decant supernatant
4. Count radioactivity of each tube using a gamma counter

Bovine GnRH radioimmunoassay (IIIV CSF)

1. Iodinated Product: Iodinated grade GnRH (Sigma, L-7134)
2. Antibody: Rabbit anti-GnRH (Dr. Caraty – BDS-037). Working dilution: 1:50,000
3. Standards (stds): Iodination grade GnRH (Sigma, L-7134). Standards were prepared fresh for every assay.
4. References (refs): GnRH added to bovine CSF
5. RIA Procedure:

   Day 1: Start assay

   1. NSB- 400 µl of PBS-0.1% Gel
   2. 0 Std – 400 µl of PBS-0.1% Gel
   3. Stds – 200 µl std + 200 µl of PBS-0.1% Gel
   4. Refs – 200 µl ref + 200 µl of PBS-0.1% Gel
   5. Unknowns – 200 µl unknown sample + 200 µl of PBS-0.1% Gel
   6. Pipette 50 µl of PBS-EDTA + 1:400 NRS without primary antibody into NSB tubes only
   7. Pipette 50 µl of anti-GnRH (diluted in PBS-EDTA + 1:400 NRS) into all tubes except NSB and TC tubes
   8. Vortex tubes briefly and incubate overnight at 4°C

   Day 2: Add tracer

   1. Pipette 50 µl ^{125}I-GnRH (12,000 cpm/50 µl diluted in PBS-0.1% Gel) into all tubes
   2. Vortex tubes briefly and incubate for 20-24 h at 4°C

Day 3: Add precipitating reagent (95% ethanol; use the walk-in cooler)

1. Add 1.5 ml of ice cold 95% ethanol to all test tubes, except TC tubes
2. Vortex tubes briefly and centrifuge for 20min at 2,000 g (3,000 rpm in Beckman J-6B) at 4°C
3. Decant supernatant into waste receptacle
4. Drain tubes by inverting them on stacks of paper towels for 15 min
5. Load tubes in the Gamma Counter and count each tube for 1 min

NPY radioimmunoassay (IIIV CSF)

RIA kit (S-2160), Peninsula Laboratories, San Carlos, CA

1. Iodination product: (^{125}I-Tyr)-NPY (bovine, porcine; H-5246)
2. Antibody: Anti-NPY (bovine, porcine; A10392)
3. Standards: NPY (bovine, porcine) added to RIA buffer (10 – 1,280 pg/ml)
4. Reference: NPY (bovine, porcine) added to bovine CSF
5. RIA Procedure:

   Day 1: Begin assay

   1. Dilute the RIA buffer (4X concentrate) with 150 ml of dd water
   2. Reconstitute the standard peptide with 1 ml of RIA buffer. Mix well.
   3. Reconstitute the rabbit anti-NPY serum with 13ml of RIA buffer
   4. Prepare dilutions of standards (10 – 1,280 pg/ml)
   5. Number tubes
   6. Pipette 200 µl of RIA buffer into each NSB tube
   7. Pipette 100 µl of RIA buffer into each TB tube
   8. Pipette 100 µl of standards into duplicate
   9. Pipette 100 µl of unknown samples into duplicate tubes
   10. Pipette 100 µl of primary antibody into all tubes, except TC and NSB tubes
   11. Reconstitute the ^{125}I-NPY with 13ml of RIA buffer and mix well. Adjust dilution for 8,000 – 10,000 cpm/100 µl
   12. Add 100 µl of tracer solution to all tubes

13. Vortex all tubes
14. Cover and incubate all tubes for 16-24 h at 4°C

Day 2: Incubate with secondary antibody and pour off assay

1. Reconstitute the Goat anti-rabbit IgG serum (GAR) with 13 ml of RIA buffer
2. Reconstitute the Normal Rabbit Serum (NRS) with 13ml of RIA buffer
3. Add 100 µl of GAR to all tubes, except TC
4. Add 100 µl of NRS to all tubes, except TC
5. Vortex well and incubate at room temperature for 90 minutes
6. Add 500 µl of RIA buffer to all tubes, except TC and vortex.
7. Centrifuge all tubes, except TC at 1,700 g (2,750 rpm in Beckman J-6B) for 20 min at 4°C
8. Carefully aspirate all the supernatant immediately following centrifugation. Do not aspirate the TC tubes
9. Count radioactivity of each tube using a gamma counter

Alpha MSH radioimmunoassay (IIIV CSF)

RIA kit (RK-043-01), Phoenix Pharmaceuticals, Inc., Burlingame, CA

1. Iodination product: (^{125}I-Tyr)-αMSH (Human, Rat, Mouse)
2. Antibody: Anti-αMSH (Human, Rat, Mouse)
3. Standards: αMSH (Human, Rat, Mouse) added to RIA buffer (10 – 1,280 pg/ml)
4. Reference: αMSH (Human, Rat, Mouse) added to bovine CSF
5. RIA Procedure:

   Day 1: Begin assay

   1. Dilute the RIA buffer (4X concentrate) with 150 ml of dd water
   2. Reconstitute the standard peptide with 1 ml of RIA buffer. Mix well.
   3. Reconstitute the rabbit anti-αMSH serum with 13ml of RIA buffer
   4. Prepare dilutions of standards (10 – 1,280 pg/ml)
   5. Number tubes
   6. Pipette 200 µl of RIA buffer into each NSB tube
   7. Pipette 100 µl of RIA buffer into each TB tube
   8. Pipette 100 µl of standards into duplicate
   9. Pipette 100 µl of unknown samples into duplicate tubes
   10. Pipette 100 µl of primary antibody into all tubes, except TC and NSB tubes
   11. Reconstitute the ^{125}I-αMSH with 13ml of RIA buffer and mix well. Adjust dilution for 8,000 – 10,000 cpm/100 µl
   12. Add 100 µl of tracer solution to all tubes

13. Vortex all tubes
14. Cover and incubate all tubes for 16-24 h at 4°C

Day 2: Incubate with secondary antibody and pour off assay

1. Reconstitute the Goat anti-rabbit IgG serum (GAR) with 13 ml of RIA buffer
2. Reconstitute the Normal Rabbit Serum (NRS) with 13ml of RIA buffer
3. Add 100 µl of GAR to all tubes, except TC
4. Add 100 µl of NRS to all tubes, except TC
5. Vortex well and incubate at room temperature for 90 minutes
6. Add 500 µl of RIA buffer to all tubes, except TC and vortex.
7. Centrifuge all tubes, except TC at 1,700 g (2,750 rpm in Beckman J-6B) for 20 min at 4°C
8. Carefully aspirate all the supernatant immediately following centrifugation. Do not aspirate the TC tubes
9. Count radioactivity of each tube using a gamma counter

Alpha MSH and GnRH dual immunofluorescence (free-floating sections)

Day 1

1. Wash tissue sections for at least 4 h in PBS (replace PBS every h) or overnight at 4^{o} C with shaking
2. Wash in 0.1 M PBS 4 X 5 min
3. Incubate in 0.1 M PBS containing 1% H_2O_2 for 10 min
4. Wash in PBS 4 X 5 min
5. Incubate in PBS + 0.4% Triton-X100 + 4% Normal Goat Serum (NGS) for 1h
6. Incubate in Mouse monoclonal anti-GnRH (Covance # SMI-41R) diluted to 1:10,000 in PBS + 0.4% TX + 4% NGS AND Guinea Pig anti-human αMSH (Biosensis Cat.#GP-030-50) diluted to 1: 40,000 in PBS + 0.4% Triton-X100 + 4% NGS overnight (~ 16 h) at RT

Day 2

1. Wash in PBS 3 X 5 min
2. Incubate in Biotinylated Goat anti-Guinea Pig IgG (Vector Labs Cat # BA-7000) diluted 1:400 in PBS + 0.4% Triton-X100 + 4% NGS for 1 h
3. Wash in PBS 3 X 5 min

   ** From this point on, keep dish containing tissue covered with foil to prevent fading of the fluorescent dyes **

4. Incubate in Alexa 555 conjugated streptavidin (Molecular Probes Cat. # S-21381) diluted to 1:250 in PBS for 30 min

5. Wash in PBS 4 X 5 min
6. Incubate in Alexa 488 conjugated goat anti-mouse IgG (Molecular Probes Cat. # A-11001) diluted to 1:200 in PBS + 0.4% Triton-X100 + 4% NGS for 1 h
7. Wash in PBS 4 X 5 min
8. Mount tissue sections on slides, air dry, and coverslip using Gelvatol
9. Store in dark at 4^{o} C

Alpha MSH and kisspeptin dual immunofluorescence (free-floating sections)

Day 1

1. Wash tissue sections for at least 4 h in PBS (replace PBS every h) or overnight at 4^{o}C with shaking
2. Wash in 0.1 M PBS 4 X 5 min
3. Incubate in a solution of Sodium Citrate (10mM in ddH_2O – pH 6), in water bath, at 90°C for 18 min
4. Let it cool down at room temperature for 20 min
5. Wash in 0.1M PBS 4 X 5 min
6. Incubate in 0.1 M PBS containing 1% H_2O_2 for 10 min
7. Wash in PBS 4 X 5 min
8. Incubate in PBS + 0.4% Triton-X100 + 4% Normal Goat Serum (NGS) for 1h
9. Incubate in Rabbit anti-Kiss 10 (Caraty, INRA # 564) diluted to 1:250,000 in PBS + 0.4% TX + 4% NGS overnight (~ 16 h) at RT

Day 2

1. Wash in PBS 3 X 5 min
2. Incubate in Biotinylated Goat anti-Rabbit IgG (Vector Labs Cat # BA-1000) diluted 1:400 in PBS + 0.4% Triton-X100 + 4% NGS for 1 h
3. Wash in PBS 3 X 5 min
4. Incubate in ABC (Vectastatin; Vector Labs Cat # PK-6100) diluted 1:600 (each A and B) in PBS for 1h

** ABC must be diluted at least 30 min before use

5. Wash in 0.1 M PBS 4 X 5 min

   ** From this point on, keep dish containing tissue covered with foil to prevent fading of the fluorescent dyes **

6. Incubate in Alexa 555 conjugated streptavidin (Molecular Probes Cat. # S-21381) diluted to 1:250 in PBS for 30 min
7. Wash in PBS 4 X 5 min
8. Incubate in PBS + 0.4% Triton-X100 + 4% NGS for 1h
9. Incubate in Guinea Pig anti αMSH (Biosensis Cat # GP-030-50) diluted to 1:20,000 in PBS + 0.4% TX + 4% NGS overnight (~ 16 h) at RT

Day 3

1. Wash in PBS 3 X 5 min
2. Incubate in Alexa 488 conjugated Goat anti-Guinea Pig IgG (Molecular Probes Cat. # A-11073) diluted to 1:200 in PBS + 0.4% TX + 4% NGS for 1h
3. Wash in PBS 4 X 5 min
4. Mount tissue sections on slides, air dry, and coverslip using Gelvatol
5. Store in dark at 4^{o} C

Isotopic in situ hybridization of frozen sections

In vitro transcription

1. MAXIscript® Procedure (Ambion/Applied Biosystems)

2. Set up probe synthesis reaction in the following order at room temperature on a 1.5 ml microtube
   * all steps should be kept free of RNase; thaw reagents on ice

   a) __ µl of RNase-free sterile H_2O to a final volume of 20 µl
   b) __ µl DNA template linearized (1 µg)
   c) 2 µl of 10X transcription buffer
   d) 2 µl of 100 mM DTT (dithiothreitol)
   e) 1 µl of RNasin
   f) 4 µl of 2.5 mM rATP + rGTP + rCTP (made by adding equal volumes of the three rNTP and H_2O)
   g) 2 µl of 100 µM rUTP (made by a 1:100 dilution of the 10 mM rUTP stock)
   h) __ µl ^{35}S-UTP (50 µCi)
   i) 1 µl of either T7 or SP6 RNA polymerase, depending on template orientation
   Final volume of 20 µl

3. Flick the tube gently to mix, centrifuge briefly to collect reaction mixture at the bottom of tube, and incubate reaction for 2 h at 37^o C
   * If running 5% acrylamide-8M urea gel to confirm synthesis, remove 2 µl aliquot from reaction after 1 h

4. Add 1 µl RNase-free TURBO DNase and 1 µl RNasin after completion of reaction
5. Mix by vortex, centrifuge briefly, and incubate at 37^o C for 15 min

6. Add 2 µl of 0.5 M EDTA; mix and place on ice

7. Remove unincorporated nucleotides using NucAway® spin columns (Ambion/Applied Biosystems; see protocol for instructions)

8. Add 1 µl tRNA (10 mg/ml); Mix by vortex and centrifuge briefly;

9. Count 1 µl diluted in 4.5 ml scintillation cocktail

* If not using probe soon after, freeze at -80^o C until use.
* Consider radioactivity decay and probe degradation - use probe within a week

Day 1 – Hybridization

Dry Slides	10 min	55°C
4% PFA in 0.1M PB	15 min	RT
0.1M PB	2 X 5 min	
Proteinase K (0.5 µg) in 0.5 mM EDTA, 50 mM Tris-HCL	30 min	37°C **Add Protienase K just before use; Mix well
0.1M PB	1 X 5 min	RT
4% PFA in 0.1M PB	5 min	RT
0.1M PB	1 X 5 min	
Dip in RNase Fee Water	2 X	
Dip in 0.1 M TEA	1 X	
0.1 M TEA + Acetic Anhydride	1 X 10 min	250ul/100ml TEA; add to dish first, then TEA
2X SSC	1 X 5 min	
70%	1 X 3 min	
95%	1 X 3 min	
100%	1 X 3 min	
Chloroform	1 X 5 min	
100%	1 X 3 min	
95%	1 X 3 min	
70%	1 X 3 min	
Air Dry Slides	A few min	Do not let slides dry out
Pre-Hybridization	1 hr can be longer if need be	37°C

1. Denature radiolabeled probe (1 X 10^6 cpm/150µl) diluted in hybridization buffer containing 100 mM DTT (with DTT added fresh from 1M stock) at 70°C for 10 min.
2. Let hybridization solution cool on ice for 5 min.
3. Add 200µl of hybridization solution to each slide.
4. Cover with parafilm coverslip on each slide --- AVOID BUBBLES!
5. Hybridize overnight at (55°C) in humidified chamber containing Whatman 3MM paper wetted with 50% formamide + 5X SSC. Hybridization temperature is dependent on probe and expression.

**Heat a water bath to 37°C. (Depending on probe and tissue, two temperatures may be needed (55°C). Steps are denoted (*))

**Make solutions to let them warm to washing temperature in advance

Day 2 – Washing

1. Remove parafilm coverslips from slides and place in 5X SSC

*Do not let slides dry

5X SSC with 10mM βME	30 min	37°C (*55°C)
1X TEN	3 X 10 min	37°C
RNase (10µg/ml) in 1X TEN	30 min	37°C **Add RNase A just before use; Mix well
1X TEN	30 min	37°C
2X SSC with 10mM βME	30 min	37°C (*55°C)
2X SSC with 10mM βME	15 min	37°C (*55°C)
0.1X SSC with 10mM βME	15 min	37°C (*55°C)
0.1X SSC	15 min	RT
70% ETOH + 0.3M NH_4Ac	2 X 5 min	RT
95% ETOH + 0.3M NH_4Ac	3 min	RT
100% ETOH	2 X 2 min	RT

- Air dry Slides or (1-3 hr at 37°C)
- If needed, Expose slides to film overnight to estimate autoradiography time

Autoradiography

- Warm photographic NBT2 emulsion in a light tight container at 44°C in a water bath (approx. 45 min) (This may take longer, Do Not want the emulsion to still in a gel form)
- Warm equal volume of ddH_2O to 44°C in a water bath (approx. 45 min)
- Place a dipping chamber into water bath

IN the DARK (with safe-light)

- Mix photographic NBT2 emulsion with water in a 1:1 dilution
- Pour emulsion into the dipping chamber and let equilibrate to 42-44°C (avoid bubbles)
- Dip each slide once and withdrawal it slowly (keep the same motion)
- Keep slides vertical and wipe excess emulsion from the back of the slide
- Let slides to stand up in a rack and air dry for 2-3 hr (place slide rack in a light-tight box)
- Place slides in a plastic slide box with desiccant
- Wrap slide box in triple foil to avoid light exposure and place at 4°C for appropriate exposure time

<u>Developing Slides</u>

- Allow slides to warm to room temperature
- Place dish(es) on ice in ice bucket
- Dilute room temperature Kodak D-19 Developer with cold ddH_2O water (4°C) in equal parts (1:1)
- Chill Kodak D-19 Developer to 15°C. *The temperature is very important.

In the DARK (with safe-light, at least 1.5 m from slides)

- Place slides into chilled D-19 Developer (diluted 1:1) for 4 min – dip approx. 10 times/min (can be more)
    - DO NOT use developer for more than one rack
- Dip in H_2O for 30 sec to stop development. Dipping rack for the whole time.
- Place slides into Kodak Fixer (with hardener, diluted 1:7 with ddH_2O) for 5 min - dip approx. 10 times/min (can be more)
- Dip in ddH_2O for 1 min – dipping the whole time
- Dip in ddH_2O for 5 min – dip throughout the allotted time. (can turn lights on)

*After each step, make sure to allow the racks to drain the excess solution off before moving to the next step.

<u>Coverslipping</u>

If counterstaining is not needed, then cover slip

- Dehydrate sections through ETHO
    - 70% ETOH for 1 min

- 95% ETOH for 1 min
- 100% ETOH for 5 min

- Place slides in Citrosol 3 times for 5 min each
- Cover slip with DPX

Reagents

DEPC-treated H_2O (diethilpyrocarbonate H_2O)

ddH_2O	1 L
DEPC	1 ml

Shake well until foam forms
Incubate overnight at 37^o C
Autoclave

1 M Phosphate Buffer (10X PB; pH 7.3) – RNase free

Sodium phosphate dibasic	54.65 g
Sodium phosphate monobasic	15.9 g

Depc H_2O to final volume of 500 ml

0.1 M PB	50 ml	300 ml
1 M PB	5 ml	30 ml
ddH_2O	45 ml	260 ml

4% Paraformaldehyde (pH 7.2-7.4)

	100 ml	300 ml
Paraformaldehyde	4 g	12 g
Sodium Hydroxide	~3g	~10g
0.1M PB	100 ml	300 ml

*Add Sodium Hydroxide to a portion of PB to dissolve first, then add Paraformaldehyde
*This is just a staring amount of Sodium Hydroxide; add slowly until Paraformaldehyde has completely dissolved
Adjust pH then add remaining PB

1 M Triethanolamine (10X TEA; pH 8) – RNase free

	1L	500 ml
Triethanolamine (98%; 149.2 MW; density 1.124 g/ml)	135.5 ml	67.9 ml
Sodium Chloride	81.8 g	40.9 g

TEA with acetic anhydride

	50 ml	300 ml
0.1 M TEA	50 ml	300 ml
Acetic anhydride	125 ul	750 ul

1M Tris-HCl (pH 8) – RNase free

Tris-HCl	78.8 g

Adjust pH with HCl
DEPC H_2O to final volume of 500 ml
Autoclave

0.5mM EDTA, 50mM TRIS-HCL

	100 ml
0.5M EDTA	100 ul
1 M TRIS-HCL	5 mL

DEPC water to 100ml
0.5mM EDTA, 50mM TRIS-HCL with 0.5 µg of Proteinase K

Proteinase K plus EDTA, TRIS-HCL	50 ml
Proteinase K	1.25 ul
0.5mM EDTA, 50mM TRIS-HCL	to 50 ml

20X Sodium Chloride Sodium Citrate (20X SSC) – RNase free

Sodium Citrate	88 g
Sodium Chloride	175 g

DEPC H2O to final volume of 1 L
Autoclave

5X SSC – 1 L
250 ml 20X SSC
750 ml DEPC ddH2O

2X SSC – 1 L
100 ml 20X SSC
900 ml DEPC ddH2O
0.1X SSC – 1 L
5 ml 20X SSC
995 ml DEPC ddH2O

10X Tris-EDTA-Sodium Chloride (TEN; 1L) – RNase free

NaCl	292.2 g
Tris-HCl	100 ml 1 M Tris-HCl (pH 8)
EDTA	100 ml 0.5 M EDTA (pH 8)
DEPC H_2O to final volume of 1 L	

RNase A (10 ug/ml) in 1X TEN

	50 ml
RNase A (20 ug/ml)	25 ul
1X TEN	to 50 ml

Pre-hybe 4X SSC, 50% formamide

	50 ml	300 ml
20X SSC	10 ml	60 ml
Deioniezed Formamide	25 ml	150 ml
DEPC water	15 ml	90 ml

0.5 M EDTA (pH 8) - RNase free

Ethylene diamine tetraacetate	93.05 g
NaOH pellets	12g
DEPC H_2O to final volume of 500 ml	
Warm to dissolve – must be at pH 8 to stay in solution	
Adjust pH with NaOH pellets	
Autoclave	

5M NaCl

	500 ml
NaCl	146.1g
ddH_2O	to 500 ml
Autoclave	

3 M Sodium Acetate, pH 5.2

	100 ml
Sodium Acetate	40.81 g
RNase Free water	80 ml
pH with glacial acetic acid	
RNase Free water	to 100 ml
Autoclave	

1M DTT

	10 ml
DTT	1.545 g
0.01M Sodium Acetate pH 5.2	10 ml
Filter sterilize, and make 1 ml aliquots	

Store at -20°C

5M NH_4AC

	500 ml
NH_4AC (FW 77.08)	192.7g
ddH_2O	to 500 ml

TE pH 8.0

	1 L
1M Tris, pH 8.0	10 ml
0.5M EDTA, pH8.0	2 ml
ddH_2O	to 1 L

Hybridization Buffer (RNase free)

	Final	Make 50 ml
Deionized Formamide	50%	25 ml of 100%
NaCl	0.3 M	3 ml of 5 M stock
Tris-HCl (pH 8)	20 mM	1 ml of 1 M stock
EDTA (pH 8)	5 mM	0.5 ml of 0.5 M stock
$NaPO_4$ (pH 8)	10 mM	0.5 ml of 1 M stock
Denhardt's solution	1X	1 ml of 50X stock
*Dextran Sulfate solution)	10%	5 g (dilute in H_2O before adding to
Yeast tRNA	0.5 mg/ml	2.5 ml of 10 mg/ml stock
DEPC H_2O		q.s. (to final volume; ~ 10 ml)

*Add ~10ml of water to conical tube, then add Dextran Sulfate. Mix this first before adding other reagents.
Before use, add 1 M DTT to a final concentration of 100 mM DTT for ^{35}S-labeled probes

Yeast tRNA (10mg/ml)
Yeast tRNA (25mg)
Dilute 25mg (one vial) in 2.5 ml sterile water.
Incubate for 1 hour at RT
Aliquot and Store at -20˚C

50X Denhardt's Solution (RNase free)

	Final	Make 100 ml
Ficoll 400	1%	1 g
Polyvinylpyrrolidone	1%	1 g
BSA (Fraction V)	1%	1 g
DEPC Water		q.s. (to final volume)

RNase A

RNase A, 50mg vial	one vial
TE	2.5 ml

-Mix in vial then aliquot
-Store at -20˚C

Kodak D-19 Developer - Store in Dark with bottle wrapped in foil

-Heat 800 ml ddH2O to 52˚C (NOT OVER!)
-Add 159 g of Developer to headed water slowly while stirring
-Final solution may have slight amber/yellow color. If dark then the developer is bad.
-Adjust to final volume of 1 L
-If Developer does not all dissolve then filter into a foil wrapped bottle.
-Allow to cool completely prior to use.
-Developer can be stored in the dark at RT for future uses as long as no color change

For developing
-Dilute Developer in equal parts (1:1) with cold (4˚C) ddH2O

Kodak Fixer with hardener

-Dilute one part fixer to eight parts ddH_2O
-Make just before use

	300 ml	600 ml
Kodak Fixer	37.5 ml	81.25 ml
ddH2O	262.5 ml	518.75 ml

Cresyl Violet

5 g Cresyl violet (Sigma C-1791)
0.5 g sodium acetate
Dissolve in approximate 800 ml ddH_2O
Adjust pH to 3.5 with glacial acetic acid
Adjust to final volume of 1 L
Filter
Adjust pH to 3.13 to 3.14 with acetic acid
* Obs: Cresyl violet solution should be filtered before each use

10X Acetate Buffer
2.5 g sodium acetate (MW 136.08)
Dissolve in approximately 450 ml dd H_2O

Adjust pH to 3.5 with glacial acetic acid (approx. 20 ml)
Adjust final volume to 500 ml

Acidified 70% Ethanol
10 ml acetic acid
490 ml 70% Ethanol

All solutions and dishware must be RNase Free!