NUTRITIONAL REGULATION OF PRECOCIOUS PUBERTY IN HEIFERS.

DISSERTATION

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By

Martin G. Maquivar, DVM, MSc

Graduate Program in Animal Sciences

The Ohio State University

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Committee:
Professor Michael L. Day, Adviser
Professor Kichoon Lee
Professor Steve Loerch
Professor Joseph Ottobre
Professor Alexandre Vaz Pires
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Abstract

Feeding a high energy diet to heifers weaned at 2 months of age hastens sexual maturation and results in precocious puberty (first ovulation before 300 days of age). Two experiments were designed to study metabolic, reproductive and molecular responses that lead to precocious puberty and in the first experiment, impact of composition of high energy diet on these responses in beef heifers. In the first experiment the objective was to test the influence of feeding either a high energy diets with a high starch content (46.4% starch, n = 10) or low starch content (14.48% starch, n = 12) and control diet (19.03% starch, n = 11) weaned at 76 ± 1.1 d of age and beginning the diets at 98 ± 1.1 d of age on age at puberty and the impact of these diets on metabolic and reproductive hormones. Precocious puberty was induced in 42% (5/12) and 60% (6/10) of heifers in the low and high starch diets, respectively and none in the control diet. The high energy diets induced increased IGF-I concentrations as compared to the control diet that contained lesser energy, and this response was only marginally influenced by composition of the high energy diets. Across the two high energy diets, heifers that experienced precocious puberty had greater (P < 0.05) IGF-I concentrations than those that did not reach puberty precociously; in the absence of differences in GH concentrations. Results suggested that energy content of the high energy diet was more
critical than composition to induce precocious puberty and strongly suggested a link between enhanced IGF-I concentrations and the occurrence of precocious puberty.

In the second study the objectives were to evaluate the impact of the high energy diet on estrogenic capacity and other molecular changes within the dominant follicle that reflect developmental capacity and their relationship to systemic estradiol and LH concentrations in the same heifer model. Within dietary treatments (high energy with high starch content [HIGH] and roughage-based control [CONT]) that were initiated at 86 ± 2 d of age and 137 ± 3.3 kg body weight, heifers were assigned to groups that were to be ovariectomized (OVX) to assess follicular function, or to remain intact (INT) for assessment of secretion of LH and age at puberty. Overall ADG (including all heifers) was greater (P < 0.01) in HIGH heifers (1.57 ± 0.71 kg/d) than CONT heifers (1.21 ± 0.74 kg/d). Likewise, body weight of the HIGH heifers was greater (P < 0.05) than CONT heifers at 186 days of age and thereafter (diet by age interaction, P < 0.01).

Comparing HIGH-INT vs. CONT-INT differences were observed at age at puberty (P < 0.05) (194.33 ± 8.95 d vs. 253.75 ± 1.25 d, respectively) and BW at puberty (P < 0.02) HIGH-INT (257.7 ± 17.02 kg) vs. CONT-INT (321.67 ± 11.65 kg). Additionally, comparing HIGH-OVX vs. CONT-OVX no differences (P > 0.05) were observed at age at puberty (235.25 ± 11.9 d vs. 219.66 ± 10.9 d, respectively) and a tendency (P = 0.07) was observed on BW at puberty, HIGH-OVX (329.07 ± 13.17 kg) vs. CONT-OVX (285.43 ± 14.08 kg). Mean LH concentrations were greater (P < 0.05) in the HIGH-INT than CONT-INT treatment at 170 and 208 days of age but did not differ at other times assessed. Neither frequency nor amplitude of LH pulses differed between treatments.
Relative expression of mRNA concentrations for insulin-like growth factor-I receptor (IGF-IR), luteinizing hormone receptor (LHR), cholesterol side-cleavage chain (CYP11A1) and aromatase (CYP19A1) were not affected by treatment. Numerically, concentrations of all genes evaluated favored the HIGH-OVX treatment over the CONT-OVX heifers. Ratio between intra-follicular fluid concentration of estradiol and progesterone was similar among treatments, CONT-OVX (3.4 ± 1.7) vs. HIGH-OVX (1.16 ± 0.57). In conclusion, the HIGH dietary treatment increased body weight gain and LH secretion and resulted in an earlier age at puberty in the intact heifers. Incidence of precocious puberty did not differ between treatments. Intra-follicular concentrations of estradiol and progesterone nor steroidogenic enzyme expression differed between dietary treatments. It is therefore plausible that heifers in both diets received metabolic signals that precociously activated the reproductive axis in this experiment and further research is needed to determine if the initial step to precocious activation of the reproductive axis is driven by increased IGF-I enhancement of follicular function.
Dedication

In Loving Memory of My Parents
With all my Love, Respect and Admiration

DVM Martin Maquivar and BA, MSc Hoppe A. Linfoot

To Kim

To my sister and brothers

To the animals that gave their lives for improvement of human and animal wellbeing
Acknowledgments

The time that I have spent here at The Ohio State University has been the hardest, hectic, most challenging, and demanding years of my life. Moving away from my family, friends and culture combined with all the sacrifices that I had to make were sometimes unbearable. However, I was blessed to find amazing people that gave me more than friendship, gave me more than words can say. I thank all people that were there for me, to support me and to offer me a hand when I needed the most. This acknowledgement section just represents a small portion of all the special people that helped me. THANKS SO MUCH!

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viii
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“There are men who struggle for a day, and they are good. There are others who struggle for a year, and they are better. There are some who struggle many years, and they are better still. But there are those who struggle all their lives, and these are the indispensable ones.”

Bertolt Brecht (1898 – 1956) German socialist, dramatist and poet
Vita

September 18, 1976  Born, Mexico City, Mexico

1995 - 2001  D.V.M. National Autonomous University of Mexico

2001 - 2003  M.Sc. National Autonomous University of Mexico

2003 - 2006  Cattle specialist. National Autonomous University of Mexico

2006 – present  Graduate research Associate, The Ohio State University

Publications

Journal Articles


Abstracts


Fields of Study

Major Field: Animal Sciences

Reproductive Physiology
Table of Contents

Abstract .......................................................................................................................... ii

Dedication ....................................................................................................................... v

Acknowledgments .......................................................................................................... vi

Vita ................................................................................................................................... x

List of Tables .................................................................................................................. xvii

List of Figures ................................................................................................................ xix

Chapter 1: Introduction ................................................................................................. 1

Chapter 2: Review of literature ...................................................................................... 5

  Introduction ..................................................................................................................... 5

  Effect of growth and development on the onset of puberty ........................................... 5

    Influence of maternal nutrition during gestation on reproductive function and
    age at puberty of offspring ............................................................................................. 5

    Influence of pre-weaning growth rate on age at puberty .............................................. 7

    Post-weaning growth and age at puberty .................................................................... 8

    Endocrinological and genetic control of puberty ......................................................... 11
Endocrine control of puberty ................................................................. 11
Endocrinology of precocious puberty .................................................. 14
Nutritional management to induce precocious puberty ......................... 16
Neuroendocrine effect of steroids on GnRH secretion ........................... 19
Steroid regulation of gonadotropin secretion ........................................ 20
Estradiol negative feedback and puberty ............................................... 23
Role of kisspeptin and other peptides on puberty ................................. 26
Kisspeptin and GPR54 ........................................................................... 26
Gonadotropin inhibitory Hormone (GnIH) ............................................. 31
Neuropeptide Y, Leptin, feed intake and control of reproduction ............ 32
Genetic selection and control of the onset of puberty ........................... 38
The somatotropic axis and precocious activation of the reproductive axis .... 41
Somatotropic axis ................................................................................. 41
Insulin-like growth factor (IGF-I) and ovarian physiology .................... 47
Statement of the problem ..................................................................... 50

Chapter 3: Effect of differing dietary starch on precocious maturation of the
reproductive and somatotropic axis in beef heifers. .............................. 52
Abstract ............................................................................................. 52
Introduction ......................................................................................... 54
Materials and methods ................................................................................................................. 56
Results............................................................................................................................................... 62
Discussion........................................................................................................................................ 64

Chapter 4: Effect of a high-energy diet on steroidogenic capacity of dominant follicles, secretion of LH and estradiol, and precocious puberty in beef heifers................................. 78

Abstract ........................................................................................................................................... 78

Introduction....................................................................................................................................... 82

Materials and methods .................................................................................................................... 84

Results.............................................................................................................................................. 94

Discussion......................................................................................................................................... 97

Chapter 5: General discussion........................................................................................................... 115

Appendix A: Growth hormone radioimmunoassay ..................................................................... 121

References......................................................................................................................................... 126
List of Tables

Table 2.1 The percentage of heifers that experienced precocious puberty and age at puberty .......................................................... 16

Table 3.1 Composition and chemical analysis of experimental diets fed to heifers (% as fed) .......................................................... 76

Table 3.2 LH characteristics at different ages in heifers fed with control diet (CONT), low-starch content (LOW-S), or high-starch content in the diet (HIGH-S). ..................... 77

Table 4.1 Composition and chemical analysis of experimental diets fed (% as fed) to beef heifers weaned at 66 ± 2 d of age, and randomized to receive one of two diets beginning at 86 ± 2 d of age and 137 ± 3.3 kg body weight ......................................................... 111

Table 4.2 Average (±SE) of age and body weight characteristics of the heifers fed either a high energy diet (HIGH) or a control diet (CONT) and randomly designated to be ovariectomized (OVX) or to remain intact (INT) ................................................................. 112

Table 4.3 Sequence of primers used for RT-PCR ......................................................... 113

Table 4.4 Mean (±SE) of LH concentration, frequency and amplitude of LH pulses in beef heifers fed either a high energy (HIGH) or a control diet (CONT) in a 12 h period ................................................................. 114
Table A.1 Recovery of bovine growth hormone (bGH) in 50 μl of bovine plasma and serum with the addition of either 50 μl of 5 ng/ml of bGH, 10 ng/ml of bGH or 20 ng/ml of bGH. ........................................................................................................................................................................... 123
List of Figures

**Figure 1.1** Endocrinological model for attainment of puberty in the beef heifer. Adapted from Day and Anderson (1998) .................................................................................................................................................. 4

**Figure 2.1** Endocrine changes during the peri-pubertal period that result in puberty in heifers ........................................................................................................................................................................................................ 13

**Figure 2.2** Ovine estrus cycle with endocrine changes in the major hormones: progesterone, estradiol, and LH, and modulation of progesterone and estradiol on the hypothalamic-hypophyseal axis. (Adapted from Clarke and Pompolo, 2005) ................. 21

**Figure 2.3** Hypothalamic areas and pituitary lobules (adapted from Reeves, 1987) ...... 24

**Figure 2.4** Kisspeptin neurons system and the neuroendocrine control of GnRH maturation neurons during the peri-pubertal period ..................................................................................................................... 31

**Figure 2.5** Physiological mechanisms and changes on the IGF system that drive the onset of puberty in cattle (Adapted from Lucy M.C., 2000) ..................................................................................................................... 49

**Figure 3.1** Body weight (mean ± SE) of pre-pubertal heifers fed either a forage based control diet (CONT), or higher energy diets containing either a low-starch (LOW-S) or high-starch content (HIGH-S) ..................................................................................................................................................... 70
Figure 3.2 Cumulative percentage of pubertal heifers fed either a forage based control diet (CONT), or higher energy diets containing either a low-starch (LOW-S) or high-starch content (HIGH-S). ................................................................. 71

Figure 3.3 Plasma concentration of insulin (mean ± SE) during a 6-h intensive sampling period at 239 days of age in heifers fed either a forage based control diet (CONT), or higher energy diets containing either a low-starch (LOW-S) or high-starch content (HIGH-S). ................................................................. 72

Figure 3.4 Glucose concentration (mean ± SE) during a 6-h intensive sampling period at 239 days of age in heifers fed either a forage based control diet (CONT), or higher energy diets containing either a low-starch (LOW-S) or high-starch content (HIGH-S). .......... 73

Figure 3.5 Serum concentration (mean ± SE) of IGF-I in heifers fed either a forage based control diet (CONT), or higher energy diets containing either a low-starch (LOW-S) or high-starch content (HIGH-S). ................................................................. 74

Figure 3.6 Serum concentration (mean ± SE) of IGF-I of heifers fed high energy diets containing either a low-starch (LOW-S) or high-starch content (HIGH-S) that reached precocious puberty (PREC; before 300 days of age) and heifers that did not reach precocious puberty (NON PREC); regardless of treatment. .................................................. 75

Figure 4.1 Body weight (means ± SE) of pre-pubertal heifers fed either a control diet (CONT), or high energy diet (HIGH). Analysis included heifers from INT and OVX groups. .................................................................................................................................................. 101

Figure 4.2 Mean (±SE) body weight and age at the onset of puberty in heifers fed either a control diet (CONT), or high-energy diet (HIGH). Heifers from each treatment were xx
divided in two subgroups: heifers that were ovariectomized (OVX) and heifers that were intact (INT).

Figure 4.3 Mean (±SE) estradiol concentration of heifers fed either a high concentrate (HIGH) or a control diet (CONT) prior to the day of ovariectomy (d 0).

Figure 4.4 Mean (±SE) length of the follicular waves preceding ovariectomy in heifers fed either a high-energy (HIGH) or a control diet (CONT) ovariectomized at 174 ± 4 d of age.

Figure 4.5 Mean (±SE) progesterone (P4) and estradiol (E2) concentration in follicular fluid (FF) extracted from dominant follicles of heifers fed either a high energy (HIGH-OVX) or a control diet (CONT-OVX) ovariectomized at 174 ± 4 d of age.

Figure 4.6 Expression of genes in terms of ratio (±SE) (gene of interest/house keeping gene) from dominant follicles of heifers fed either a high-energy (HIGH-OVX) or a control diet (CONT-OVX) ovariectomized at 174 ± 4 d of age.

Figure 4.7 Expression of insulin-like growth factor –I (IGF-IR) expressed in dominant follicles from heifers fed either a high-energy (HIGH-OVX) or a control diet (CONT-OVX) and ovariectomized at 174 ± 4 d of age.

Figure 4.8 Expression of luteinizing hormone receptor (LHR) expressed in dominant follicles from heifers fed either a high-energy (HIGH-OVX) or a control diet (CONT-OVX) and ovariectomized at 174 ± 4 d of age.

Figure 4.9 Expression of cholesterol side-cleavage chain (CYP11A1) expressed in dominant follicles from heifers fed either a high-energy (HIGH-OVX) or a control diet (CONT-OVX) and ovariectomized at 174 ± 4 d of age.
Figure 4.10 Expression of aromatase (CYP19A1) expressed in dominant follicles from heifers fed either a high–energy (HIGH-OVX) or a control diet (CONT-OVX) and ovariectomized at 174 ± 4 d of age.

Figure A.1 Typical fraction curve collected from purified GH iodinated with NaI$^{125}$ by the chloramine-T. After 40 seconds of reaction the mix is separated in the Sephadex 75 column and the fractions are collected.

Figure A.2 Parallelism of displacement of labeled hormone by serial dilutions of bovine known concentration samples.
CHAPTER 1

INTRODUCTION

The reproductive efficiency of beef production is determined by particular physiological events such as age at puberty, estrus and the length of postpartum anestrus. Additionally, reproductive as well productive performance is influenced by season of birth, growth rate, nutritional management and genetics of the animal. In particular, age at puberty has an important impact on the reproductive and economic efficiency of the herd and during the productive life of the cow (Larson, 2007). The occurrence of this physiological event implies an adequate growth rate and development of the animal that supports the endocrinological mechanisms that lead to sexual maturity. Age at the onset of puberty and first conception will influence lifetime reproductive performance in cattle, reflected in the likelihood of calving on an annual basis and longevity in the herd (Bagley, 1993). Beef heifers that conceived early during their initial breeding season and calved as 2 year old females had a greater probability to remain in the herd and increase the number of calves during their lifetime (Lesmeister et al., 1973).

In spite of the importance of this physiological endpoint, the metabolic signals that activate the endocrine mechanisms that trigger puberty are still poorly understood. Sexual
maturation involves a series of metabolic and endocrine changes that leads to the activation of the hypothalamic – hypophysis and ovarian axis, a process that starts with increased gonadotropin secretion (follicle stimulating hormone, FSH; and luteinizing hormone, LH) in response to increased gonadotropin releasing hormone (GnRH) release by the hypothalamus which leads to increased growth, maturation and eventually ovulation of a follicle that contains a viable oocyte (Day and Anderson, 1998). While these endocrine mechanisms that lead to puberty have been well established, the cellular pathways and hormones involved in the activation and the relationship with nutritional signals that activate these pathways are less well known.

From a reproductive standpoint, puberty is defined as the manifestation of reproductive capability that occurs when an animal acquires the ability to ovulate an oocyte with presentation of estrous behavior (Kinder et al., 1987). In other words, the endocrine changes prior to the onset of puberty such as increase of LH pulses, and decrease of negative feedback to estradiol in the hypothalamus result in the ovulation of a dominant follicle accompanied by development and maintenance of a functional corpus luteum of normal lifespan (Kinder et al., 1987). Puberty in cattle, specifically, is characterized by maturation of the reproductive axis that occurs gradually and is associated with changes in body composition, endocrinological modifications in the reproductive axis (hypothalamus-hypophysis-ovary) (Day and Anderson, 1998) and somatotropic axis (growth hormone, insulin-like growth factor-I and its binding proteins) (Velazquez et al., 2008).
From the practical standpoint, age at puberty has been associated with productive variables such as body weight, adipose tissue reserves and genetics of the animal (Patterson et al., 1992). In order to understand these changes associated with growth of the animal and its relationship to maturation of the reproductive axis (Figure 1.1), the pre-pubertal period (birth to onset of puberty) has been divided in four phases: infantile period (birth to 2 months of age), developmental period (2 to 6 months of age) a static phase (6 to 10 months of age) and, finally the peri-pubertal period (after 10 months of age; Day and Anderson, 1998). At the end of the developmental phase it has been suggested that the hypothalamus and reproductive tissues as individual organs are fully mature (Day and Anderson, 1998). The developmental phase is characterized by an increase in LH secretion (presumptively to cause maturation of the ovary reflected by acquisition of antral follicles) followed by a decrease that is sustained through the static phase due to negative feedback by ovarian estradiol on the hypothalamo - hypophyseal axis. The static phase is characterized by gradual maturation of the reproductive axis, reflected primarily in gradual increases in diameter of the dominant follicle of each successive wave, until initiation of the peri-pubertal phase. During the peri-pubertal period there is a decrease in the negative estradiol feedback on GnRH secretion, resulting in a substantial increase in frequency of LH pulses. The enhanced stimulation of ovarian follicles results in further growth of each successive dominant ovarian follicle. The resulting increase in systemic concentrations of estradiol will in turn activate the positive estradiol feedback loop that results in induction of the initial pre-ovulatory LH surge and ovulation.
Figure 1.1 Endocrinological model for attainment of puberty in the beef heifer. Adapted from Day and Anderson (1998).
CHAPTER 2

REVIEW OF LITERATURE

Introduction

This review of literature emphasizes endocrinological and nutritional mechanisms that are involved in puberty as well organs involved in the maturation of the reproductive axis. Additionally some of the developmental and genetic aspects that affect puberty in cattle are discussed. Finally, a conceptual model depicting the link between metabolic status of the heifer and activation of the reproductive axis as well the intra-ovarian IGF system involving follicular growth and maturation and the possible role in the establishment of precocious puberty is proposed.

Effect of growth and development on the onset of puberty

_Influence of maternal nutrition during gestation on reproductive function and age at puberty of offspring._

Recently studies have demonstrated that maternal nutrition and uterine environment during gestation plays an important role in performance of offspring. In fact, during the
last third of gestation in ewes, 75% of fetal growth occurs and nutritional deficiency during this time has been demonstrated to negatively impact subsequent performance of offspring (Robinson et al., 1995). It has also been reported, that impaired intrauterine nutrition as a result of inadequate maternal nutrition is associated with health abnormalities in adult humans. In an epidemiological study, Barker et al., (1994) reported that low weight at birth as a result of maternal under-nutrition is associated with hypertension and cardiovascular diseases as well neurological and reproductive dysfunctions in adult humans (Barker et al., 1994; Fowden et al., 2008). This evidence suggests that intrauterine environment might affect the development of the organs and also impact physiological functions and incidence of common adult diseases.

The uterus has diverse functions during fetal development, providing adequate nutrients and oxygen for the correct development and growth of the organs and tissues, and also providing essential hormones that modulate developmental processes (Fowden et al., 2004). The importance of the uterine environment and the programming of metabolic functions while offspring are gestating relative to development of pathological conditions such as diabetes, obesity, etc, after birth and more recently, subsequent growth and reproductive efficiency in domestic animals of economic importance has been the focus of intense investigation in the last decade.

There is limited information regarding the impact of late gestation nutrition of cow dams on the age at puberty in the female calves. Recently, Martin et al., (2007) tested the effect of protein supplementation during late gestation on heifer performance. Results from this
study suggest that no differences in terms of age at puberty, birth weight or weaning weight were observed between heifers from supplemented dams and heifers in the control treatment, however, it was observed that heifers from supplemented dams had greater pregnancy rates than heifers from control dams (93% vs. 80%, respectively). Similarly, Corah et al., (1975) did not observed differences in age at puberty in heifers from dams that were restricted to 65% of the NRC requirements for energy intake during the last 100 days of gestation.

There is no doubt that maternal nutrition during pregnancy impacts the development of offspring, although, the specific physiological mechanisms that are involved (e.g. impact on age at puberty, incidence of diseases) are poorly understood. Recently it has been suggested by Belkacemi et al., (2010) that altered uterine environment in response to nutritional excess or deficit can affect the fetus at three different levels, which include abnormal development of organs and tissues, modification of endocrine physiological functions (predisposition to adult diseases) and modulation of gene expression activity. More research is needed in order to determine these effects and modulate this important phase of development and growth and assess the impact on adult life.

*Influence of pre-weaning growth rate on age at puberty*

During the first three months of life, a beef heifer primarily receives its nutrition from the dam’s milk, however, in most production systems, forage also plays an important role in nutrition during early development. In traditional management in the USA, heifers
remain with their dam until weaning (between 6 – 10 months of age). Limited emphasis has been placed on the influence of pre-weaning growth rate on puberty, although it was demonstrated over 4 decades ago that nutritional impacts on growth during this period have significant impact on age at puberty. Wiltbank et al., (1966) reported that pre-weaning BW gain influenced age at puberty in heifers more consistently than post-weaning BW gain, with increased pre-weaning BW gain resulting in earlier onset of puberty. Others have also observed a reduction in age at puberty with increased weaning weight (Arije and Wiltbank, 1971; Greer et al., 1983). Recently, Roberts et al., (2007) found that age at puberty before breeding season and fertility is affected more by growth rate pre-weaning (birth to weaning) and the growth rate during the first 140 days after weaning than growth in the period just before breeding. Much of the emphasis on the effect of pre-weaning BW and on productive variables has focused on mammary gland development in dairy breeds (Brown et al., 2005).

Post-weaning growth and age at puberty

The age at which cattle reach puberty is associated with genetic and nutritional factors. Weight gain after weaning is a major variable that influences age and weight at puberty (for review, see Patterson et al., 1992). Beef heifers that conceived early during the breeding season and calved at 2 years of age had a greater productive and reproductive lifetime (Lesmesiter et al., 1973). The influence of different diets during the post-weaning period on age of puberty has been extensively investigated. Growth rate between
weaning (6 to 8 months of age) and puberty is positively associated with age at puberty (Smith et al., 1976). Target weights at breeding have been developed to reflect these relationships and suggest that heifers should achieve a specific proportion of mature body weight by the beginning of the breeding season in order to achieve high pregnancy rates (Lamond, 1970, Wiltbank et al., 1969). Early reports established that heifers should be fed to achieve 60 to 65% of their projected mature weight before their first breeding season to ensure high pregnancy rates (Lamond 1970; Taylor and Fitzhugh, 1971). Ferrell (1982) evaluated the effect of feeding to achieve different ADG (low 0.4 kg/d, medium 0.6 kg/d and high 0.8 kg/d) in different beef cattle breeds. It was demonstrated that both breed and post-weaning ADG were significant sources of variation for both age and weight at puberty. Across breeds, heifers that were fed to achieve the lowest ADG tended to be older and lighter at puberty. More recently, Thallman et al., (1999) investigated the effect of breed on weight at puberty in different breeds in the USA. Across breeds, age and weight at puberty were approximately 357 days of age and 320 kg BW, respectively, although breed differences were noted. Clanton et al., (1983) demonstrated in a series of experiments that the plane of nutrition in which heifers are developed is flexible, in other words, delaying the weight gain until 3 months before breeding season (fed to achieve 0.91 kg/d) resulted in same age at puberty and same BW as heifers fed from weaning to breeding season (fed to achieve 0.45 kg/d). Additionally, it was observed that the high nutrition plane in beef heifers three months before breeding season, did not affect conception rates. For example, Lynch et al., (1997) reported that heifers fed a greater plane of nutrition promoted greater average daily gain during the peri-pubertal phase (60
days before breeding season, 0.91 kg/d) and increase the number of heifers cycling at the beginning of the breeding season, and minimized feed costs using less amount of feed in comparison with the heifers that were fed from weaning to beginning of the breeding season (fed to achieve 0.45 Kg/d). Collectively, these data suggest that the timing at which nutritional strategies to promote an accelerated is flexible and different strategies can be used to help minimize feed costs and hasten the reproductive maturation. Also, these data emphasize that heifers can be stimulated to achieve puberty during the period before breeding season (Figure 1.1).
Endocrinological and genetic control of puberty

Endocrine control of puberty

In cattle, maturation of the reproductive axis (Figure 2.1) occurs in a gradual fashion and this process requires endocrine and metabolic changes that occur in a harmonious fashion (Day and Anderson, 1998). From an endocrine mechanism standpoint, the occurrence of puberty is the result of a decrease in estradiol negative feedback in the hypothalamus on secretion of gonadotropin releasing hormone which leads to an increase in the secretion of LH in response to an increment of GnRH release (Rodriguez and Wise, 1989), resulting in final growth and maturation of ovarian follicles leading to a successful ovulation (Day et al., 1984; Day et al., 1986, Kinder et al., 1987). The change in the frequency pattern of the GnRH release during the early pre-pubertal development drives the increase in the secretion of gonadotropins that is critical for the activation and proper follicle growth and development (Madgwick et al., 2005; Whitlock et al., 2006).

Madwick et al., 2005 observed that animals treated with GnRH twice a day from 4 to 8 weeks of age, reached the puberty earlier (56.8 ± 1.7 weeks of age) than the control animals (62.8 ± 2.4 weeks of age) suggesting that an administration of GnRH in heifers as early as 4 weeks of age (infantile phase) can stimulate the release of LH and FSH. Even though these authors did not measure ovarian dynamics during the administration of GnRH and LH sampling, it is possible that the effect of the treatment was directly related to the maturation of follicles and these structures start synthesizing estradiol and driving the activation of the GnRH neurons. Foster et al., 2006) suggested that an early
programming of the GnRH secretion may conduct to an early activation of the reproductive axis and early onset of puberty. Early studies have shown that the components of the reproductive axis (hypothalamus, hypophysis and ovaries) are responsive to appropriate stimuli (exogenous hormone administration) between 1 and 6 mo of age in heifers depending upon component. The ovary becomes responsive to exogenous gonadotropins at 1-2 months of age (Howe et al., 1962, Seidel et al., 1971), and the hypothalamo - hypophyseal axis becomes responsive to exogenous estradiol-17β (E2), in terms of the estradiol induced LH surge at 5 - 6 months of age (Staigmiller et al., 1979, Schillo et al., 1983). Administration of estradiol in pre-pubertal heifers hastened the onset of puberty, as Dyer et al., (1990) demonstrated that estradiol administration early in the pre-pubertal phase (288 days of age) advances the increase in LH secretion. However, after stimulation with exogenous hormones that may induce an ovulation in pre-pubertal heifers, estrous cycles do not typically continue (Evans et al., 1995) unless heifers are treated in the peri-pubertal period. Therefore while independent systems can respond to exogenous stimuli, the restraint exerted by estradiol negative feedback prevents the coordinated function of this axis and hence continuation of estrous cycles or puberty. It has been described previously that removal of the restraint exerted by ovarian estradiol on the hypothalamus results in increased LH secretion. For example, Swanson et al., (1971) suggested that after ovariectomy in heifers an increase in plasma concentration of LH is observed due a removal of the negative feedback that estradiol exerts in the hypothalamus. Kiser et al., (1981) ovariectomized pre-pubertal Hereford heifers at 10 to 14 months of age. Pre-pubertal heifers had an increase of mean LH and an increase in the
number of pulses after surgery confirming the hypothesis of the negative feedback of estradiol on the secretion of gonadotropins.

**Figure 2.1** Endocrine changes during the peri-pubertal period that results in puberty in heifers. The peri-pubertal period includes the 50 to 60 days preceding puberty in heifers. The dashed line represents estradiol negative feedback on secretion of LH. The secretion of GnRH is highly sensitive to estradiol negative feedback during the pre-pubertal period. As the peri-pubertal period and the associated decline in estradiol negative feedback begins, secretion of GnRH, and hence LH, increases, resulting in increased growth and estradiol secretion by dominant ovarian follicles. As a result of the progressive decline in estradiol negative feedback and increase in LH secretion during the peri-pubertal period,
estradiol concentrations eventually attain levels sufficient to induce the pubertal surge of LH.

*Endocrinology of precocious puberty*

The occurrence of spontaneous precocious puberty in beef heifers has been reported previously by Wehrman et al., (1996) in which as many as 25% of the heifers in a herd experienced luteolytic activity indicative of puberty before 300 days of age. Feeding a high energy diet during the developmental phase (2 to 6 months of age) hastens puberty in beef heifers. Studies from our lab clearly demonstrated that when heifers are weaned early and fed a high energy diet, the timing of the events that culminate in puberty is observed before 300 days of age (Gasser et al., 2006a, b, c, d; Table 2.1) in most heifers. Gasser et al., 2006 (a, b, c and d) investigated changes in various components of the reproductive axis in three of the previously cited experiments. In the first experiment (Gasser et al., 2006a) secretion of LH was greater in the early weaned high energy diet treatment (EWH), beginning at 190 days of age up to the time of puberty. In the second experiment (Gasser et al., 2006b) follicular growth was enhanced in the EWH treatment by 196 days of age, as evidenced by a greater diameter of the dominant ovarian follicle. In the same study, it was demonstrated that animals which experience precocious puberty had a larger dominant ovarian follicle, a longer, follicular wave, and greater estradiol concentrations than heifers that reached puberty after 300 days of age. These differences were evident as early as 120 days of age between heifers that experienced precocious
puberty and those that did not. Finally, Gasser et al., (2006c) demonstrated that timing of the peri-pubertal decline in estradiol negative feedback on LH secretion was hastened in the EWH treatment. It was demonstrated in this series of experiments that key changes that occur in the reproductive axis between 10 and 12 months of age (peri-pubertal period) in traditionally managed heifers were instead occurring between 6 and 8 months in heifers that were weaned early and fed a high energy diet. From this body of information it can be concluded that the combined effect of weaned early and fed a high energy diet induce a shift in the timing of the maturation of the reproductive axis. It appears that with this dietary manipulation, heifers forego the static phase described previously, moving directly from the developmental phase to the peri-pubertal period.
Table 2.1. The percentage of heifers that experienced precocious puberty and age at puberty

<table>
<thead>
<tr>
<th>Experiment</th>
<th>n</th>
<th>% Precocious puberty</th>
<th>Age at Puberty (d)</th>
<th>% Precocious puberty</th>
<th>Age at Puberty (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gasser et al., 2006a</td>
<td>18</td>
<td>89 (8/9)</td>
<td>262 ± 10</td>
<td>0 (0/9)</td>
<td>368 ± 10</td>
</tr>
<tr>
<td>Gasser et al., 2006b</td>
<td>18</td>
<td>100 (9/9)</td>
<td>252 ± 9</td>
<td>56 (5/9)</td>
<td>308 ± 26</td>
</tr>
<tr>
<td>Gasser et al., 2006c</td>
<td>10</td>
<td>80 (4/5)</td>
<td>275 ± 30</td>
<td>0 (0/5)</td>
<td>385 ± 14</td>
</tr>
<tr>
<td>Gasser et al., 2006d</td>
<td>30</td>
<td>67 (10/15)</td>
<td>271 ± 17</td>
<td>20 (3/15)</td>
<td>331 ± 11</td>
</tr>
</tbody>
</table>

aData from Gasser et al., 2006a; 2006b; 2006c; and 2006d.

Nutritional management to induce precocious puberty

It has been demonstrated that feeding of a high energy diet precociously activates the reproductive axis as mentioned before. It appears that this nutritional stimulus is most profound between 3 and 6 months of age (Gasser et al., 2006a). Radcliff et al., (1997;
2004) evaluated the effect of two different diets from 120 days of age until puberty in Holstein heifers, high diet (containing 75% grain and 25% haylage) and control diet (10% grain and 90% haylage). Heifers fed the high diet showed puberty at 266 ± 9 days of age while puberty occurred at 313 ± 10 days of age when fed the control diet (Radcliff et al., 1997). Therefore, similar to findings in beef heifers (Gasser et al., 2006a, b, c, d) feeding a high concentrate, high energy diet early in life induced precocious puberty in dairy heifers. The effect of feeding a high energy diet during development on the somatotropic axis was also reported by these authors (Radcliff et al., 2004). Heifers fed the high diet had greater peripheral IGF-I concentrations, and lesser IGFBP-2 concentrations than those fed the control diet. Concentrations of GH were unaffected by diet. However, the liver concentrations of growth hormone receptor 1A (GHR 1A) and IGF-I mRNA were increased, and concentration of IGFBP-2 mRNA was decreased in heifers fed the high energy diet; explaining the effect of diet on peripheral concentrations of IGF-I and IGFBP-2. Since IGFBP-2 has been suggested to inhibit IGF-I activity (Clemmons, 1998), the relative influence of the high vs. control diet on IGF-I may be even greater than that reflected by concentrations of IGF-I in circulation.

Yelich et al., (1996) working with beef heifers from nine months of age until the onset of puberty, supplemented with two diets (high gain; 2.04 Mcal/kg NEm and 1.31 Mcal/kg NEg and low gain; 1.23 Mcal/kg NEm and 0.61 Mcal/kg NEg fed for 16 weeks followed by the high gain diet). Heifers that received a high energy diet reached the puberty earlier than animals in the low-high gain diet (369 ± 16 vs. 460 ±17 days of age, respectively).
Heifers in the high gain treatment had greater concentrations of LH, IGF-I, and insulin during the first 84 days of treatment than the heifers in the low gain treatment. Across treatments, serum GH concentrations were greater at 1 week before the onset of puberty than at 3 weeks before puberty. Their results suggest that a change in the GH concentrations occurs near to the onset of puberty and could play a role in this process. Results of this study support the hypothesis that the nutrition influences age at puberty through regulation of IGF-I concentrations and that GH may be involved in final maturation of the reproduction axis.
Neuroendocrine effect of steroids on GnRH secretion

Increase of GnRH secretion is critical for the establishment of normal estrus cycles in pre-pubertal animals. Secretion of GnRH is mediated greatly by the ovarian steroids estrogen and progesterone, however other hormones and peptides also affect its secretion such as leptin, IGF-I, GABA, glutamate, neuropeptide Y, activin and inhibin (Clarke, 2011). Recently, a new peptide, kisspeptin and regulation of its receptor, GPR54 have emerged as possible key regulators of function of GnRH neurons (Clarkson and Herbison, 2006; Ojeda et al., 2010). Early studies pinpointed that decreased negative feedback of estradiol on LH secretion, and hence GnRH secretion, is necessary for occurrence of puberty (Ramirez and McCann, 1963). Increase in the pulsatile pattern of GnRH secretion needs to occur for the induction of gonadotropin (LH and FSH) secretion, and consequently, enhanced follicular growth, maturation of the dominant follicle and finally ovulation. However, the exact mechanisms that are involved in the neuronal network that leads the decrease of negative feedback of estradiol in the hypothalamus and the disinhibition of the GnRH neurons to initiate puberty are poorly understood. It was demonstrated in ewes that these neurons do not express estradiol receptors (Lehman and Karsch, 1993; Lehman et al., 1993). Therefore, the idea of the existence of a network of communication between other (non-GnRH) neurons that are regulated by estradiol, which in turn regulate GnRH neurons via other hormones within the hypothalamus to modulate the frequency of GnRH pulses has been advanced (Ojeda et al., 2010).
Steroid regulation of gonadotropin secretion.

In cyclic female cattle, estradiol is an important modulator of LH and FSH secretion through positive actions stimulating GnRH release and through direct actions on the pituitary gland. In contrast, progesterone is inhibitory to the expression of GnRH receptors in the hypophysis and reduces the frequency of GnRH decreasing the sensitivity of the hypothalamus to the estradiol action (Nett et al., 2002). It has been hypothesized that estradiol has two different actions at the neuronal level: the first is to induce a preovulatory surge of LH, increasing the secretion of GnRH. Secondly, it has been reported that at hypophyseal tissue estradiol stimulates the expression and translocation of GnRH receptors in the gonadotropes resulting in an release of gonadotropins. (Nett et al., 2002; Clarke and Pompolo, 2005). Turzillo et al., (1998) administered exogenous estradiol during the luteal phase in the sheep, observing that estradiol was not capable of increasing the number of GnRH receptors in the hypophysis in the presence of elevated progesterone. Confirming this finding, during the estrous cycle in the ewe (Figure 2.2), when progesterone concentrations are elevated (luteal phase), GnRH receptor expression at hypophyseal level is low and LH secretion is characterized by low frequency of pulses and high amplitude due the inhibitory actions of progesterone (Clarke and Pompolo, 2005), whereas once the corpus luteum regresses and progesterone concentrations decline, the follicular phase progresses resulting in increase of estradiol concentration and the frequency of GnRH and LH pulses raises substantially (Moenter et al., 1991).
Figure 2.2 Ovine estrus cycle with endocrine changes in the major hormones: progesterone, estradiol, and LH, and modulation of progesterone and estradiol on the hypothalamic-hypophyseal axis (Adapted from Clarke and Pompolo, 2005).

In the study cited previously (Turzillo et al., 1998) it was reported that expression of GnRH receptors increased in response to decreasing progesterone concentrations, but interestingly, this increase in GnRH receptors occurred before a detectable increase in endogenous estradiol concentrations. It has been speculated that other factors exist that modulate GnRH neurons (Herbison, 1997). Alternatively, it has been hypothesized that basal estradiol concentration may be sufficient to induce the expression of GnRH
receptors in the hypophysis, therefore once progesterone declines, the suppressive effect on the GnRH pulse generator is released and the neurons are responsive again (Rispoli and Nett, 2005). In fact, Turzillo and Nett, (1995) observed in sheep that treatment with exogenous estradiol incremented the expression of GnRH receptors in the hypophysis in the absence of progesterone and this response was in a dose dependent fashion.

As mentioned before, progesterone has inhibitory actions at hypothalamic level, reducing the frequency of GnRH pulses and decreasing the sensitivity of the hypophysis to GnRH, however this steroid is also essential in cycling animals due its effect on “priming” the hypothalamus to achieve full positive feedback actions of estradiol to induce the LH surge at estrus (Clarke and Scott, 1993). Caraty and Skinner (1999) tested the effect of progesterone priming in ovariectomized sheep treated with exogenous estradiol. Results from this study indicate that estradiol-induced LH surge was greater in ewes primed with progesterone, indicating the critical aspect of the cycle of progesterone and estradiol concentrations that occur during an estrous cycle. This finding reinforces the fact that although estradiol and progesterone have opposite actions, there is an intimate relationship between these hormones in regulation of GnRH secretion during the estrous cycle. In pre-pubertal heifers, progesterone is not present until occurrence of a short-lived corpus luteum that typically occurs with their first spontaneous ovulation. Therefore, progesterone priming occurs before the first normal length estrous cycle in heifers (Nogueira et al., 2003).
In the brain two major progesterone receptors (PR\textsubscript{A} and PR\textsubscript{B}) have been identified, however the biological functions are poorly understood. Recently, Richter et al., (2005) demonstrated in ewes that progesterone inhibits neurons at hypothalamic areas responsible for the GnRH surge. Skinner et al., (2001) demonstrated that GnRH neurons do not express progesterone receptors, therefore it has been hypothesized that progesterone may exert GnRH inhibitory secretion through various non classical pathways positively affecting inhibitory neuro-modulators such as γ-aminobutyric acid (GABA) and opioids such as β-endorphin (Pluchino et al., 2009)

**Estradiol negative feedback and puberty**

The role of negative feedback of estradiol on LH secretion in regulating puberty has been studied extensively. A possible mechanism of additional mechanisms by which estradiol can positively influence puberty in heifers is through alterations of synaptic connections between GnRH neurons and other neurons that express estradiol receptors (For review see Day and Anderson, 1998). In fact, recent evidence suggests that estradiol may regulate GnRH neurons through modulation of GABA and glutamate cells that express estradiol receptor α (ER\textsubscript{α}; for review see Christian and Moenter, 2010). Experimental evidence suggest that GABA concentration decrease during the LH surge in sheep during the breeding season (Clarke and Scott, 1993). Moreover, GABA and glutamate responsive neurons are located in the hypothalamic anteroventral periventricular area (AVPV, which is located in the periventricular nuclei) and are directly upstream of
GnRH neurons and are able to respond to ovarian estrogen. Additionally, these neurons receive input from other areas of the hypothalamus such as medial pre-optic nuclei, arcuate nucleus (ARC), and the amygdala which contain neurons that express estrogen receptors. Progesterone receptors are localized in the ventromedial nucleus (VMN) and the arcuate nucleus and also in several zones of the medial pre-optic area (mPOA) in addition to the AVPV area, suprachiasmatic nuclei (SCN). Figure 2.2 depicts the main hypothalamic nuclei and the hypophysis lobules in the bovine brain.

Figure 2.3 Hypothalamic areas and pituitary lobules. Adapted from Reeves (1987)

AHA, anterior hypothalamus; ARC, arcuate nuclei; DHA, dorsal hypothalamic area; DMN, dorsal medial nuclei; ME, median eminence; MB, mammillary bodies; PM, premammillary nucleus; OC, optic chiasm; PVN, paraventricular nuclei; PON, preoptic
nuclei; PHA, posterior hypothalamic area; PT, pars tuberalis; SCN, suprachiasmatic nuclei; SON, supraoptic nuclei; VMN, ventromedial nuclei; AH, adenohypophysis; NH, neurohypophysis.

Early studies characterized the pattern of estradiol receptor expression in regions of the hypothalamus such as anterior hypothalamus (AHA), and medial basal area that includes the arcuate (ARC) and ventromedial nuclei (VMN). Day et al., (1987) reported that during the peri-pubertal period expression of estradiol receptors in the AHA and medial basal area was observed, suggesting that this decrement could reflect the decline in inhibitory actions of estradiol on GnRH secretion. This decline was associated with increased LH pulse secretion which culminated in puberty. It has been suggested, counter intuitively, that increased systemic estradiol, while inhibiting GnRH secretion, also plays a critical role in promoting the decline in estradiol negative feedback on GnRH neurons. The peri-pubertal decline in the estradiol receptor expression observed in the medial pre-optic area (the region of the hypothalamus thought to be responsible for modulation of sensitivity of GnRH neurons to estradiol) was responsible for the decline in estradiol negative feedback on GnRH secretion by the hypothalamus (Goodman, 1996).

Döcke et al., (1984) proposed that, in female rats, an increase of estrogen concentrations in the MPOA inactivates medial pre-optic neurons (that exert a restraining influence on tonic LH and FSH secretion). This action is mediated by the sensitization of the mediobasal hypothalamus area (ARC and VMN) to the negative feedback action of
estrogen. Recently it has been suggested that estradiol negative feedback in the hypothalamus is mediated by kisspeptin neurons and regulation of GPR54 expression and other peptides (Clarke, 2011).

**Role of kisspeptin and other peptides on puberty**

*Kisspeptin and GPR54*

With the recent discovery of the kiss-1 gene that encodes for kisspeptin and its receptor (GPR54), and its localization in the hypothalamus and ovary it has been hypothesized to be involved in the modulation of reproductive events such as puberty (Smith, 2008). GPR54 receptor is co-localized with GnRH neurons in the mouse hypothalamus (Messager et al., 2005) and also it has been reported that around 90% of the GnRH neurons from the sheep express the GPR54 receptor. In fact, it has been proven that GPR54 is expressed in the medial pre-optic area which also constitutes an area of a vast number of GnRH neurons (Smith et al., 2009). Kisspeptin neurons and its receptor have been localized in the arcuate nucleus, dorso-medial, paraventricular and ventromedial nuclei, and the pre-optic area. These areas are important in the hypothalamic regulation of the secretion of pituitary hormones (LH and FSH) and more specifically during the mechanisms involved during the pre-ovulatory release of GnRH by the action of estradiol (Clarke, 2011). Murphy (2005) suggested a possible role of kisspeptin in the regulation of gonadotropin secretion. The kiss-1 gene expression is regulated by peripheral
concentrations of estradiol, since kisspeptin neurons express ERα and in the ewe it has been demonstrated that the mediobasal hypothalamus area is the principal area of estradiol negative feedback action (Caraty et al., 1998). These neurons are responsive to changes in estradiol affecting the expression of kisspeptin. Kiss neurons communicate with the GnRH neurons to activate the secretion of GnRH thru the GPR54 and consequently the secretion of LH/FSH (Smith, 2008).

Experimental evidence suggests that kisspeptin does not trigger the onset of puberty *per se*, but the kisspeptin is a mediator between neurons that serve to establish the neuronal network for the activation of the GPR54 receptors and GnRH neurons (Pineda et al., 2010). One of the possible mechanisms by which kisspeptin regulates the reproductive axis is that it’s receptor was also found in the hypophysis, and in addition, expression is also observed in the ovaries and testes of rats (Roa and Tena Sampere, 2007), however the specific function of the peptide and its receptor on these tissues is unknown. Roa and Tena-Sampere (2007) summarized some of the specific roles of kisspeptin on reproduction that have been reported: a) Kisspeptin is a potent stimulator if the GnRH/gonadotropic axis, b)It has been demonstrated that kisspeptin is involved in regulation of timing of puberty in rodents, humans, and sheep c)Stimulatory effects are mainly driven by activation of GnRH neurons, d)Two main sites of expression of Kiss-1 system are the arcuate nuclei and the anteroventral periventricular area (AVPV) which is located in the periventricular area of the preoptic nuclei (PON) (Sumida et al., 1993). and e) expression of the Kiss-1 gene is controlled by estradiol, therefore sex steroids are
strong candidates for modulating the negative and positive feedback effects of estradiol on GnRH and consequently on the gonadotropins FSH/LH.

Finally recent evidence suggests that kiss neurons are dependent on estrogenic input from the gonads. In rats and mice, estrogen stimulates expression of Kiss-1 system in the hypothalamus in the AVPV area whereas it inhibits kisspeptin in the ARC nuclei (Garcia-Galiano et al., 2011). The biological difference for the mode of action of estrogens is due its dual negative and positive feedback on Kiss-1 gene expression and gonadotropin secretion in rodents (Glidewell-Kenney et al., 2007). In addition, it has been reported that pre-ovulatory peak of estradiol in the sheep activates the transcription of the Kiss-1 gene, inducing an increase in the expression if kisspeptin tone/secretion, resulting in an increase of kiss neurons projection to GnRH neurons and secretion of GnRH into the hypophysis to release LH and FSH (Smith et al., 2011)

On the other hand, nutritional status of the animal, influence the expression of the kisspeptin and its receptor. In fact, long term sub-nutrition and negative energy balance have been shown to induce inhibition of the mRNA concentration of kiss-1 system (Castellano et al., 2010). Recently, Castellano et al., (2011) demonstrated in female rats that manipulation of nutrition during the early postnatal period (first 30 days in life) affects the timing of puberty. Female rats were raised in litters of different sizes to achieve different nutritional status; small litter size (four pups per dam: to promote overfeeding), normal size litter (12 pups per dam to promote normal nutritional development), and large litter size (20 pups per litter to induce a stage of underfeeding). Their results demonstrated that the manipulation of the size of the litter promotes a
greater BW in the animals from the small litter size; this was accompanied by a decrease in the age of vaginal opening which indicates puberty. In addition, overfeeding also induced greater concentration of leptin and a greater expression of mRNA of kisspeptin. This study demonstrated that manipulation of the nutritional status in the rats affected the age at puberty and more importantly, affected expression of kisspeptin in the hypothalamus. This response is similar to results from our lab (Gasser et al., a, b, c, d) demonstrating that feeding a high energy diet to heifers early in life induced precocious puberty and raises the interesting question of whether effects of the high energy diet also have positive effects on kisspeptin and its receptor at the hypothalamic level. Additionally, it has been demonstrated that administration of leptin in the ewe, which is produced by adipocytes, induces expression of the Kiss-1 gene (Backholer et al., 2010).

Since leptin has been demonstrated to induce GnRH and LH/FSH secretion, this represents a potential mechanism of action for signaling between the adipose and GnRH system (Barb and Kraeling, 2004). Another hormone that has been demonstrated to be increased with greater nutritional inputs, IGF-I, has also been reported to induce the expression of Kiss-1 gene in the AVPV in female rats (Hiney et al., 2010). Conversely, administration of ghrelin, stimulates food intake and GH release and inhibits GnRH/LH secretion in sheep (Harrison et al., 2008) and it has been demonstrated in rats that ghrelin inhibits the expression of Kiss-1 system in the hypothalamus (Forbes et al., 2009). No evidence has been reported that insulin has direct control of hypothalamic expression of Kiss-1 system in rats (Castellano, et al., 2006).
Additional evidence in primates has demonstrated that kisspeptin and GPR54 decline during the pre-pubertal period, however an increase is observed during the peri-pubertal period (Shahab et al., 2005). Kisspeptin and its receptor GPR54 change according to the sexual maturation, in fact, it has been observed that GPR54 expression is increased during the transition from pre-pubertal to pubertal state in Rhesus monkeys (Shahab et al., 2005). In addition, in the same study it was observed expression of kisspeptin and neuronal projections to GnRH neurons also increases during this transition in the ARC nuclei and AVPV area. It has been suggested (Castellano et al., 2009) that kiss-1 system in the hypothalamus is activated during the neonatal/infantile period in rats, and that this development is modulated by estrogens produced by the ovary in these immature females.
Figure 2.4 Kisspeptin neurons system and the neuroendocrine control of GnRH maturation neurons during the peri-pubertal period. A simplistic model for the maturational and functional changes of GnRH neurons and release of gonadotropins (Adapted from Pineda et al., 2010 and Pralong, 2010).

**Gonadotropin inhibitory Hormone (GnIH)**

Gonadotropin inhibitory hormone was discovered first in birds (Tsutsui et al., 2000) as a peptide involved in the regulation and secretion of gonadotropins and also as a modulator of reproductive function and environmental cues (season). Later, it was found that this peptide is also present in the hypothalamus of mammals including ruminants (Clarke et
al., 2008) and more specifically in the paraventricular (PVN) and dorsomedial nuclei (DMN), suprachiasmatic (SCN) and supraoptic nuclei (SON) of the hypothalamus as well in the hypophysis. Interestingly, it has been reported that between 40 and 80% of the GnRH neurons show receptors to GnIH (Smith et al., 2008).

In steers, it has been demonstrated that systemic administration of GnIH reduced the LH pulses frequency and mean concentration (Kadokawa et al., 2009). In addition to this effect, it has been shown that GnIH neurons also might be involved in the mechanism of the negative feedback of estradiol as GnIH expressing neurons also express estrogen receptor α and these cells are able to respond to estradiol in sheep (Kadokawa et al., 2009). In addition, it is suggested that GnIH may play a role regulating energy balance due its position on the hypothalamus (PVN, DMN) and also to its projections to neuropeptide Y (NPY) neurons (Bentley et al., 2010). The indirect evidence that is accumulating regarding GnIH suggests a putative role in controlling negative feedback of sex steroids on the hypothalamus (Bentley et al., 2010). While this hormone could potentially be involved in regulation of puberty, its role, if it exists, remains to be elucidated.

**Neuropeptide Y, Leptin, feed intake and control of reproduction**

The metabolic status of the animal also plays a critical role in the onset of puberty, it has been suggested that hormones such as leptin and NPY affect the function and release of GnRH. Prolong et al., (2010) suggested that leptin exerts regulation on NPY, GABA and
kiss neurons. Neuropeptide Y is composed of 36 amino acids (Diskin et al., 2003) and among its functions is increase food intake and also has been involved in reproductive functions. Repeated or chronic administration of NPY stimulates feeding, demonstrating that NPY is capable of overriding both short- and long-term mechanisms of satiety and body weight regulation (Williams et al., 2001). For example, studies conducted in rats that were experimentally induced to develop obesity were achieved with multiple NPY injections (Zarjevski et al., 1993). The localization of NPY in the hypothalamus and the effects of feed components on NPY activity in ruminants are not fully known. General feed restriction increased levels of NPY mRNA in the infundibular nucleus of ovariectomized ewes (McShane et al. 1993). The synthesis and release of NPY in the hypothalamus are regulated by several factors, including leptin and insulin (inhibitory) and glucocorticoids (stimulatory) (Wojcik-Gladysz and Polkowska, 2006). The primary physiological role of the neurons in the ARC nuclei that express NPY/agouti-related protein (AGRP) may be to sense and respond to states of negative energy balance (Wagner et al., 2004). As well NPY, AGRP neurons are co-localized in the ARC nuclei and it has been proved that both NPY and AGRP neurons becomes overactive following a critical fall in the body’s energy stores (Henry, 2003). There is much potential for the NPY/AGRP neurons to interact with other neuronal populations that control energy balance. Overall, NPY produces a shift to positive energy balance by increasing food intake, reducing energy expenditure and facilitating triglyceride deposition through increased insulin levels. (Williams et al, 2001). In contrast, long-term restrictions of feed
resulted in an increase of NPY expression in the infundibular nucleus and PVN in the sheep (Barker-Gibb and Clarke, 1995; Barker-Gibb et al., 1996).

In addition to its role in intake regulation, NPY has also been shown to regulate function of GnRH neurons in different species including ruminants (Kalra, 1993) due an abundant population of NPY terminals located in the ARC nuclei (Lehman et al., 1986; Caldani et al., 1988). These common neuroanatomical connections suggest a neuroendocrinological interaction between neurons NYP and GnRH neurons (Wojcik-Gladysz and Polkowska, 2006). In the rat, NPY stimulates GnRH release from the hypothalamus in the presence of estrogen, whereas it inhibits GnRH under conditions of estrogen deficiency (Kalra et al., 1992). Exogenous NPY was demonstrated to suppress release of LH in both ovariectomized and ovariectomized + estrogen treated sheep (McShane et al., 1992), however, others (Porter et al., 1993) suggested that NPY exhibits a stimulatory action on the preovulatory surge of LH in the ewe.

ARC neurons express three different types of peptides: 1) NPY which mediates the increase in feed intake and decrease in energy expenditure but also some studies reported the effect of NPY on the reproductive performance inhibiting the secretion of LH (Polkowska and Gladysz, 2001; Henry, 2003); 2) AGRP is a powerful appetite stimulator, but, the specific role of AGRP in regulating body weight and feed intake is well established in non-ruminant species, but has not been extensively studied in ruminants (Henry, 2003) and 3) Neuropeptide precursor pro-opiomelanocortin (POMC) this neurons has the opposite effect of the NPY cells, decrease food intake and increase energy expenditure. (Borberger, 2005).
Another hormone that has been shown to regulate metabolic functions as well reproductive events is leptin. Leptin is a 16kDa protein that has diverse functions, regulates feed intake and also it has been linked to reproductive processes such as puberty (Zieba et al., 2005). However, a direct relationship between GnRH neurons and leptin receptor has not been observed since GnRH neurons do not express receptor for leptin (LepR) (Donato et al., 2011), therefore the existence of other mediators has been hypothesized (NPY and POMC). The expression of the LepR has been found in both the arcuate and ventromedial nuclei of sheep (Cunningham et al., 1999).

Leptin is synthesized and secreted from the adipose tissue and was first described by Zhang et al., (1994) in mice lacking the leptin gene (ob/ob) or the leptin receptor (LepR). These mice exhibited metabolic dysfunctions such as hyperphagia, obesity and diabetes and reproductive disorders such as low gonadotropin concentrations, incomplete development of reproductive organs and failure to reach sexual maturity. Treatment of ob/ob mice with leptin stimulated gonadal development, restored gonadotropin secretion and resulted in puberty in males but not in females (Chehab et al., 1996).

Early reports indicated that the onset of puberty has been linked to a “critical” amount of energy reserves or body fat (Lamond 1970; Taylor and Fitzhugh, 1971), as discussed earlier in this review. Leptin due its site of synthesis and role in reproductive processes has been linked to the establishment of puberty. In fact, Garcia et al., (2002) and Amstalden et al., (2000) reported strong correlation between circulating concentrations of leptin and the IGF-I increase in heifers as puberty approaches and that in pre-pubertal
heifers subjected to fasting, leptin and IGF-I concentrations decreased and this was associated with a reduction in frequency of LH pulses.

Leptin has diverse functions in the body, LepR is expressed in different organs and tissues, including the hypothalamus and pituitary (Zieba et al., 2008), and therefore is a potential candidate to modulate the onset of puberty due the relationship with the amount of adiposity or body condition score. Williams et al., (2002) in a review suggest that body weight of the heifers is highly correlated with circulating leptin concentration. Furthermore, leptin concentration increases around four months prior to the onset of puberty or first ovulation (Garcia et al., 2003). However, it has been demonstrated that leptin when administered exogenously is incapable to promote an increase of the frequency of LH pulses in pre-pubertal heifers regardless of nutritional status (Zieba et al., 2005). Another study, administering leptin exogenously in pre-pubertal heifers (Zieba et al., 2004) did not promote an increase in LH pulses, however the average concentration was slightly increased after leptin administration. Results from these studies suggested that leptin alone is not enough to initiate puberty in pre-pubertal heifers. Conversely, exogenous administration of leptin induces an acceleration of the age at puberty in mice (Ahima et al., 1997).

Finally, the control of feed intake in ruminants requires the integration of many signals and often these signals have been related to reproductive processes due to its localization in the hypothalamic areas (Baile and McLaughlin, 1987). Ruminant species receive a continuous influx of nutrient molecules in the form of volatile fatty acids. There is some
evidence in ruminants (sheep model) that neurotransmitters regulate feed intake by different mechanisms than those of non-ruminants and that these mechanisms can affect reproductive functions. (Henry, 2003)

For a ruminant to regulate energy balance there must be a mechanism for monitoring energy status that includes signals of 1) body energy content, 2) energy intake, and 3) energy utilization. (Miner, 1992). These signals presumably would be communicated to an integrator that determines hunger or the drive to eat. The regulation of energy metabolism presents dynamic and complex system of neuronal network and hormone communication, with hypothalamic areas such as ARC nuclei acting as the central coordinator (Broberger, 2005). The hypothalamus contains other nuclei associated also with feed intake. Electrical stimulation of the ventromedial area inhibits eating in hungry ewes, but ablation of the area produces hyperphagia and obesity. Although, much remains unknown about how the brain processes peripheral information in terms of metabolic hormones. Early work in sheep and goats suggested that the VMH mediates satiety and AHA mediates hunger (Baile and McLaughlin, 1987). In fact, recent studies have shown the existence of receptors for hormonal signals located in the arcuate nuclei in the ventromedial hypothalamus (Wegner et al., 2004). The ARC also has extensively reciprocal connections with other hypothalamic regions, including the paraventricular nucleus, dorsomedial hypothalamic nucleus, ventromedial hypothalamic nucleus and lateral hypothalamic area.
In conclusion, the regulation of the feed intake and energy balance involves a complex interaction of hormones such as leptin and neuropeptides such as NPY. Furthermore, experimental evidence indicated that these hormones and peptides communicate with the intricate reproductive mechanisms since the proximity of the hypothalamic nuclei that contain neurons permissive to the metabolic function of these hormones and peptides. This complexity has been demonstrated in different animal models (mice, rats, sheep), however, the specific mechanisms are not well understood, and also the existence of other peptides seems to be possible.

**Genetic selection and control of the onset of puberty**

Genetic selection is an important tool to improve productivity and to increase the profitability in the dairy and beef industry. Unfortunately, reproductive variables in general have been shown to have low inheritance and to be controlled by a variety of genes, as a result, it has been difficult to establish appropriate methods of selection to promote an improvement in reproductive efficiency to the same degree as other productive variables such as growth rate.

Selection of heifers based upon on variables such as ADG, growth rate from weaning to the onset of puberty, growth from birth to weaning, weaning weight (adjusted weight at 205 days), feed efficiency, etc. have shown variable results in terms of age at puberty (Wolfe et al., 1990). Furthermore, Smith et al., (1976) estimated that puberty heritability index is -.64 suggesting that there is a large component affected by other factors other
than the heifer, however, recently, others scientists reported lower index values (-0.16, Martinez-Velazquez et al., 2003; and -0.42, Vargas et al., 1998). In this regard, Smith et al., (1976) reported favorable genetic correlations between age at puberty and birth weight, weaning weight and yearling weight with this association greater for weaning and yearling weight than birth weight (Martin et al., 1992). These correlations indicate a favorable genetic relationship between growth/ADG and measurements of puberty (age and weight). Also, other variables such as milk production have been associated with age at puberty in cattle across breeds (-.88, Laster et al., 1979). For example, lighter breeds of cattle reached puberty earlier than heavy breeds, additionally, other variables such as birth weight (-.16, Werre and Brinks, 1986), weaning weight (-.52, Smith et al., 1976) and yearling weight (-.29, Smith et al., 1976) have been reported to impact the age at puberty in beef cattle. In other words, growth selection in heifers has been consistently reducing age at puberty. Results from this series of experiments suggested that the sire breed influences greatly the age at which heifers reached puberty and also growth rate of the heifer influences the age at puberty.

The most investigated genetic correlation relative to puberty has been the relationship between the sire scrotal circumference (SC), of a sire and age at puberty of his daughters. A favorable relationship between age at puberty and SC has been demonstrated (Brinks et al., 1978). Later, Toelle and Robinson (1985) reported a similar genetic correlation by sire-daughter analysis between SC and age at first breeding (-0.46); age at first calving and SC (-0.44). More studies about the genetic control of puberty have been performed recently in human populations. Much insight about the genetic regulation of the onset of
puberty was gained from abnormal gene disorders. For example abnormalities in the GNRHr gene (GnRH receptor) and GPR54 gene (G protein couple receptor 54 for the kisspeptin system) results in delayed or lack of the onset of puberty. Other genes such as deletion of the ob/ob gene encoding for leptin and LepR also resulted in lack of puberty as discussed previously.

As described previously, puberty is a complex mechanism that involves several systems and organs that are driven by a strict genetic control. In humans for example, several authors reported that suggested that 70 to 80% of variance in age at puberty can be attributed to genetic effects (Kaprio et al., 1995; Parent et al., 2003; Palmert et al., 2003) with the remaining variance the result of social and environmental cues. Epidemiological investigations have demonstrated that age at puberty in daughters is highly associated with the age at which the mother reached puberty (Slyper, 2006) and is linked to ethnicity and geographic location (Parent et al., 2003).

Recent sequencing of the bovine genome has allowed the detection of genes and DNA markers that potentially affect a variety of production traits in cattle (Goddard and Hayes, 2009; Beuzen et al., 2000) leading to a process coined “genomic selection”. Phenotypic traits are the result of different combinations of alleles and the interaction with the environment and differences between alleles are called single nucleotide polymorphisms (SNPs). The use of modern quantitative and molecular genetics allows distinguishing and validating SNPs that influence phenotypes, and subsequent use of the SNPs as markers for genes that influence productive traits. Recently, Fortes et al., (2010) using a genome wide association analysis examined the relationship between genes and puberty in beef
cattle, defined in this analysis as age of occurrence of the first corpus luteum (AGECL). Results from this study suggested that AGECL was linked to 2,799 SNPs. Among these SNPs were genes such as NMDAR2B (Glutamate receptor from the NMDA class of receptors, which are involved in pubertal brain development), PROP1 (differentiation of gonadotropes and modulator of genes for growth hormone and prolactin) and ESRRG (which corresponds to a variety of receptors such as FSHr, GABAr1, and also regulates NMDAR2B). In addition, genes involved with energy balance were included and tested in the matrix, and it was found that PPARG (regulator of energy balance) showed a strong relationship with ESRRG confirming the relationship between energy status of the animal and the genes modulating puberty and reproductive processes. Interestingly, these genes are also been identified as candidates for age at menarche in humans; suggesting that genetic control of puberty is well conserved among species. While in the future it will be possible determine which genes are involved in the activation of the reproductive axis commercial SNPs assays are being used to select for reproductive traits in the field in Angus (McClure et al., 2010) and Holstein (Huang et al., 2010) cattle with successful results.

The somatotropic axis and precocious activation of the reproductive axis

Somatotropic axis

The somatotropic axis is an important complex of organs and hormones that form a system that regulates growth and adipose tissue mobilization, mammary development and
a variety of other physiological functions related to growth and metabolism (Renaville et al., 2002). Metabolic hormones that are regulated by the somatotropic axis have also been demonstrated to regulate aspects of reproductive performance. For example, growth hormone (GH), insulin-like growth factor -I (IGF-I), and IGF binding proteins (IGFBP) are involved in the regulation of follicular growth and development (Webb et al., 1999; Renaville et al., 2002). During the post partum anestrous period, the resumption of ovarian activity is a function of energetic balance and the impact of metabolic hormones on the reproductive axis (Wetteman and Bossis, 2000). Additionally, several nutritionally regulated factors or hormones such as opioids (β-endorphin, poopiomelanocortin), NPY, leptin, glucose and insulin discussed previously) serve as messengers (positive and negative feedback controls) that may regulate the reproductive axis (Hess et al., 2005; Keisler and Lucy, 1996).

The IGF system is necessary for a normal growth and development (Clemmons, 1998). IGF-I is a potent mitogen synthesized and secreted by the liver in response to growth hormone (Yakar et al., 1999). This hormone plays an important role in regulating ovarian events. In a review, Zulu et al., (2002) reported that the effect of the IGF -I can be found in different organs and tissues in the reproductive axis such as the granulosa and theca cells in the ovaries and in the median eminence. In the ovary, IGF-I increased proliferation and differentiation of granulosa cells and promoted synthesis of steroid hormones through stimulating the aromatase enzymatic system (Zulu et al., 2002). However, the function and effect of IGF-I depends on the abundance of insulin-like growth factor binding proteins. These IGFBP regulate the amount of free IGF-I that is
available to receptors and transport IGF-I in the bloodstream and the existence of six different IFGBP has been reported in cattle (Clemmons, 1998; Schams et al., 1999). The primary IGF-I binding protein in blood of cattle is IGFBP-3, however every tissue expresses specific IGFB proteins to modulate the availability of the hormone at that site. Govoni et al., (2003) evaluated IGF-I, IGFBP-2 and IGFBP-3 in male and female Hereford calves during their first year of life. In the first 16 weeks of age, no differences were observed between male and female calves in the concentrations of IGF-I but concentrations were greater in males than females from the 17th week of age until 1 year of age. Concentrations of IGFBP-3 were greater in males than in females however, after 17 weeks of age concentrations of IGFBP-3 decreased and remained constant until concentrations of IGF-I reached a plateau. Concentrations of IGFBP-2 were greater in females than in males from 17 week to 1 year. No heifers had reached puberty by the conclusion of the study therefore correlations between puberty and IGF-I and GFBP concentrations could not be determined. Later, Govoni et al., (2004) manipulated IGF-I concentrations by administering exogenous bovine somatotropin (bST) to male and female Hereford calves from birth to one year of age. They reported that IGF-I concentration increased after bST treatment from 50 to 350 days. At 250 days of age concentrations of IGFBP-3 increased following administration of bST and at this age, an increase in growth rate was observed in animals receiving bST. The somatotropic axis appeared to be more responsiveness to exogenous treatment of bST at 250 days of age but reproductive performance was not evaluated
In pre-pubertal heifers, restricted feeding programs delayed the onset of puberty (Day et al., 1986). In fact, synthesis and secretion of IGF-I is decreased and GH concentrations remains unaltered in nutritionally restricted heifers (Breier et al., 1986, Breier, 1999), suggesting that decreased IGF-I concentrations are the result of the inability of the liver to express GHR in animals under nutritional restriction (Oldham et al., 1999; Radcliff et al., 2006). Hepatic GH receptor expression is positively correlated with the level of nutrition. Studies in dairy cattle under nutritional restriction have shown that the expression and abundance of the growth hormone receptor (GHR) is reduced (Rhoads et al., 2007). Additionally, the expression of GHR mRNA is decreased in the liver in postpartum cows with an energetic balance deficit (Jiang et al., 2005). Radcliff et al., (2006) reported that a decrease in dry matter intake reduced the expression of GHR 1A mRNA by 21 days postpartum in dairy cows. Rhoads et al., (2004) demonstrated that an insulin infusion in postpartum dairy cows increased liver GHR and IGF mRNA.

The relationship of IGF-I, GH and reproductive axis has been extensively investigated in cyclic cattle and nutritional restriction. Many models of restriction have been employed, for example, in beef cows under nutritional restriction, serum LH and IGF-I concentrations are low resulting in a prolonged anestrous period (Richards et al., 1991). Bossis et al., (2000) working with beef heifers, evaluated the effect of two different gain rates (1.5 kg/d and 0.6 kg/d) during re-alimentation in heifers that were in nutritionally-induced anestrous. Animals with the higher rate of gain resumed ovarian activity 23 days earlier than heifers with the low rate of gain. It was observed that a positive association
between increase of serum concentrations of IGF-I and estradiol was observed close to the resumption of ovarian activity, suggesting that IGF-I may be one of the possible metabolic signals that influence secretion of LH and ovarian function.

In an early attempt to elucidate the function of GH on the onset of puberty, Jones et al., (1991) evaluated changes in GH and IGF-I in four different breeds of cattle (Angus, Braford, Charolais and Simmental). Concentrations of IGF-I increased in the Angus heifers from day -56 to day 0 (onset of puberty) but not in other breeds. A decreased frequency of GH pulses occurred in Angus heifers from day -40 to day -17 before puberty, but in Charolais and Braford heifers, frequency of GH pulses increased. The inconsistency of response between breeds made interpretation of these findings difficult. Another approach used to study the effect of the somatotropic axis on puberty was the immunization against GH releasing factor (GHRF). Simpson et al., (1991) evaluated the effect of serial active immunization against GHRF in 6 month-old beef heifers. They demonstrated that in immunized animals, growth rate, ADG, and feed intake were lower but fatness was greater in immunized heifers. Concentrations of IGF-I at 253 days of age were lower and frequency and amplitude of GH pulses were lesser in the immunized than non-immunized heifers. By 12.9 months of age, 81% of the non-immunized and 40% of immunized heifers were pubertal. At 18.5 months of age, only 45% of the immunized heifers had reached puberty. The impact of GHRF immunization beginning at 104 days of age on a variety of reproductive characteristics was investigated by Schoppee et al., (1996). Decreased IGF-I concentrations were detected by 169 days of age in the
immunized heifers. At 6 months of age, serum estradiol concentrations and intra-follicular concentrations of IGF-I were lower in immunized than non-immunized heifers. By 393 days of age, 32% of immunized heifers had reached puberty whereas 71% of non-immunized heifers were pubertal by this time. These authors suggested that the deficiency in IGF-I induced by immunization against GHRF impaired the ability of ovarian follicles to synthesize estradiol and thereby delayed puberty. Stanko et al., (1994) characterized the secretion pattern of GH, IGF-I and IGFBP2 in response to a 52 day treatment (starting at 18.1 months of age) with bovine somatotropin (bST) in 11 heifers that remained pre-pubertal in response to serial immunization against GHRF beginning at 6 months of age. Treatment with bST did not induce puberty, even though GH and IGF-I were increased and IGFBP-2 concentrations decreased by bST administration.

Across reports in which pre-pubertal concentrations of IGF-I have been evaluated, age at puberty has been demonstrated to be negatively associated with concentrations of IGF-I. Radcliff et al., (1997; 2004) also demonstrated that precocious puberty was associated with increased concentrations of IGF-I and decreased concentrations of IGFBP-2 when high energy diets were fed to heifers at 3 months of age. Consistent with these data, Gasser et al., (2006 a, b, c) demonstrated that feeding of a high energy diet beginning at three months of age enhanced follicular development and induced precocious puberty: although, IGF-I concentrations were not measured in these studies. When diets were manipulated later in the pre-pubertal period, Granger et al., (1989) observed that heifers fed the higher energy diet reached puberty earlier and had greater concentration of IGF-I. As mentioned before, Yelich et al., (1996) working with beef heifers from nine months of
age until the onset of puberty observed an association between feeding a high energy diet and greater concentrations of LH, IGF-I, and insulin during the first 84 days of treatment than the animals in the low gain treatment. These reports suggest that IGF-I may have a crucial role in determining age at puberty.

*Insulin-like growth factor – I (IGF-I) and ovarian physiology*

As discussed previously, somatotropic hormones (GH and IGF-I) have important roles controlling reproductive processes, and particularly several reports indicated the importance of the IGF-I in the ovary, controlling steroid synthesis, follicular development and ovulation (Diskin et al., 2003; Fortune et al., 2004; Lucy, 2000). In cattle it has been reported that the ovary synthesized IGF-I in the granulosa cells and IGF-II in the theca cells in response to systemic GH and also intra-follicular synthesis of IGF-I (Lucy, 2000). However the synthesis depends on the stage of the follicular growth and appears that IGF-I system (consisting of the hormone, receptor and binding proteins) are modulators of cell differentiation and survival of follicular cells (Guidice, 1992). In particular, IGF-I acts as stimulator of steroidogenesis and also potentiates the effect of the gonadotropins (Spicer et al., 1993, 1995). In fact, it has been demonstrated that IGF-I promotes the expression of FSH/LH receptors (Adashi, 1998) and also it has been reported these hormones also induce the synthesis of IGF-I in the granulosa cells (Spicer et al., 1995).
Regulation at follicular level of IGF-I availability is mediated by IGFBP-2 and -4 that is expressed in granulosa and theca cells (Guidice, 1992), however the synthesis of these IGFBPs depend on the follicular stage. In fact, follicles that are undergoing atresia contain more mRNA for IGFBP-2, thought to prevent binding of IGF-I in granulosa cells to avoid further differentiation of these cells (Armstrong et al., 1998). Greater intra-follicular synthesis of IGF-I stimulate proliferation and differentiation of granulosa cells and promotes estradiol synthesis in rats, humans and cattle. However in cattle also it has been reported that insulin is a potent stimulator of estradiol synthesis (Spicer et al., 1994). It is obvious that IGF-I concentrations can be associated with energy intake and puberty and this hormone has critical actions at the ovarian follicle that could represent the mechanism for induction of precocious puberty that we have previously observed. In summary, IGF-I is an important hormone that appears to modulate the follicular growth and parallel the endocrinological changes that occurs previous to the onset of puberty. Our proposed model for the link between metabolic status and activation of the reproductive axis in heifers is shown in figure 2.5. In heifers fed a diet with high energy content beginning at 3 – 4 months of age, hepatic GH receptor expression is stimulated and consequently concentrations of IGF-I are increased. Higher concentrations of IGF-I stimulate proliferation and differentiation of granulosa cells in the follicle and promote estradiol synthesis. Estradiol in turn, acts at the hypothalamic level to accelerate the decline in the negative feedback, resulting in more LH secretion, which further stimulates follicular maturation. This process progresses to the point that ovulation and precocious puberty occurs.
In conclusion, IGF-I hormones that regulate essential roles during growth and development of the follicle, therefore, more research is needed to determine the impact of nutrients on this micro-endocrine environment.

**Figure 2.5** Physiological mechanisms and changes on the IGF system that drive the onset of puberty in cattle (Adapted from Lucy M.C., 2000).
Statement of the problem

Age at puberty is an important reproductive trait in developing heifers due its direct influences on the reproductive performance of the animal during her productive life in the herd. Heifers that reached puberty before the breeding season have more opportunity to become pregnant and give birth early during the subsequent breeding season which might be advantageous in terms of management. However, the establishment of puberty requires maturation of the reproductive axis as well maturation of the reproductive organs. Early studies clearly demonstrated that nutritional status of the animal plays an important role in determining the onset of puberty and maturation of the reproductive organs such as the uterus and ovaries. However, the signal(s) associated with nutritional status that activates the reproductive axis to trigger puberty have not been identified.

The somatotropic axis and its relationship with feed intake and growth regulates rate of maturation of the reproductive axis. The hormone IGF-I, plays a role in a variety of reproductive processes, particularly follicular function, and greater concentrations are consistently linked with earlier age at puberty. We have previously shown that feeding a high-concentrate diet beginning at 3 to 4 months of age will induce precocious puberty in heifers, and is the result of premature activation of the cascade of endocrine events that induce the first ovulation, including increased LH secretion and follicular growth and
systemic estradiol concentrations and a hastened decline in the negative feedback of estradiol to the hypothalamus.

Nonetheless, the signal related to this feeding regimen that reduces age at puberty from 12 to 8 months is unknown. Furthermore, it is unknown whether this signal is driven by the metabolic hormones that influence the activation of reproductive tissues. Due to the magnitude of the effect of nutrients on premature activation of the reproductive axis, the present series of studies were design to identify some of the nutrients and mechanisms associated with the onset of precocious puberty in beef heifers.

Results from these experiments will reveal new insights about the effect of the diet on physiological changes that occur before the onset of puberty as well molecular aspects of the activation of follicular development by the influence of diet. A potential application of the results would be the implementation and design of diets that promote and stimulate follicular development, thereby regulating timing of puberty to increase the productivity of the beef cattle through decreasing the cost of heifer development. This will provide economic stimulus to the beef cattle industry. Most importantly, the study will use a unique animal model to begin to determine how nutritional signals, via their influences on the somatotropic axis, may regulate activation of the reproductive axis.
CHAPTER 3

Effect of differing dietary starch on precocious maturation of the reproductive and somatotropic axis in beef heifers.

Abstract

Feeding a high concentrate diet to heifers weaned at 3 months of age (early-weaned) induces precocious puberty (before 300 d of age) in a high proportion of animals. The objective of the present experiment was to test the influence of feeding either a high or low starch diet to early-weaned heifers on age and body weight (BW) at puberty and to assess the impact of these diets on metabolic and reproductive hormones. Heifers (n = 33) weaned at 76.5 ± 1.1 d of age received 1 of 3 diets beginning at 98 ± 1.1 d of age. The control diet (CONT, n = 11) was hay-based and targeted for 0.75 kg/d ADG. Experimental diets, targeting for 1.5 kg/d ADG but differing in starch (S) content were designated as HIGH-S (46.4% starch, n = 10) and LOW-S (14.48% starch, n = 12). Blood samples were collected weekly and analyzed for progesterone. In addition, IGF-I, and GH concentrations were determined at 174, 184, 212, 239, 269, 297 and 323 d of age. Blood samples collected 0, 1, 2, 3, 4, and 6 h post feeding (onset of feeding = hour 0) at
239 d of age were analyzed for insulin and glucose concentrations. The LOW-S and HIGH-S treatments did not differ in ADG (1.21 ± 0.34 vs. 1.27 ± 0.39 kg/d respectively) and were greater (P < 0.05) than the CONT treatment (0.77 ± 0.41 kg/d). Concentrations of insulin did not differ between treatments at hour 0 but were greater in the HIGH-S and LOW-S than the CONT treatment from hour 1 to 4 (treatment by hour interaction, P = 0.06) while glucose concentrations did not differ among treatments from hour 0 to 6. Concentration of GH in the CONT treatment was greater (P < 0.05) at 212 days of age compared to LOW-S and HIGH-S treatments, and by 297 days of age, the HIGH-S treatment had lesser GH concentrations than the LOW-S treatment. Concentrations of IGF-I were greater (P < 0.05) in the HIGH-S and LOW-S than in the CONT treatment from d 174 to 239, were greater (P < 0.05) in the HIGH-S than LOW-S on d 212 and 269, and did not differ between treatments on d 297 or 323. Precocious puberty was induced in 42% (5/12) and 60% (6/10) of heifers in the LOW-S and HIGH-S and in 0/11 heifers in the CONT treatment. Age and BW at puberty of heifers that reached precocious puberty were similar among high energy treatment HIGH-S; 260 ± 20 d of age; and LOW-S, 269 ± 24 d of age and BW, at puberty was similar among diets, HIGH-S, 334.9 ± 33.3 kg; LOW-S, 348.3 ± 30.1 kg. Across the heifers fed the HIGH diet (HIGH-S and LOW-S), heifers that experienced precocious puberty had greater (P < 0.05) IGF-I concentrations from d 174 to 239 than those that did not have precocious puberty, but GH concentrations did not differ between these groups. These data suggest a role of IGF-I in precocious puberty and support the conclusion that high dietary starch is not essential to induce precocious puberty in early weaned heifers.
Sexual maturation and consequently the onset of puberty involve a series of metabolic and endocrine changes that leads to the activation of the reproductive axis (Day and Anderson, 1998). However, the specific nutritional signals that drive the occurrence of puberty and more specifically, the relationship between specific nutrients and the reproductive axis, are still unclear.

Somatotropic hormones have been implicated in this process, and particularly an increased concentration of insulin–like growth factor I (IGF-I) has been associated with puberty (Lucy, 2000; Radcliff et al., 1997, 2004). Data from our laboratory indicate that the cascade of endocrine events that culminate in puberty can be hastened by increased energy intake between 3 and 7 months of age (Gasser et al., 2006a, b, c, d). In these experiments, it was demonstrated that a majority of heifers weaned between 2.5 to 4 months of age and fed a diet that was concentrate-based and formulated for BW gains of 1.5 kg/d had precocious puberty (onset of puberty before 300 days of age). Heifers fed the high concentrate diet at this early age, showed greater LH pulse frequency at 190 days of age (Gasser et al., 2006a), greater diameter of the dominant follicle beginning at 196 d of age (Gasser et al., 2006b), and animals that experienced precocious puberty had a larger dominant ovarian follicle accompanied with a longer period of dominance, greater estradiol concentrations, and a greater duration of follicular waves (Gasser et al., 2006b). Additionally, Gasser et al., (2006c) demonstrated that precocious puberty is associated
with an early decline in estradiol inhibition of LH secretion preceding occurrence of
precocious puberty.

We hypothesized that the high starch content of the diet in the work of Gasser (2006 a, b, c, d) was responsible for activation of the reproductive axis and precocious puberty through actions to enhance IGF-I concentrations. To test this hypothesis, we performed an experiment with the objective to compare incidence of precocious puberty, IGF-I, GH, insulin and glucose concentrations between heifers fed two diets that resulted in similar and rapid BW gains, but that differed greatly in starch content when initiated at approximately 3 months of age in early weaned beef heifers.
Materials and methods

All animal handling and experimental procedures described were approved by The Ohio State University Agricultural Animal Care and Use Committee (protocol #08-AG012).

Animals and treatments

Thirty-three beef heifers were weaned at 76.5 ± 1.09 d of age and 126.7 ± 6.71 kg. Heifers were fed a receiving diet (17.2% CP, 1.93 Mcal/kg NEm and 1.29 Mcal/kg NEg) for two weeks. After the receiving period, heifers were divided randomly into three groups and were transitioned onto one of three experimental diets for a period of seven days beginning at 91 ± 1.1 d of age. Experimental diets were fed from 98 ± 1.1 d of age through 310 ± 1.1 d of age and consisted of a high starch diet (HIGH-S, n = 10), low starch diet (LOW-S, n = 12), and a control diet (CONT, n = 11) (Table 3.1). Diets fed in the HIGH-S and LOW-S treatment were formulated to achieve an ADG of 1.50 kg/d. The CONT diet was formulated for BW gains of 0.75 kg/d. Body weight was assessed every 2 weeks beginning at 76 days of age and ending at 345 days of age for all heifers. All diets were initially fed at a rate of 2.5% of BW on an “as fed” basis. Amount of feed offered was adjusted biweekly based upon BW gain during the preceding two weeks. The amount of feed as a proportion of body weight that was fed was also adjusted within a range of 2.5% to 3.5% of BW, within treatment, to attempt to sustain targeted ADG within that treatment.
Sample collection

Blood samples were collected via jugular venipuncture once weekly, beginning at 174 days of age and finishing at 345 days of age to assess progesterone concentration and determine luteal activity. Blood samples were collected in K2-EDTA vacutainer tubes (BD, New Jersey, USA) and centrifuged at 2,785 x g for 20 min, plasma harvested, and samples frozen at -20°C until analyzed. Age at puberty was defined as 7 days before the date of collection of the first blood sample that contained > 2 ng/ml progesterone or 7 days before the date of collection of the first of 2 consecutive weekly blood samples with each having >1 ng/ml progesterone. A heifer was classified as having precocious puberty if this date was before 300 d of age. As puberty was confirmed in an individual heifer, evidenced by continue luteal phase activity after the initial phase, they were removed from the experiment.

Samples to be analyzed for IGF-I, and GH concentrations were collected at 174, 184, 212, 239, 269 and 297 days of age with the same K2 EDTA tubes and the same procedure described for progesterone samples.

Additionally, heifers were randomly selected within each treatment at 226 d of age (CONT n = 5, HIGH-S n = 4 and LOW-S n = 4) and serial blood samples were collected to assess patterns of LH secretion at 226, 253 and 281 d of age. The same heifers were sampled at each time point. Heifers were fitted with indwelling jugular catheters and blood samples were collected at 15-min intervals for 12 h. Blood samples for LH analysis
were allowed to clot for 48 hours at 4°C, and then centrifuged at 2,785 x g for 20 min, serum was harvested, and samples frozen at -20°C until analyses were performed. Concomitant with the serial blood sample collection at 239 days of age, samples to assess concentrations of insulin and glucose in response to feeding of the diet were collected. Blood samples were collected just before feed was offered (designated as hour 0) and at hour 1, 2, 3, 4 and 6. Samples for insulin analysis were collected in K2 EDTA Vacutainer tubes (BD, New Jersey, USA) whereas glucose samples were collected in sodium fluoride/potassium oxalate vacutainer tubes. In both cases, samples were centrifuged at 2,785 x g for 20 min, plasma harvested, and samples frozen at -20°C until analyzed.

_Hormone Analyses_

Concentrations of progesterone in plasma were analyzed using a commercial radioimmunoassay analysis (RIA) kit (Coat-a-Count, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) according to the procedures validated in our laboratory by Burke et al., (2003). The inter-assay CV for the low pool (0.15 ng/ml) was 4.64%, medium pool (1.0 ng/ml) was 5.57% and high pool (3.0 ng/ml) was 3.46%. The intra-assay CV was 4.62% and the average sensitivity was 0.78 ng/ml. Quantification of IGF-I concentrations were performed in duplicate in one assay from samples at 174, 184, 212, 239, 269 and 297 days of age using a validated RIA in Dr. Dennis Hallford’s lab (Shirley et al., 2001). The intra-assay CV was 4.8% and inter-assay CV 14.8% for the IGF-I analyses. Concentration of LH was determined in duplicate for all serial samples.
using a double-antibody system previously validated in our laboratory (Anderson et al., 1996). The intra-assay CV was 3.0% and inter-assay for low (1.6 ng/ml) and high (5.5 ng/ml) pools were 15.0 and 17.9%, respectively. The sensitivity of the assay was 0.17 ng/ml. A LH pulse was defined as an increased concentration that occurred within two samples of the previous nadir, and an increase that exceeded two standard deviations of the assay; LH pulse amplitude was defined as the concentration of LH at the peak minus the concentration at the previous nadir and mean LH concentrations was determined as the average of the LH concentration during the 12 hours of sampling (Goodman and Karsch, 1980).

Growth hormone was assessed by RIA using a double antibody system (Apendix A). Briefly, purified growth hormone (AFP9884C) as well the first antibody (AFPB55) were obtained from the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA, USA). The second antibody (IR-HU-IGG, Goat anti human IgG antiserum) was obtained from Innovative Research (Novi, MI). Iodination of purified GH was performed using the chloramine-T method, and validation was performed via parallel displacement of labeled hormone by serial dilutions of bovine plasma samples (see appendix A). The inter-assay CV for a low pool (5.0 ng/ml) was 1.89%, and for a high pool (40.0 ng/ml) was 7.81%. The intra-assay CV was 4.85% and the average sensitivity was 1.39 ng/ml. Finally, concentration of insulin in plasma was determined using a RIA following the procedures by Benson and Reynolds (2001) and validated in our lab (Relling and Reynolds, 2007). All insulin samples were in duplicate and analyzed in a
single assay. The intra-assay CV was 7.5%. Glucose concentration was determined using an enzymatic colorimetric procedure (#1070 Glucose Trinder, Stanbio Laboratory, Boerne, TX). The average inter-assay CV was 4.10% for a pool containing 69.2 mg/dl. The average intra-assay CV was 1.81%.

Statistical analysis

The effect of treatment, days and the interaction of these variables on body weight, mean LH concentration, LH pulse frequency and amplitude, mean concentration of IGF-I, GH, insulin, and glucose were analyzed by ANOVA using the MIXED procedure in SAS (2004) with a compound symmetry covariance structure included in the model with repeated measures analysis included in the model. The model used was:

\[ Y_{ijk} = \mu + T_i + h_{j:i} + D_k + (TD)_{ik} + \epsilon_{ijk}, \]

where:

- \( Y_{ijk} \): Observation of the \( j \)th animal in the \( i \)th treatment on the \( k \)th day
- \( \mu \): grand mean
- \( T_i \): \( i \)th treatment
- \( h_{j:i} \): random effect of the \( j \)th animal within the \( i \)th treatment (\( h_{j:i} \sim N[0, \sigma^2_h] \))
- \( D_k \): \( k \)th day
- \( (TD)_{ik} \): interaction between treatment and day
- \( \epsilon_{ijk} \): error

Overall average daily gain, body weight and age at puberty among treatments (heifers from HIGH-S and LOW-S that did not reached precocious puberty were not included in the analysis) were analyzed with a PROC GLM procedure of SAS using animal within treatment as the error term. The model used was:

\[ Y_{ijk} = \mu + T_i + h_{j:i} + \epsilon_{ij}, \]

where:
Cumulative percentage of animals becoming pubertal for each treatment was analyzed with a survival curve test by the PROC LIFETEST procedure in SAS (2004).

Finally, to further characterize the relationships of precocious puberty, IGF-I and GH, heifers from the HIGH-S and LOW-S treatments were grouped into those that experienced puberty before 300 days of age (PREC) and those that did not (NON PREC). The effect of group (PREC and NON PREC), days and the interaction of these variables on IGF-I and GH concentration were analyzed by ANOVA using the MIXED procedure in SAS (2004) with a compound symmetry covariance structure included in the model with repeated measure analysis. The model used was:  

\[ Y_{ijk} = \mu + T_i + h_{j:i} + D_k + (TD)_{ik} + \varepsilon_{ijk}, \]

where:

\( Y_{ijk} = \) Observation of the \( j \)th animal in the \( i \)th treatment and the \( k \)th day; \( \mu = \) grand mean, \( T_i = \) \( i \)th treatment, \( h_{j:i} = \) random effect of the \( j \)th animal within the \( i \)th treatment (\( h_{j:i} \sim N(0, \sigma^2_h) \)), \( D_k = k \)th day, \( (TD)_{ik} = \) interaction between treatment and day, \( \varepsilon_{ijk} = \) error

Results are presented as means ± SE.
Results

Overall ADG and BW in the LOW-S and HIGH-S treatments did not differ (1.21 ± 0.34 kg/d vs. 1.27 ± 0.39 kg/d, respectively; Figure 3.1) and ADG in these treatments was greater (P < 0.05) than in the CONT treatment (0.77 ± 0.41 kg/d). Body weight was greater (P < 0.01) by 170 days of age in the LOW-S and HIGH-S treatments than the body weight observed in the CONT treatment.

No animals in the CONT treatment reached precocious puberty, whereas 42% (5/12) and 60% (6/10) in the LOW-S and the HIGH-S treatments, respectively, reached puberty by 300 d of age (Figure 3.2). Two heifers in the LOW-S treatment were pubertal at the initiation of blood collection for progesterone concentrations at 174 d of age and 174 d of age was assigned as age at puberty in these females. At 345 d of age (cessation of blood sample collection for progesterone analyses), 80% (8/10) of HIGH-S, 66.6% (8/12) in the LOW-S and 9% (1/11) in the CONT treatment were pubertal (Figure 3.2). Average age at puberty of heifers that reached precocious puberty (before 300 d of age) in the HIGH-S and LOW-S was 260 ± 20 d and 269 ± 24 d, respectively. Likewise, BW at puberty was similar among heifers that reached precocious puberty in the HIGH-S and LOW-S (334.9 ± 33.3 kg, and 348.3 ± 30.1 kg, respectively).

The acute response of insulin to dietary treatment is shown in figure 3.3. Concentrations did not differ between treatments at hour 0, but were lesser (P < 0.05) in the CONT than HIGH-S and LOW-S treatments at hour 1, 2, 3 and 4 (treatment by hour, P < 0.03). Insulin concentrations did not differ between the HIGH-S and LOW-S treatments. Blood
glucose concentration did not differ among treatments (Figure 3.4) at any point during the 6 hours of blood collection after feeding.

Concentrations of IGF-I were greater (P < 0.05) in both the HIGH-S and LOW-S treatments than in the CONT treatment throughout a majority of the period preceding occurrence of precocious puberty (174 to 239 d of age; treatment by age, P < 0.01; Figure 3.5). Later, as heifers were removed due to attainment of puberty, differences between the CONT and HIGH-S and LOW-S treatments were varied, and were eventually non-existent by 297 days. Comparison of heifers that did, and did not experience precocious puberty, across the HIGH-S and LOW-S treatments revealed that heifers that had precocious puberty had greater (P < 0.05, treatment by age, P < 0.05) IGF-I concentrations from d 174 to 239 than those that did not reach precocious puberty (Figure 3.6). Concentrations of GH in the CONT treatment were greater (P < 0.05) than the LOW-S and HIGH-S at 212 and 297 d of age (treatment by age interaction P < 0.05). Concentrations of GH did not differ between the precocious and non-precocious heifers (data not shown).

Mean LH concentration, LH pulse frequency and amplitude of LH pulses did not differ between treatments in the sub-group of heifers sampled for this purpose (Table 3.2). Precocious puberty was not observed in any of the heifers that were sampled for LH.
Discussion

No animals in the CONT treatment reached precocious puberty, whereas 60% and 42% in the HIGH-S and the LOW-S treatments reached puberty by 300 d of age. Concentrations of IGF-I were greater in both the HIGH-S and LOW-S treatments than in the CONT treatment throughout the period of time preceding precocious puberty in these treatments. Comparison of heifers that did, and did not experience precocious puberty, across the HIGH-S and LOW-S treatments revealed that heifers that had precocious puberty had greater IGF-I concentrations. We theorized that precocious puberty was linked with the IGF-I response that was driven by metabolites of starch digestion. Since similar proportions of heifers in the high and low starch diets had precocious puberty, we not accept our hypothesis and conclude that precocious puberty is a result of the accelerated growth rate achieved with a high energy diet, rather than signals provided by starch content of the diet. In addition, while, minor differences in IGF-I were noted due to starch content, it appears this response is driven predominantly by energy, rather than starch content of the diet. The most compelling result of this experiment was that in heifers that had precocious puberty, IGF-I was greater than in heifers fed similar diets that did not reach precocious puberty.

Dry distillers grain with solubles (DDGS) are a by-product of corn use for ethanol production (Klopfenstein et al., 2008). Due to this process the majority of starch is removed. Additionally, soybean hulls (a by-product of the soybean processing industry)
has been used widely substituting corn in cattle diets, with a maximum of 2.9% of starch content (Poore et al., 2002; Ipharraguerre and Clark, 2003). In the present experiment, the high energy diet with low starch content was achieved through feeding a diet consisting predominantly of DDGS and soybean hulls. Recent findings relative to the impact of these co-products on propionate and glucose suggest that starch content of diets derived by chemical analyses may not accurately reflect the impact of the diet on propionate production by the rumen and subsequent impacts on gluconeogenesis. The majority of emphasis of recent studies has been placed on the relative impact of DDGS as compared to traditional high starch feed components. For example, DDGS induce the same amount of propionate as soy bean meal in lactating dairy cows (Christen et al., 2010). Moreover, in a recent study conducted in beef cows during the late gestation period, Radunz et al., (2010) observed that glucose concentration did not differ in cows fed corn vs. DDGS. Consistent with these finding by Radunz et al., (2010), no differences in insulin and glucose were observed in heifers fed high energy diets composed in one case primarily of corn, and in the other case DDGS and soybean hulls. Hence, it is possible that in the present study differences in putative signaling due to systemic glucose/propionate synthesis in circulation were much less than reflected by the chemical analysis of starch content of the diet. This putative action of gluconeogenic precursors on age at puberty has previously been proposed in experiments using monensin infusion (Lalman et al., 1993) to enhance glucose concentration, this approach caused an increase the amount of propionate and a reduction of age at puberty. In fact, Susin et al., (1995b) showed that lambs fed with corn grain had higher ruminal concentration of propionate and lesser
concentration of acetate, however age at puberty was similar among lambs fed high grain and lambs fed high forage. Furthermore, Susin et al., (1995a) demonstrated that feeding a high grain diet promoted increased concentration of insulin in lambs compared to the high forage diet, however diets (high grain diet or high forage diet) did not differ in the concentration of glucose. Finally, recently it was demonstrated by Felix (2011) that DDGS fed up to 60% to lambs resulted in decreased digestion of dry matter and fat which affected the productive performance (final BW, ADG and gain: feed ratio) of lambs on feedlot conditions. Therefore, site of digestion of the starch impact greatly the efficiency of cattle, therefore, when starch is processed in the rumen instead of the small intestine raises the question of whether the present experiment fully tested the impact of high and low starch on precocious puberty.

Gasser et al., (2006a, b, c and d) demonstrated that heifers weaned at 2 to 3 months of age and fed a diet with a high energy and starch content, had an earlier age at puberty compared to heifers fed a roughage-based diet that supported gains similar to those that remained with their dams. With the exception of Gasser et al., (2006a), BW at puberty was similar between heifers fed the high energy and control diets (Gasser et al., 2006 b, c and d). In the present study, BW at puberty did not differ between heifers fed the high energy diets. Because the lack of response of induction of puberty in the CONT treatment as only one heifer reached puberty by the termination of the experiment, body weight at puberty is only based on one measurement.
Radcliff et al., (1997, 2004) reported the influences of feeding a high energy diet to Holstein heifers beginning at 4 months of age on age at puberty and metabolic responses. When compared to the control ration, age at puberty was decreased by 47 days (high 266 d and control 313 d of age), similar to results reported by Gasser et al., (2006a, 2006 b, 2006c and 2006d). In the study of Radcliff et al., (1997) the high energy contained a high proportion of ground corn (75%) and it enhanced peripheral IGF-I concentrations within 100 days after starting the diets but concentrations of GH were unaffected by diet. They suggested a strong association between precocious puberty and IGF-I concentrations. This relationship has been suggested with other dietary feeding regimens. For example, Yelich et al., (1996) demonstrated that IGF-I concentrations were increased by a high energy diet compared to a low energy diet when initiated at 9 months of age, and puberty occurred earlier in heifers fed a high energy. In fact, a recent report indicated an increase of IGF-I concentration as puberty approached (Velazquez et al., 2008). In the present study, IGF-I was first measured at 174 days of age and it was observed that IGF-I concentrations were already greater in heifers fed high energy than the control diet and largely unaffected by the starch content of the diet. Since approximately 50% of the heifers fed on the two high energy diets had precocious puberty, we also compared IGF-I between precocious and non-precocious heifers, and demonstrated that heifers that had precocious puberty had substantially greater concentrations of IGF-I preceding precocious puberty. This finding supports the idea that the extent of IGF-I response is related to whether or not precocious puberty is realized. Further research is needed to
determine if a threshold of IGF-I concentrations necessary to induce precocious activation of the reproductive axis exists in heifers.

Although not an objective of the present study, considerable evidence exists to suggest a mechanism for IGF-I to stimulate earlier puberty. The target of IGF-I most studied in cattle is the ovarian follicle. It is known that IGF-I in the follicle increases proliferation and differentiation of granulosa cells and promotes the synthesis of steroid hormones by stimulating the aromatase enzymatic system (Zulu et al, 2002). Spicer et al., (2000) observed that intra-follicular injection of IGF-I increased estradiol production. Furthermore, across many reports (Yelich et al., 1996; Radcliff et al., 1997; Granger et al., 1989) in which a variety of models have been used to assess IGF-I concentrations and age at puberty, increased IGF-I concentrations were associated with earlier puberty. More specific to a putative mechanism of action, Schoppee et al., (1996) reported that when lower concentrations of IGF-I were experimentally induced by immunization against growth hormone releasing factor or chronic feed restriction beginning at 3.5 months of age, follicular development at 6 months of age was impaired and puberty was delayed. Therefore the action of enhanced IGF-I concentrations in precocious heifers in the present experiment may have been to enhance follicular development that lead to a cascade of events resulting in precocious puberty. Accordingly, Gasser et al., (2006 b) showed that the high energy enhanced follicular development within 75 days of treatment. Further analysis between heifers that did and did not reach precocious puberty, showed that follicular wave length, maximum diameter and number of follicles differed
among these heifers. Therefore, our current hypothesis is that augmentation of pre-pubertal follicular development as a result of increased IGF-I concentrations is the initial mechanism by which precocious puberty is induced in our early weaning, high energy model in beef heifers.

In conclusion, heifers weaned early and fed either a high or low starch diet showed increased concentration of IGF-I that induced precocious puberty in some heifers. These data suggest a role of IGF-I, but not GH, in the establishment of precocious puberty and support the conclusion that high starch from the diet is not essential to induce precocious puberty in early-weaned heifers.
Figure 3.1 Body weight (mean ± SE) of pre-pubertal heifers fed either a forage based control diet (CONT), or higher energy diets containing either a low-starch (LOW-S) or high-starch content (HIGH-S). Body weight was greater in LOW-S and HIGH-S (P < 0.05) than CONT treatment from 170 days of age until the end of the evaluation (treatment by age, P < 0.001). *groups differ within age (P < 0.05), **groups differ within age (P < 0.10).
Figure 3.2 Cumulative percentage of pubertal heifers fed either a forage based control diet (CONT), or higher energy diets containing either a low-starch (LOW-S) or high-starch content (HIGH-S). At 345 d of age (cessation of blood sample collection for progesterone analyses), 80% (8/10) of HIGH-S heifers were pubertal, 66.6% (8/12) (P < 0.05) in the LOW-S and 9% (1/11) in the CONT treatment.
Figure 3.3 Plasma concentration of insulin (mean ± SE) during a 6-h intensive sampling period at 239 days of age in heifers fed either a forage based control diet (CONT), or higher energy diets containing either a low-starch (LOW-S) or high-starch content (HIGH-S). Insulin concentration was lesser in CONT treatment compared to LOW-S and HIGH-S from hour 1 to hour 4 (treatment by hour interaction, P = 0.03) *CONT treatment differ from LOW-S and HIGH-S within hour (P < 0.05).
Figure 3.4 Glucose concentration (mean ± SE) during a 6-h intensive sampling period at 239 days of age in heifers fed either a forage based control diet (CONT), or higher energy diets containing either a low-starch (LOW-S) or high-starch content (HIGH-S).
Figure 3.5 Serum concentration (mean ± SE) of IGF-I in heifers fed either a forage based control diet (CONT), or higher energy diets containing either a low-starch (LOW-S) or high-starch content (HIGH-S). Diet by age interaction (P < 0.01). Different letters within age differ P < 0.05.
Figure 3.6 Serum concentration (mean ± SE) of IGF-I of heifers fed high energy diets containing either a low-starch (LOW-S) or high-starch content (HIGH-S) that reached precocious puberty (PREC; before 300 days of age) and heifers that did not reach precocious puberty (NON PREC); regardless of treatment. Group by age interaction (P < 0.05). Different letters within ages differ P < 0.05.
Table 3.1 Composition and chemical analysis of experimental supplements fed to heifers (% as fed).

<table>
<thead>
<tr>
<th>Item</th>
<th>HIGH-S</th>
<th>LOW-S</th>
<th>CONT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole shelled corn</td>
<td>50.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Orchard grass hay</td>
<td>30.00</td>
<td>23.00</td>
<td>70.00</td>
</tr>
<tr>
<td>DDGS</td>
<td>37.00</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Soy hulls</td>
<td>-</td>
<td>20.00</td>
<td>-</td>
</tr>
</tbody>
</table>

**Supplement**

<table>
<thead>
<tr>
<th>Item</th>
<th>HIGH-S</th>
<th>LOW-S</th>
<th>CONT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ground corn</td>
<td>4.10</td>
<td>17.80</td>
<td>17.90</td>
</tr>
<tr>
<td>Limestone</td>
<td>1.40</td>
<td>1.30</td>
<td>1.00</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>13.14</td>
<td>-</td>
<td>10.00</td>
</tr>
<tr>
<td>Urea</td>
<td>0.46</td>
<td>-</td>
<td>0.20</td>
</tr>
<tr>
<td>Trace mineral salt</td>
<td>0.50</td>
<td>0.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Vitamin A</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
</tr>
<tr>
<td>Vitavet Selenium</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>A-V Blend</td>
<td>0.30</td>
<td>0.30</td>
<td>0.30</td>
</tr>
</tbody>
</table>

**Chemical analysis**

<table>
<thead>
<tr>
<th>Item</th>
<th>HIGH-S</th>
<th>LOW-S</th>
<th>CONT</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP, %</td>
<td>15.99</td>
<td>16.14</td>
<td>12.85</td>
</tr>
<tr>
<td>Starch,%</td>
<td>46.40</td>
<td>14.48</td>
<td>19.03</td>
</tr>
<tr>
<td>Fat,%</td>
<td>4.03</td>
<td>6.43</td>
<td>3.73</td>
</tr>
<tr>
<td>NEm, Mcal/kg</td>
<td>1.830</td>
<td>1.836</td>
<td>1.398</td>
</tr>
<tr>
<td>NEg, Mcal/kg</td>
<td>1.198</td>
<td>1.197</td>
<td>0.813</td>
</tr>
</tbody>
</table>

HIGH-S=high-starch diet, LOW-S=low-starch diet, CONT=control diet.
Table 3.2 LH characteristics (mean ± SE) at different ages in heifers fed either a forage based control diet (CONT), or higher energy diets containing either a low-starch (LOW-S) or high-starch content (HIGH-S).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Age (d)</th>
<th>n</th>
<th>Mean LH, ng/ml</th>
<th>Average number of peaks/12 h</th>
<th>Magnitude of LH pulse, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONT</td>
<td>226</td>
<td>5</td>
<td>0.50 ± 0.03</td>
<td>2.20 ± 0.20</td>
<td>1.23 ± 0.30</td>
</tr>
<tr>
<td></td>
<td>253</td>
<td>5</td>
<td>0.54 ± 0.03</td>
<td>2.80 ± 0.86</td>
<td>1.05 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>281</td>
<td>5</td>
<td>0.60 ± 0.03</td>
<td>2.40 ± 0.40</td>
<td>1.54 ± 0.23</td>
</tr>
<tr>
<td>LOW-S</td>
<td>226</td>
<td>4</td>
<td>0.58 ± 0.06</td>
<td>4.00 ± 1.08</td>
<td>1.96 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>253</td>
<td>4</td>
<td>0.80 ± 0.06</td>
<td>3.75 ± 0.85</td>
<td>1.59 ± 0.33</td>
</tr>
<tr>
<td></td>
<td>281</td>
<td>4</td>
<td>0.89 ± 0.08</td>
<td>3.50 ± 0.64</td>
<td>2.44 ± 0.58</td>
</tr>
<tr>
<td>HIGH-S</td>
<td>226</td>
<td>4</td>
<td>1.03 ± 0.06</td>
<td>2.75 ± 0.25</td>
<td>2.20 ± 0.57</td>
</tr>
<tr>
<td></td>
<td>253</td>
<td>4</td>
<td>0.74 ± 0.03</td>
<td>2.50 ± 0.95</td>
<td>1.24 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>281</td>
<td>4</td>
<td>0.85 ± 0.04</td>
<td>2.75 ± 0.62</td>
<td>1.47 ± 0.20</td>
</tr>
</tbody>
</table>
CHAPTER 4

Effect of a high-energy diet on steroidogenic capacity of dominant follicles, secretion of LH and estradiol, and precocious puberty in beef heifers.

Abstract

The objectives of this experiment were to evaluate the impact of feeding a high-energy diet, beginning at 3 months of age in beef heifers on estrogenic capacity of the dominant follicle and changes in systemic hormone concentrations. Twenty heifer calves were weaned at 66 ± 2 d of age, and randomized to receive one of two diets beginning at 86 ± 2 d of age and 137 ± 3.3 kg body weight, (BW). Diets were iso-nitrogenous (14.1% crude protein) and contained either a high-energy content (HIGH; 2.2 Mcal/kg NEm and 1.37 Mcal/kg NEg; n = 10) or a lesser-energy content (control diet; CONT; 1.70 Mcal/kg NEm and 1.09 Mcal/kg NEg; n = 10) and were fed until 301 days of age. Target rates of gain were 1.50 kg/d for the HIGH and 0.75 kg/d for the CONT treatment. Within diet treatments, heifers were randomly assigned to be hemi-ovariectomized (OVX) or to remain intact (INT). In heifers to be OVX, daily ultrasonographic evaluation of ovarian
follicles was initiated at $155 \pm 4$ d of age, or at 70 d after initiation of diets and continued through two complete follicular waves. During the 3\textsuperscript{rd} wave after initiation of ultrasonography, the ovary bearing the dominant follicle was removed ($174 \pm 4$ d of age, d 0) one day after it achieved a diameter of 9 mm in order to assess intra-follicular estradiol (E2) and progesterone (P4) concentrations and granulosa/theca cell gene expression. Daily blood samples were collected during ultrasonography and systemic estradiol concentrations were evaluated in the OVX groups for the 11 days preceding ovariectomy. In the CONT-INT and HIGH-INT treatments, serial blood samples were collected at 15-min intervals for 12 hr at 145, 170, 208 and 236 days of age to assess secretion of LH. Weekly blood samples were collected during the feeding period on all heifers and analyzed for progesterone concentrations to determine age at puberty. An initial analysis for body weight changes over time, which included all heifers from each diet, demonstrated that ADG was greater ($P < 0.01$) in the HIGH ($1.57 \pm 0.71$ kg/d) than CONT treatment ($1.21 \pm 0.74$ kg/d). Body weight of the HIGH diet was greater ($P < 0.05$) than CONT diet at 186 d of age and thereafter (diet by age interaction, $P < 0.01$). Precocious puberty occurred in 50% (3/6) heifers in the HIGH-INT and 80% (4/5) heifers in the CONT-INT treatment. Differences were observed at age at puberty ($P < 0.05$) comparing HIGH-INT vs. CONT-INT, ($194.33 \pm 8.95$ d vs. $253.75 \pm 1.25$ d, respectively) and BW at puberty was different ($P < 0.02$) between HIGH-INT and CONT-INT ($257.7 \pm 17.02$ kg, $321.67 \pm 11.65$ kg, respectively). In the intact heifers, mean LH concentration was greater ($P < 0.05$) in the HIGH-INT than the CONT-INT treatment at 170 days and 208 days of age. In the OVX heifers, systemic estradiol
concentration was not different from d -11 and d -3; however, concentrations were greater (P < 0.05) in the CONT-OVX than HIGH INT on d -1 and d 0 (treatment by day interaction, P < 0.05). No differences between treatments were observed in wave length of the two follicular waves preceding ovariectomy. Progesterone and estradiol concentration in the follicular fluid were not different (P > 0.05) among treatments. Likewise, E2/P4 ratio was similar among treatments, HIGH-OVX (1.16 ± 0.57) vs. CONT-OVX (3.4 ± 1.7). Expression of the genes studied was not affected by diet, but were numerically greater in the HIGH-OVX than CONT-OVX treatment for insulin-like growth factor –I receptor (IGF-IR), luteinizing hormone receptor (LHR), and for steroidogenesis pathway enzymes: cholesterol side-cleavage chain (CYP11A1) and aromatase (CYP19A1). On the other hand, 100% (4/4) in the HIGH OVX and 60% (3/5) heifers in the CONT-OVX reached precocious puberty. However, no differences (P > 0.05) were observed at age at puberty (235.25 ± 11.9 d vs. 219.66 ± 10.9 d, respectively) and a tendency (P = 0.07) was observed for BW at puberty between HIGH-OVX and CONT-OVX (329.07 ± 13.17 kg, 285.43 ± 14.08 kg, respectively). In conclusion, the high energy diet increased body weight gain and LH secretion by 170 and 208 days of age and resulted in an earlier age at puberty in the heifers that were ovary intact. However, the incidence of precocious puberty did not differ between treatments. Intra-follicular concentrations of estradiol and progesterone as well as the ratio E2/P4 nor expression of IGF-IR, LHR, CYP11A1 and CYP19A1 differed between dietary treatments, indicating no direct dietary effects at the level of individual follicles from heifers ovariectomized at 174 ± 4 d of age. It is plausible that heifers in both diets
received metabolic signals that precociously activated the reproductive axis in this experiment as weight gains in the CONT treatment exceeded target ADG and further research is needed to determine if the initial step to precocious activation of the reproductive axis is driven by increased IGF-I enhancement of follicular function.
**Introduction**

Maturation of the reproductive axis in cattle occurs in a gradual fashion and is intimately linked with nutrition, growth, metabolism and somatotropic stimuli. The current understanding of the endocrinology of puberty is that it is driven by a decrease in estradiol negative feedback on secretion of gonadotropin releasing hormone (GnRH, Rodriguez and Wise 1989), leading to increased secretion of luteinizing hormone (LH) which drives final growth and maturation of ovarian follicles and culminates with ovulation (Kinder et al., 1987; Day and Anderson, 1998). The specific nutritional and/or somatotropic signals that activate this process are unclear at present.

In a series of experiments (Gasser et al., 2006a; b, c and d) demonstrated that onset of puberty is hastened by approximately 80 days when heifers were fed a high energy diet beginning at approximately 3 months of age. Advancement of the pre-pubertal increase in LH (Gasser et al., 2006a), the decline in estradiol negative feedback (Gasser et al., 2006c), and enhancement of follicular growth (Gasser et al., 2006b) occurred with this treatment regimen, leading to the conclusion that overall, the mechanisms that are typically activated during the peri-pubertal period (around 10 to 12 months of age) were advanced to reach puberty between 8 to 10 months. Interestingly, in Gasser et al., experiments, it appeared that the initial reproductive response to diet was expressed through enhanced follicular growth beginning approximately 180 days of age followed by increased LH secretion and the decline in estradiol negative feedback at later intervals after initiation of dietary treatments. Hence, across these previous experiments it appeared that the initial response to the high energy diet was at the level of ovarian
follicles. Therefore, our aim in the present experiment was to test the hypothesis that feeding of a high energy diet beginning at 3 months of age would first augment follicular development, with this response followed subsequently by increased secretion of LH and precocious puberty.

The rationale for this hypothesis was augmented by our recent findings that heifers which experience precocious puberty had greater IGF-I concentrations than heifers that did not reach puberty precociously, even though all heifers were fed high energy diets (Maquivar et al., 2009). A similar association of IGF-I and advancement of puberty has been reported in dairy heifers fed a high energy diet beginning at 4 months of age (Radcliff et al., 1997; 2004). A primary target of IGF-I is ovarian follicles (Webb et al., 1999; Velazquez et al., 2008) and it has been reported to induce expression of the enzymes required for the synthesis of estradiol (Gutierrez et al., 1997), promote differentiation and proliferation of granulosa cells in the dominant follicle (Mazerbourg et al., 2000) and enhance expression of LH receptors (Adashi, 1998).

Therefore, we further hypothesized that the augmentation of follicular development would be consistent with actions of IGF-I to stimulate steroid synthesis. Specific objectives of the present experiment were to assess the impact of a high energy diet, initiated at 3 months of age, on intra-follicular changes in mRNA expression for insulin-like growth factor –I receptor (IGF-IR), luteinizing hormone receptor (LHR), and steroidogenesis pathway enzymes: cholesterol side-cleavage chain (CYP11A1) and aromatase (CYP19A1), and the association of these changes with previously described influences on secretion of LH.
Material and methods

All animal handling and experimental procedures were conducted in accordance with the The Ohio State University Agricultural Animal Care and Use Committee.

Animals and treatments

Twenty commercial beef heifers (Angus x Simmental) were weaned at an average of 66 ± 2 days of age and 136 ± 3.3 kg body weight and were then fed a receiving diet (17.2% CP, 1.93 Mcal/kg NEm and 1.29 Mcal/kg NEg) for 13 days before initiation of treatment diets. Heifers were randomly assigned to one of two diets (10 heifers/diet) and transitioned onto the experimental diets over a 7 day interval that was initiated at 86 ± 2 d of age. Diets were formulated to contain the same amount of protein (14.1% crude protein) but to differ in energy content. The high-energy diet (HIGH) provided 2.2 Mcal/kg NEm and 1.37 Mcal/kg NEg and the control diet (CONT) contained 1.70 Mcal/kg NEm and 1.09 Mcal/kg NEg (Table 4.1). The target rate of average daily gain was 1.50 kg/d and 0.75 kg/d for the HIGH diet and CONT diet respectively. Diets were formulated to be fed at a rate between 2.5% and 3.5% of BW to meet these targets. Heifers were group-fed twice daily at 8:00 and 16:00 h within treatment. All diets were initially fed at a rate of 2.5% of BW on an “as fed” basis. Amount of feed offered was adjusted biweekly based upon BW gain during the preceding two weeks. The amount of feed as a proportion of body weight that was fed was also adjusted within a range of 2.5% to 3.5% of BW, within treatment, to attempt to sustain targeted ADG within that treatment. Intake was never adjusted to less than 2.5% of BW.
Within dietary treatment, heifers were randomized by BW to be hemi-ovariectomized (OVX) at 174 ± 4 d of age (HIGH-OVX, n = 4; CONT-OVX, n = 5) or to remain intact (INT) and serve as a “sentinel” group (HIGH-INT, n = 6; CONT-INT, n = 5) for heifers subjected to OVX. Thus, OVX groups were used to assess the influence of diet on molecular characteristics of follicular development whereas the INT treatments were used to assess the impact of diet on pre-pubertal LH secretion and age at puberty in response to diets (Table 2).

**Blood sample collection**

Blood samples (10 ml) were collected by jugular venipuncture once weekly from all heifers to assess progesterone concentrations, beginning at 140 days of age and continuing until an individual heifer was determined to have reached puberty or termination of the experiment at 301 d of age. Blood samples were collected in K2 EDTA vacutainer tubes (BD, New Jersey, USA) and centrifuged at 2,785 x g for 20 min, plasma harvested, and samples frozen at -20°C until analyzed.

Age at puberty was defined one week before the date of collection of the first plasma sample that contained > 2 ng/ml progesterone or one week before the date of collection of the first of 2 consecutive weekly blood samples that each had > 1 ng/ml progesterone. Precocious puberty was defined as an animal that reached puberty before 300 days of age. After heifers were confirmed to be pubertal based on continuous luteal phases, they were removed from the study.
To evaluate changes in secretion of LH, serial blood samples were obtained from INT heifers via indwelling jugular catheters every 15 min for 12 h at 140, 165, 190 and 215 days of age. Blood samples for LH analysis were allowed to clot in plastic tubes for 48 hours at 4ºC, and then centrifuged at 2,785 x g for 20 min, serum harvested, and samples frozen at -20ºC until analyses were performed. A LH pulse was defined as an increase in LH that occurred within two samples of the previous nadir with this increase exceeding two standard deviations from nadir; LH pulse amplitude was defined as the concentration of LH at the peak minus the concentration at the previous nadir and mean LH concentrations was determined as the average of the LH concentration during the 12 hours of sampling (Goodman and Karsch, 1980).

Finally, to evaluate changes in estradiol concentration, blood samples were obtained from OVX heifers from 155 ± 4 d of age until 174 ± 4 d when hemi-ovariectomy was performed (d 0). Blood samples were collected in K2 EDTA vacutainer tubes (BD, New Jersey, USA) and centrifuged at 2,785 x g for 20 min, plasma harvested, and samples frozen at -20ºC until analyzed. Only concentrations of estradiol for the 11 d preceding ovariectomy were determined.

Ultrasonography and Ovariectomy

Daily ultrasonography was initiated at 155 ± 4 d of age, or at 70 d after initiation of diet treatments in the OVX groups. The diameter of all ovarian follicles > 3mm in diameter were mapped for both ovaries on each day. Within a heifer, two complete follicular waves were assessed. The follicular wave length was defined as the time (d) from
emergence of follicles in both ovaries to the emergence of new follicles in the subsequent follicular wave. Upon initiation of the third wave of development the dominant follicle was assessed daily until its size reached at least 9 mm in diameter. At this time, the heifer was scheduled for hemi-ovariectomy the following day, with only the ovary bearing the dominant follicle removed at that time. This procedure occurred on average at 174 ± 4 d of age. The hemi-ovariectomy was performed via high lumbar laparotomy (Moser et al., 1989) and following an approved animal use protocol (protocol #09-AG012). Following hemi-ovariectomy, all follicles greater than or equal to 9 mm of diameter were dissected from the ovary (the atretic follicle from the previous follicular wave in some ovaries was present). All follicular fluid contained in the dominant follicle(s) was immediately collected with a 1 ml syringe to assess intra-follicular estradiol and progesterone concentrations. Then, the dominant follicle(s) was dissected from the remainder of the ovary and snap frozen in liquid nitrogen.

**RNA isolation and real time PCR analysis**

Total RNA was extracted from the whole follicle using Trizol reagent (Invitrogen, Life Technologies, Rockville, MD) according to the manufacturer’s instructions. Concentration and purity of the RNA extracted from each sample was quantified using a NanoDrop instrument (ND-1000; NanoDrop Technologies, Wilmington, DE) using 260nm and 260/280 nm of wavelength ratio. Additionally, the integrity of the RNA was assessed using a 1.2% formaldehyde agarose gel by electrophoresis and assessment of the 28s to 18s ribosomal RNA ratio.
The standard curve method (Larionov et al., 2005) was used to determine the absolute quantity of mRNA in the real time RT-PCR for glyceraldehyde 3-phosphate dehydrogenase (GAPDH, used as a house keeping gene) IGF-IR, LHR, CYP11A1, and CYP19A1. Reverse transcription was performed using a TaqMan Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA) using random hexamers as primers during the reverse transcription reaction (25°C for 10 min, 48°C for 30 min, 95°C for 5 min and cooling down samples at 5°C). For all samples, an additional reaction was performed using water substituting reverse transcriptase enzyme as a negative control. The standard curve was constructed from a pool of combined samples from heifers across treatments. One microgram of total RNA for reverse transcription was used in the reaction. The resulting cDNA was serially diluted to create five standards ranging 1:1 to 1:10,000-fold. Samples were assessed at 200 ng of total RNA per 25 micro liters of reaction volume. Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA) was used and concentration of primer was validated for each gene. Primers for detection of IGF-IR, LHR, CYP11A1, CYP19A1, GAPDH are presented in table 3. Each sample was run in duplicate. Amplification of cDNA was performed by incubating the samples and reagents in 96-well plates for 10 min at 95 °C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute in a 7500 Real Time PCR machine (Applied Biosystems, Foster City, CA). Dissociation curve analysis was performed for each run to verify the specific amplification of the expected products. Additionally, in order to confirm specific amplification of the genes after RT-PCR, each sample was run in a 3% agarose gel with ethidium bromide as a staining agent.
**Hormone Analyses**

Concentration of progesterone in plasma were analyzed in duplicate using a commercial radioimmunoassay analysis (RIA) kit (Coat-a-Count, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA) according to the procedures described by Burke et al., (2003). Intra-assay CV was 8.5% and inter-assay CV for low pool (0.15 ng/ml) was 2.16%, for the medium pool (1.0 ng/ml) 5.5%, and for the high pool (3 ng/ml), inter-assay CV was 4.4%. The average sensitivity of the assays was 0.01 ng/ml.

Concentration of luteinizing hormone in serum was assessed in duplicate for all samples using a double-antibody system previously validated in our laboratory (Anderson et al., 1996). The intra-assay CV was 6.8% and the inter-assay CV for low (1.6 ng/ml) and high (5.5 ng/ml) pools was 6.2 and 7.5%, respectively. The sensitivity of the assay was 0.16 ng/ml.

Estradiol concentrations in plasma were analyzed using a double antibody assay previously validated in our lab (Souto et al., 2010). Average intra-assay CV was 5.2% and inter-assay CVs for low (1.5 pg/ml), medium, (8 pg/ml), and high (15 pg/ml) pools were 7.7%, 6.7%, and 2.0%, respectively. The sensitivity of the assay was 1.35 pg/ml.
**Follicular fluid hormone analyses**

Samples of follicular fluid were serially diluted (1:1, 1:10, 1:100, 1:1,000, 1:10,000) in phosphate buffer solution (pH= 7.0) containing 0.1% gel (Type A: From porcine skin, SIGMA G-2625, St. Louis, MO, USA). All samples were analyzed in duplicate and in a single assay. The progesterone assay included 1:10, 1:100 and 1:1000 dilutions of the samples, whereas for estradiol assay the 1:1000 and 1:10,000 dilutions were used in the assay. Progesterone inter-assay CV for the low pool (0.15 ng/ml) was 3.55%, for the medium pool (1.0 ng/ml) 1.25%, and for the high pool (3 ng/ml), inter-assay CV was 1.59%. The sensitivity of the assay was 0.17 ng/ml. Estradiol inter-assay CV for low pool (10 ng/ml) was 1.51%, and for high pool (15 ng/ml) was 2.82%. The sensitivity of the assay was 0.84 ng/ml. Finally, the estradiol/progesterone ratio was calculated to estimate the steroidogenic status of each follicle (Ireland and Roche, 1982).

**Statistical analysis**

**Overall analysis**

The effect of treatment, day and ovarian status (INT or OVX) and the interaction of treatment by day, treatment by ovarian status and the interaction of treatment by day by ovarian status on body weight on average daily gain, which included all heifers, were analyzed by ANOVA using the MIXED procedure in SAS (2004) with a 2 x 2 factorial arrangement (HIGH and CONT; INT and OVX) with repeated measures analysis with a
first order ante-dependence covariance structure included in the model. The model was as follows:

\[ Y_{ijk} = \mu + T_i + h_{ji} + O_m + D_k + (TO)_{im} + (TD)_{ik} + (TOD)_{imk} + \varepsilon_{ijk}, \]

where:

- \( Y_{ijk} \) = observation of the \( j \)th animal in the \( i \)th treatment on the \( m \)th ovarian status on the \( k \)th day, \( \mu \) = grand mean, \( T_i \) = \( i \)th treatment, \( h_{ji} \) = random effect of the \( j \)th animal within the \( i \)th treatment (\( h_{ji} \sim N[0, \sigma^2] \)), \( O_m \) = \( m \)th ovarian status, \( D_k \) = \( k \)th day, \( (TO)_{im} \) = interaction between treatment and ovarian status, \( (TD)_{ik} \) = interaction between treatment and day, \( (TOD)_{imk} \) = interaction between treatment, ovarian status and day, \( \varepsilon_{ijk} \) = error \( \sim N(0, \sigma^2) \).

The effect of treatment on body weight and age at puberty (all heifers) was analyzed by ANOVA using the GLM procedure in SAS using animal within treatment as the error term. The model used was:

\[ Y_{ijk} = \mu + T_i + \varepsilon_{ij} \]

where:

- \( Y_{ijk} \) = observation of the \( j \)th animal in the \( i \)th treatment, \( \mu \) = grand mean, \( T_i \) = \( i \)th treatment, \( \varepsilon_{ij} \) = error \( \sim N(0, \sigma^2) \).

**Intact heifer analysis**

In the sentinel heifers (INT), mean LH concentration, LH pulse frequency and LH pulse amplitude were analyzed by ANOVA using the MIXED procedure in SAS (2004) with repeated measures analysis using a first order ante-dependence covariance structure included in the model. The model was as follows:

\[ Y_{ijk} = \mu + T_i + h_{ji} + D_k + (TD)_{ik} + \varepsilon_{ijk}, \]

where:
\( Y_{ijk} \) = observation of the \( j \)th animal in the \( i \)th treatment on the \( k \)th day, \( \mu \) = grand mean, \( T_i \) = \( i \)th treatment, \( h_{ji} \) = random effect of the \( j \)th animal within the \( i \)th treatment (\( h_{ji} \sim N[0, \sigma^2 h] \)), \( D_k \) = \( k \)th day, \( (TD)_{ik} \) = interaction between treatment and day, \( \varepsilon_{ijk} \) = error i.i.d \( \sim N(0, \sigma^2) \).

The effect of treatment on body weight and age at puberty was analyzed by ANOVA using the GLM procedure in SAS using animal within treatment as the error term. The model used was: \( Y_{ijk} = \mu + T_i + \varepsilon_{ij} \) where:

\( Y_{ijk} \) = observation of the \( j \)th animal in the \( i \)th treatment, \( \mu \) = grand mean, \( T_i \) = \( i \)th treatment, \( \varepsilon_{ij} \) = error i.i.d \( \sim N(0, \sigma^2) \).

Finally, proportion and timing at puberty for each treatment was analyzed with a survival curve test by the PROC LIFETEST procedure in SAS v. 9.1, (2004).

**Ovariectomized heifer analysis**

The effect of treatment, days previous to ovariectomy and the interaction of treatment and days on estradiol concentration, were analyzed by ANOVA using the MIXED procedure in SAS (2004) with repeated measures analysis with a first order ante-dependence covariance structure included in the model. The model was as follows:

\( Y_{ijk} = \mu + T_i + h_{ji} + D_k + (TD)_{ik} + \varepsilon_{ijk} \), where

\( Y_{ijk} \) = observation of the \( j \)th animal in the \( i \)th treatment on the \( k \)th day, \( \mu \) = grand mean, \( T_i \) = \( i \)th treatment, \( h_{ji} \) = random effect of the \( j \)th animal within the \( i \)th treatment (\( h_{ji} \sim N[0, \sigma^2 h] \)), \( D_k \) = \( k \)th day, \( (TD)_{ih} \) = interaction between treatment and day, \( \varepsilon_{ijk} \) = error i.i.d \( \sim N(0, \sigma^2) \).
The effect of treatment on follicular wave length, and intra-follicular estradiol/progesterone ratio, BW and age at puberty were analyzed by ANOVA using the GLM procedure in SAS using animal within treatment as the error term. The model used was: $Y_{ijk} = \mu + T_i + \epsilon_{ij}$, where:

$Y_{ijk} =$ observation of the $j$th animal in the $i$th treatment, $\mu =$ grand mean, $T_i =$ $i$th treatment, $\epsilon_{ij} =$ error $i.i.d \sim N(0, \sigma^2)$.

The expression of GAPDH was tested for homogeneity of variance among plates using the GLM procedure of SAS, the model included treatment and plate. Expression of IGF-IR, LHR, CYP11A1 and CYP19A1, were adjusted by dividing the expression of the gene of interest by the expression of GAPDH. Adjusted expression of the genes was analyzed for the main effect of treatment (diet) with the GLM procedure in SAS.

The model used was: $Y_{ijk} = \mu + T_i + \epsilon_{ij}$, where:

$Y_{ijk} =$ observation of the $j$th animal in the $i$th treatment, $\mu =$ grand mean, $T_i =$ $i$th treatment, $\epsilon_{ij} =$ error $i.i.d \sim N(0, \sigma^2)$.

Data are presented in means ± SE.
Results

All heifers ADG, BW

Overall ADG was greater (P < 0.01) in the HIGH (1.57 ± 0.71 kg/d) than CONT diet (1.21 ± 0.74 kg/d) and no interaction was observed between treatment and ovarian status (P = 0.74). Body weight of the heifers fed the HIGH diet was greater (P < 0.05) than those fed the CONT diet at 186 days of age and thereafter (treatment by day interaction, P < 0.01; Figure 4.1). Body weight at puberty was not different (P = 0.71) between CONT and HIGH diets (306.1 ± 11 kg and 298.5 ± 17.3 kg, respectively) and age at puberty was not different (P = 0.14) between diets (HIGH, 217.7 ± 10.9 d and CONT, 239.1 ± 8.05 d).

INT heifers:

BW and age at puberty

Precocious puberty occurred in 50% (3/6) of heifers in the HIGH-INT and 80% (4/5) of heifers in the CONT-INT treatment. Age at puberty was earlier in the HIGH-INT than CONT-INT treatment (P < 0.05) (194.33 ± 8.95 d vs. 253.75 ± 1.25 d, respectively) and BW at puberty was less (P < 0.02) in the HIGH-INT (257.7 ± 17.02 kg) than CONT-INT diet (321.67 ± 11.65 kg; Figure 4.2).

LH characteristics

Mean LH concentration, LH pulse amplitude and frequency of pulses detected in 12 hours are presented in table 4.4. Mean LH concentration was greater (P < 0.05) in the HIGH-INT than the CONT-INT treatment at 170 d and 208 d of age. The greater mean
LH concentration, was not reflected in either greater frequency or amplitude of LH pulses at neither 170 nor 208 days of age in HIGH-INT heifers than CONT-INT heifers.

OVX heifers:

*Systemic estradiol concentration and follicular wave length*

Figure 4.3 shows the systemic estradiol concentration of heifers for the eleven days before OVX. There were no differences between d -11 and d -3; however, estradiol concentrations were greater in the CONT-OVX than HIGH INT on d -1 and d 0 (treatment by day interaction P < 0.05). No differences were observed in wave length of the two follicular waves preceding ovariectomy (Figure 4.4).

*Gene expression and follicular steroid concentration*

Only follicles with apparent expression of CYP11A1 and CYP19A1 were included in the final analysis. Therefore, one follicle on each animal was included in the analysis. Finally, one of the heifers that were destined to be ovariectomized in the HIGH treatment had a progesterone value above 1 ng/ml previously to the surgery, and later that animal became pubertal, and one heifer in the CONT treatment was also excluded from the analysis due a complications reaching the ovary bearing the dominant follicle during surgery, therefore, final analysis included four animals in each treatment.

Figure 4.5 depicts progesterone concentration and estradiol concentration in the follicular fluid extracted from each dominant follicle; no differences (P > 0.05) were observed among treatments. The ratio E2/P4 was similar among treatments, HIGH-OVX (1.16 ±
0.57) vs. CONT-OVX (3.4 ± 1.7). Expression of the genes studied was not affected by diet, but were numerically greater in the HIGH-OVX than CONT-OVX treatment for all genes evaluated. Figure 4.6 shows the expression in terms of ratio of the genes studied to the housekeeping gene. Figures 4.7 through 4.10 depict electrophoretic gels and the amplification of the genes from each treatment.

**BW and age at puberty**

Precocious puberty was observed in 100% (4/4) of the HIGH OVX heifers and 60% (3/5) of the heifers in the CONT-OVX treatment. No differences (P > 0.05) were observed at age at puberty among HIGH-OVX and CONT-OVX (235.25 ± 11.9 d, 219.66 ± 10.9 d, respectively) and a tendency (P = 0.07) to be greater was observed on BW at puberty in the HIGH-OVX than CONT-OVX (329.07 ± 13.17 kg, 285.43 ± 14.08 kg, respectively) (Figure 4.2).
Discussion

In INT heifers in the present study, age at puberty was advanced by approximately 2 months with the high energy diet for the heifers that reached puberty during the experiment. However, incidence of precocious puberty was only 50% (3/6) in the HIGH-INT but was 80% (4/5) in the CONT-INT treatment. Furthermore, only subtle differences were detected in secretion of LH between diet treatments. It appears that in this experiment, the BW gains achieved with the CONT diet induced precocious puberty in a majority of heifers and indeed, were approximately equal to gains achieved in previous experiments (Gasser et al., 2006 a, b, c, and d, Maquivar et al., 2009) with a high energy diet.

Furthermore, expression of critical genes in the dominant follicle (IGF-IR, LHR, CYP11A1 and CYP19A1) that would indicate advancement in the maturation of the dominant follicle was not detected in heifers fed the HIGH diet. Only minor or no differences were detected in follicular characteristics evaluated (follicle wave length and systemic estradiol concentration) between diets. Similarity of follicle characteristics between OVX heifers fed the high energy and control diets was reflected in the lack of difference in age at puberty of heifers that were subjected to hemi-ovariectomy at approximately 6 months of age. It is speculated that in the present experiment, both diets precociously advanced maturation of the reproductive axis; resulting in similar outcomes between diet for many variables.
Mean concentration of luteinizing hormone was greater in the HIGH-INT than CONT-INT treatment at 170 and 208 days of age, but this increase was not reflected in greater LH pulse frequency or LH pulse amplitude. Gasser et al., (2006a) demonstrated a robust increase in frequency of LH pulses by 190 days of age in heifers fed similar high energy and control rations, and this large difference remained through termination of the experiment. In the same study, approximately 90% of heifers fed the high energy diet reached precocious puberty whereas no heifers fed the control diet had precocious puberty. Frequency of LH pulses at similar ages in the present study appeared to be equal to, or greater than, those observed in the high energy treatment in the previous report (Gasser et al., 2006a) at similar ages. This comparison supports that both the HIGH and CONT diet in the present study precociously activated the reproductive axis. It has been shown that the molecular effect of LH in the follicle is induce the synthesis of androgens in the theca cells and at the same time increase the expression of CYP19A1 in the granulosa cells, promoting increased estradiol synthesis (Fortune et al., 2001). In addition to the effect of LH, nutritional status has been shown to regulate ovarian follicle maturation and endocrine patterns. For example, under-nutrition delays the onset of puberty by affecting the development of follicles (Bergfeld et al., 1994), and the secretion of LH (Day et al., 1986), FSH (Bossis et al., 1999), and IGF-I (Richards et al., 1991). Contrary to the hypothesis formulated for the present study, there was no difference in gene expression of IGF-IR, LHR and the steroidogenic enzymes (CYP11A1 and CYP19A1) among treatments. Although, we were successful in collecting dominant follicles that were estrogentially active based upon on the E2/P4 ratio and in detecting
the mRNA of the genes studied, the numerically greater expression in the HIGH-OVX treatment may have been significant with more animals, the lack of difference in other variables studied, makes hard to interpret the results. Although, there was a numerical increase in the expression of steroidogenic enzymes (CYP11A1 and CYP19A1), which catalyze the conversion of cholesterol into pregnenolone and testosterone/androstendione into estradiol/estrone, respectively; progesterone or estradiol synthesis in the follicle did not differ. Progesterone and estradiol concentration in the follicular fluid were similar among treatments.

Gasser et al., (2006b) observed that the follicle wave length in pre-pubertal heifers under the same model at 160 days of age is between 6.5 (for non precocious heifers) and 7.5 days (for heifers that had precocious puberty). The follicular wave length in the present experiment ranged from 6 to 8 days. Looking at systemic estradiol concentrations during the follicular wave previous to the date of surgery, it seems that the period of high estradiol synthesis in the HIGH-OVX is around 2 days (d -10 and d -9) similar to CONT-OVX (d -10 to d -8), therefore it is possible that those follicles were not fully estrogenic when they were removed. However, the concentration of E2/P4 ratio was greater than 1.0 in both treatments (HIGH-OVX 1.16 ± 0.57 and CONT-OVX 3.4 ± 1.7). A ratio superior to 1.0 indicates that follicles were dominant at the time of removal and were estrogenically active; indicative of a non atretic healthy follicle (Nishimoto et al., 2009).

An interesting finding in the present study was that removal of one ovary did not impair the capacity of rapid BW gains to induce precocious puberty in heifers. In fact, 78% (7/9, including all OVX heifers) subjected to this procedure attained precocious puberty as
compared to 64% (7/11, including all INT heifers) that remained intact. Swanson et al., (1971) suggested that after ovariectomy in heifers, increased plasma concentration of LH is due to a removal of the negative feedback that estradiol exerts in the hypothalamus. Kiser et al., (1981) ovariectomized pre-pubertal Hereford heifers and an increase of LH secretion shortly after surgery confirmed the hypothesis that removal of ovarian negative feedback enhanced secretion of gonadotropins in pre-pubertal heifers. Grass and Hauser (1981) reported that unilateral ovariectomy of 2 month old heifers resulted in an earlier age at puberty compared to intact heifers (363 vs. 390 days). Perhaps hemi-ovariectomy reduced the negative feedback of ovarian steroids on the hypothalamus resulting in increased secretion of FSH and LH in the present study and this enhanced stimulus overcame any short term negative effects of surgery on performance of heifers in the OVX group.

In conclusion, a high energy diet increased body weight gain and LH secretion at 170 and 208 days of age and resulted in an earlier age at puberty in the heifers that were ovary intact. However, the incidence of precocious puberty did not differ between treatments. Intra-follicular concentrations of estradiol and progesterone nor steroidogenic enzyme expression differed between dietary treatments, indicating no direct dietary effects at the level of individual follicles. It is therefore plausible that heifers in both diets received metabolic signals that precociously activated the reproductive axis in this experiment. Further research is needed to determine if the initial step to precocious activation of the reproductive axis is driven by increased IGF-I enhancement of follicular function.
Figure 4.1 Body weight (means ± SE) of pre-pubertal heifers fed either a control diet (CONT), or high energy diet (HIGH). Analysis included heifers from INT and OVX groups. The diet by age interaction was significant (P<0.001). *groups differ within age (P < 0.05).
Figure 4.2 Mean (±SE) body weight and age at the onset of puberty in heifers fed either a control diet (CONT), or high-energy diet (HIGH). Heifers from each treatment were divided in two subgroups: heifers that were ovariectomized (OVX) and heifers that were intact (INT). Different letters within ovarian status indicate statistical difference (\(^{ab} \ P<0.05; \ ^{cd} \ P = 0.07\)).
**Figure 4.3** Mean (±SE) estradiol concentration of heifers fed either a high concentrate (HIGH) or a control diet (CONT) prior to the day of ovariectomy (d 0). *treatments differ within day (P < 0.05) (treatment by day interaction P < 0.05)
Figure 4.4 Mean (±SE) length of the follicular waves preceding ovariectomy in heifers fed either a high-energy (HIGH) or a control diet (CONT) ovariectomized at 174 ± 4 d of age. There were no differences among groups (P > 0.05).
Figure 4.5 Mean (±SE) progesterone (P4) and estradiol (E2) concentration in follicular fluid (FF) extracted from dominant follicles of heifers fed either a high energy (HIGH-OVX) or a control diet (CONT-OVX) ovariectomized at 174 ± 4 d of age.
Figure 4.6 Expression of genes in terms of ratio (±SE) (gene of interest/house keeping gene) from dominant follicles of heifers fed either a high–energy (HIGH-OVX) or a control diet (CONT-OVX) ovariectomized at 174 ± 4 d of age.
Figure 4.7 Expression of insulin-like growth factor –I (IGF-IR, 101 bp amplicon size) expressed in dominant follicles from heifers fed either a high-energy (HIGH-OVX) or a control diet (CONT-OVX) and ovariectomized at 174 ± 4 d of age. (3% Agarose gel with ethidium bromide as staining agent. First well is the 25 bp DNA ladder)
**Figure 4.8** Expression of luteinizing hormone receptor (LHR, 157 bp amplicon size) expressed in dominant follicles from heifers fed either a high-energy (HIGH-OVX) or a control diet (CONT-OVX) and ovariectomized at 174 ± 4 d of age. (3% Agarose gel with ethidium bromide as staining agent. First well is the 25 bp DNA ladder.)
**Figure 4.9** Expression of cholesterol side-cleavage chain (CYP11A1, 117 bp amplicon size) expressed in dominant follicles from heifers fed either a high-energy (HIGH-OVX) or a control diet (CONT-OVX) and ovariectomized at 174 ± 4 d of age. (3% Agarose gel with ethidium bromide as staining agent. First well is the 25 bp DNA ladder.)
Figure 4.10 Expression of aromatase (CYP19A1, 141 bp amplicon size) expressed in dominant follicles from heifers fed either a high–energy (HIGH-OVX) or a control diet (CONT-OVX) and ovariectomized at 174 ± 4 d of age. (3% Agarose gel with ethidium bromide as staining agent. First well is the 25 bp DNA ladder.)
Table 4.1 Composition and chemical analysis of experimental diets fed (% as fed) to beef heifers weaned at 66 ± 2 d of age, and randomized to receive one of two diets beginning at 86 ± 2 d of age and 137 ± 3.3 kg body weight.

<table>
<thead>
<tr>
<th>Ingredient, % as-fed</th>
<th>Diet</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>High energy</td>
<td>Control</td>
</tr>
<tr>
<td>Whole shelled corn</td>
<td></td>
<td>60</td>
<td>30</td>
</tr>
<tr>
<td>13% CP alfalfa pellets</td>
<td></td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>Pelleted soybean hulls</td>
<td></td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>Supplement</td>
<td></td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td><strong>Nutritional analyses</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP, %</td>
<td></td>
<td>14.1</td>
<td>14.1</td>
</tr>
<tr>
<td>NEm, Mcal/kg</td>
<td></td>
<td>2.02</td>
<td>1.70</td>
</tr>
<tr>
<td>NEg, Mcal/kg</td>
<td></td>
<td>1.37</td>
<td>1.09</td>
</tr>
<tr>
<td><strong>Supplement composition</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corn ground</td>
<td></td>
<td>36.9</td>
<td>21.6</td>
</tr>
<tr>
<td>Soybean meal</td>
<td></td>
<td>45.7</td>
<td>51.3</td>
</tr>
<tr>
<td>Urea</td>
<td></td>
<td>2.0</td>
<td>4.0</td>
</tr>
<tr>
<td>Limestone</td>
<td></td>
<td>3.5</td>
<td>-</td>
</tr>
<tr>
<td>Dicalcium phosphate</td>
<td></td>
<td>1.8</td>
<td>2.7</td>
</tr>
<tr>
<td>Trace mineral salt</td>
<td></td>
<td>2.2</td>
<td>4.5</td>
</tr>
<tr>
<td>Vitamin A-30</td>
<td></td>
<td>0.04</td>
<td>0.09</td>
</tr>
<tr>
<td>Vitamin D-3</td>
<td></td>
<td>0.04</td>
<td>0.09</td>
</tr>
<tr>
<td>Vitamin E</td>
<td></td>
<td>0.13</td>
<td>0.27</td>
</tr>
<tr>
<td>Selenium</td>
<td></td>
<td>0.22</td>
<td>0.45</td>
</tr>
<tr>
<td>Rumensin 80</td>
<td></td>
<td>0.07</td>
<td>0.15</td>
</tr>
<tr>
<td>KCl (Dyna K)</td>
<td></td>
<td>0.67</td>
<td>1.34</td>
</tr>
<tr>
<td>Dynamate</td>
<td></td>
<td>1.8</td>
<td>3.6</td>
</tr>
<tr>
<td>Animal-vegetable fat</td>
<td></td>
<td>4.5</td>
<td>10.0</td>
</tr>
</tbody>
</table>
Table 4.2 Average (±SE) of age and body weight characteristics of the heifers fed either a high energy diet (HIGH) or a control diet (CONT) and randomly designated to be ovariectomized (OVX) or to remain intact (INT).

<table>
<thead>
<tr>
<th></th>
<th>CONT-INT (n = 5)</th>
<th>CONT-OVX (n = 5)</th>
<th>HIGH-INT (n = 6)</th>
<th>HIGH-OVX (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (d)a</td>
<td>85.8 ± 4.53</td>
<td>87.0 ± 4.78</td>
<td>86.2 ± 4.46</td>
<td>84.0 ± 5.31</td>
</tr>
<tr>
<td>BW (Kg)b</td>
<td>142.6 ± 6.39</td>
<td>135.5 ± 10.35</td>
<td>136.10 ± 2.72</td>
<td>132.90 ± 7.82</td>
</tr>
<tr>
<td>BW (Kg) at birth</td>
<td>37.2 ± 1.93</td>
<td>38.4 ± 2.92</td>
<td>36.2 ± 2.43</td>
<td>35.2 ± 1.74</td>
</tr>
<tr>
<td>ADG (Kg/d)c</td>
<td>1.22 ± 0.05</td>
<td>1.10 ± 0.06</td>
<td>1.17 ± 0.03</td>
<td>1.16 ± 0.04</td>
</tr>
</tbody>
</table>

a Average age at the beginning of the feeding
b Average body weight at initiation of the diet (86 ± 2 days of age).
c Average daily gain calculated from birth to initiation of diet treatments.
**Table 4.3** Sequence of primers used for RT-PCR.

<table>
<thead>
<tr>
<th>Gene&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PCR Product size (bp)</th>
<th>Forward</th>
<th>Reverse</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP19A1</td>
<td>141</td>
<td>ggctatgtgagcttgacc</td>
<td>tgagaagagagttgcag</td>
<td>NM_174305</td>
</tr>
<tr>
<td>CYP11A1</td>
<td>117</td>
<td>agacttggagggaccagctagc</td>
<td>tgccctggtaattcctaaattc</td>
<td>ENSBTAT0000009106</td>
</tr>
<tr>
<td>IGF-IR</td>
<td>101</td>
<td>gatccgccgtgtcttctcagttc</td>
<td>aagcctccactatcaacagaa</td>
<td>X54980</td>
</tr>
<tr>
<td>LHR</td>
<td>157</td>
<td>gccactctgtgttgttttagaa</td>
<td>ccagccactcagttcatctca</td>
<td>U87230</td>
</tr>
<tr>
<td>GAPDH</td>
<td>221</td>
<td>agcgagatctgccaacatcaag</td>
<td>gcaggggctgtgcaatct</td>
<td>AJ000039</td>
</tr>
</tbody>
</table>

<sup>a</sup>Steroidogenesis pathway enzymes: aromatase (CYP19A1), cholesterol side-cleavage chain (CYP11A1); Insulin-like growth factor –I receptor (IGF-IR), luteinizing hormone receptor (LHR), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a house keeping gene.
Table 4.4 Mean (±SE) LH concentration and frequency and amplitude of LH pulses in beef heifers fed either a high energy (HIGH) or a control diet (CONT) in a 12 h period.

<table>
<thead>
<tr>
<th></th>
<th>Group</th>
<th>145</th>
<th>170</th>
<th>208</th>
<th>236</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LH</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (ng/ml)</td>
<td>HIGH</td>
<td>0.81 ± 0.05</td>
<td>0.81 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.28 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.87 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>CONT</td>
<td>0.79 ± 0.03</td>
<td>0.65 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.04 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.78 ± 0.05</td>
</tr>
<tr>
<td><strong>Pulse amplitude</strong> (ng/ml)</td>
<td>HIGH</td>
<td>2.48 ± 0.28</td>
<td>3.75 ± 0.38</td>
<td>3.19 ± 0.51</td>
<td>2.82 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>CONT</td>
<td>2.12 ± 0.18</td>
<td>2.35 ± 0.18</td>
<td>2.75 ± 0.22</td>
<td>1.68 ± 0.33</td>
</tr>
<tr>
<td><strong>Pulse frequency / 12 h</strong></td>
<td>HIGH</td>
<td>3.40 ± 0.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.20 ± 0.20</td>
<td>5.50 ± 1.84</td>
<td>3.33 ± 0.33</td>
</tr>
<tr>
<td></td>
<td>CONT</td>
<td>1.80 ± 0.20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.40 ± 0.40</td>
<td>3.60 ± 0.60</td>
<td>3.60 ± 0.40</td>
</tr>
</tbody>
</table>

<sup>ab</sup> P<0.05 Values with different superscripts within age and between treatments are different.
CHAPTER 5
GENERAL DISCUSSION

Age at puberty and age at first calving in cattle is an important reproductive and economic trait that will determine greatly the productive efficiency of the cow (Patterson et al., 1992). The physiological mechanisms involved during the onset of puberty are gated by a gradual transition between the pre-pubertal phase of development and puberty, modification in the endocrine system of the reproductive and somatotropic axis, and communication between nutritional factors and the reproductive axis. One of the advantages of advancement of puberty before the first breeding season on reproductive efficiency and lifetime production in beef heifers was demonstrated in several early reports. The use of nutritional regimes to promote heifers to reach puberty before the start of the breeding season has been an important goal for beef producers. One approach to advance puberty even further is feeding a high energy diet to heifers weaned at 2 – 3 months of age to induce precocious puberty. While advancing puberty to 8 months of age is not a desired production outcome in most US beef herds, use of this technology in environments such as Brazil with Bos indicus cattle is increasing substantially in order to reduce breeding age from 2 to 3 years of age, down to 14 to 15 months of age. Furthermore, this capacity to manipulate age at puberty with nutrition provides an
excellent model to study the mechanisms by which nutrition regulates timing of puberty, and was the basis and approach taken in the research described in this dissertation.

The main physiological hypothesis for these series of experiments (Figure 1.1) was that in heifers fed a high energy diet beginning at 3 – 4 months of age would result in hepatic GH receptor expression stimulation and consequently increased systemic concentrations of IGF-I. Higher concentrations of IGF-I, in conjunction with basal GH concentrations, would then stimulate proliferation and differentiation of granulosa cells in the follicle and promote estradiol synthesis. Furthermore, both IGF-I and GH, acting through their respective receptors in the granulosa cells, would stimulate additional synthesis of IGF-I from the follicle. This locally produced IGF-I, in concert with the increased systemic IGF-I, would further stimulate the expression of the enzymes required for estradiol synthesis. Increased concentrations of estradiol would subsequently act at the hypothalamus to accelerate the decline in the negative feedback, resulting in more frequent LH secretion, which further stimulates follicular maturation through action at the follicular LH receptors. This process progresses to the point that precocious puberty and ovulation occurs.

The hypothesis in the first experiment was that the high starch content of the high energy diet that had previously been used to induce precocious puberty was playing a major role in inducing precocious activation of the reproductive axis. The hypothesis was rejected based upon on the findings of the first experiment (chapter 3), in which induction of precocious puberty was similar when heifers were fed high energy diets that were similar
in energy content, but differed greatly in chemical analysis of starch content. Concentrations of IGF-I were increased equally by the high energy diets as compared to the lesser energy control diet. However, across the high and low starch diets, heifers that experienced precocious puberty had substantially greater IGF-I concentrations preceding precocious puberty than those that did not reach precocious puberty. These data implicate IGF-I as a potential signal for precocious activation of the reproductive axis, and furthermore, suggests a threshold of IGF-I concentration to cause this effect.

Based upon on findings for IGF-I concentrations in the first study, we hypothesized for the second experiment that when heifers are fed a high energy diet to induce precocious puberty, the accompanying IGF-I response would induce increased expression of IGF-I receptor, LH receptor, and steroidogenic enzymes (CYP11A1 and CYP19A1) in the dominant follicle within 50 to 70 days, and this response could precede the previously demonstrated increase in LH secretion that precedes precocious puberty. While some subtle differences were noted between the high energy and control diet treatments in this experiment, induction of precocious puberty was unexpectedly high in the control group. This was likely due to these heifers gaining much more weight than expected during the experiment. In fact, it appeared that both diets equally induced precocious activation of the reproductive axis when reproductive responses were assessed. Accordingly, neither intra-follicular concentrations of estradiol and progesterone, nor gene expression, differed between the heifers fed the high energy or control diet. It remains unknown whether the lack of difference due to diet was because incidence of precocious puberty was similar
between the high energy and control treatment, or if the mechanism of action of the high energy diet is exerted at alternative sites within the reproductive axis or at latter age of the animal.

Collectively, these experiments have shown that energy is one of the principal nutrients from the diet that regulates the onset of puberty. Additionally, it appears that diet affected different organs and tissues that were involved in the reproductive maturation. In these studies we focused on the effect at the ovarian level and the impact of diet composition relative to starch content.

It has been demonstrated that many organs in the body act harmoniously during the sexual maturation of the animal, for example in humans and cattle it is suggested that the amount of body fat is associated with the timing at which females and males achieve puberty and adipose tissue also acts as an endocrine gland producing hormones such as leptin, adiponectins and steroids (see chapter 2) that function at the hypothalamic-hypophyseal axis, gonads and in females, at mammary gland level. All of the complex interactions between the metabolism of the animal and the organs involved in the reproduction require much future investigation.

From the animal production standpoint, the animal model tested in this series of experiments might be more advantageous to *Bos indicus* cattle. The underlying mechanisms that control puberty in *Bos indicus* heifers appear to be similar to those that have been described for *Bos taurus* females above (for review, see Nogueira, 2004; Galina and Arthur, 1989, Maquivar and Galina 2009). In a review, Abeygunawardena and Dematawewa (2004) reported that the mature body weight of tropical Zebu breeds
ranged from 280 to 650 kg, and the range in age at puberty (16 to 40 months) and age at first calving ranging from 24 to 62 months for zebu cattle. This is much greater than for their *Bos taurus* counterparts in temperate climates. Magaña and Segura-Corra (2006) suggested that environmental factors (such as season of birth) are an important source of variation for pre- and post-weaning growth traits; calves born in the windy, cold and rainy season of the Mexican tropics had lower weaning weights at 240 days and gained less weight, which may impact age at puberty as compared to calves born in the dry season or at the onset of the rainy season. Early reports suggest that a lower growth rate is associated with a delay in the onset of puberty (Plasse, 1978, 1979; Mukassa-Mugerwa, 1989). Genetic, environmental and nutritional differences have all been identified as significant factors that influence the relative age at puberty between *Bos indicus* and *Bos taurus* cattle. However, much like has been observed in *Bos taurus* cattle in temperate climates, some Zebu cattle breeds have the potential to exhibit precocious puberty. In fact, it was observed that 32% of Nellore heifers became pregnant at 15 months of age and it was concluded that these heifers experienced precocious puberty (Nogueira et al., 2003). It has been demonstrated that early weaning of Zebu heifers (3 months), and feeding to achieve body weight gains typical of those in *Bos taurus* in temperate climates resulted in puberty at a mean age of 12.3 months and BW of 233 kg (Fajersson et al., 1991). Therefore, the possibility exists that the management approach of early weaning and feeding of a high concentrate diet at the appropriate time during sexual maturation could increase the incidence of calving at 2 years of age in *Bos indicus* cattle. Additionally, it has been demonstrated that nutritive signals responsible for precocious
activation of the reproductive axis are most important between 3 to 6 months of age for
*Bos taurus* breeds. Recent data regarding qualitative and quantitative aspects of diets fed
beginning at 3 months of age indicate that diets that differ widely in composition can
induce precocious puberty. However, the appropriate timing and duration of this increase
nutrient intake which would hypothetically result in precocious activation of the
reproductive system remains to be tested in *Bos indicus* heifers. If Zebu heifers in Latin-
American countries could be predictably induced to reach puberty at 12 to 15 months of
age and calve at 2 years of age, the impact on future productivity within the production
systems in tropical environments would warrant careful consideration. The value of
induction of “precocious puberty” at 12-15 months of age in cattle of this type might vary
between region, environment and the nutritional resources available.
APENDIX A; BOVINE GROWTH HORMONE RADIOIMMUNOASSAY
This appendix describes iodinating bovine growth hormone (bGH) using NaI\(^{125}\) and chloramine-T and the use of the resulting labeled hormone for validation of a radioimmunoassay (RIA) for growth hormone (GH).

General Concept: Purified GH is mixed with NaI\(^{125}\) and the reaction started with chloramine-T. After 40 seconds, the reaction is stopped and the reaction mixture is placed onto a column of Sephadex G-75 Coarse (GR Healthcare, Uppsala, Sweden). Fractions are then collected and subsamples of each fraction are counted. Iodinated GH eluted in the first peak (fractions 7 to 9, and free \(^{125}\)I eluted in the second peak observed) (Figure A.1). Growth hormone was assessed by RIA using a double antibody system. Purified bovine growth hormone (AFP9884C) as well the first antibody (AFPB55) was obtained from the National Hormone and Peptide Program (Harbor-UCLA Medical Center, Torrance, CA). The second antibody (IR-HU-IGG, Goat anti human IgG antiserum) was obtained from Innovative Research (Novi, MI). Iodination of purified GH was performed using the chloramine-T method, and validation was performed via parallel displacement of labeled hormone by serial dilutions of bovine plasma samples (Figure A.2). Table A.2 depicts the recovery of bGH in samples from serum and plasma spike them with known concentrations of bGH.
Table A.1 Recovery of bovine growth hormone (bGH) in 50 μl of bovine plasma and serum with the addition of either 50 μl of 5 ng/ml of bGH, 10 ng/ml of bGH or 20 ng/ml of bGH.

<table>
<thead>
<tr>
<th>Sample (50 μl)</th>
<th>Spike (50 μl)</th>
<th>Concentration bGH (ng/ml)</th>
<th>Expect</th>
<th>%Recov</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(ng/ml)</td>
<td>(ng/ml)</td>
<td>(Obs/Exp)*100</td>
</tr>
<tr>
<td>Cow #206 Plasma</td>
<td>-</td>
<td>48.65</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cow #162 Serum</td>
<td>-</td>
<td>15.38</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>206</td>
<td>5</td>
<td>53.06</td>
<td>53.65</td>
<td>98.91</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>56.59</td>
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<td>96.49</td>
</tr>
<tr>
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Figure A.1 Typical fraction curve collected from purified GH iodinated with NaI¹²⁵ by the chloramine-T. After 40 seconds of reaction the mix is separated in the Sephadex 75 column and the fractions are collected.
Figure A.2 Parallelism of displacement of labeled hormone by serial dilutions of bovine known concentration samples.
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151


152
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