The Effects of Nutritional and Endocrine Manipulation on Testicular Development, Attainment of Puberty, and Sperm Production of Bulls

THESIS

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ABSTRACT

A limiting factor to genetic progress in the cattle industries is that the most popular, and oftentimes most genetically superior, sires cannot produce enough semen at an early enough age to meet demand. The growing trend of using genomically-tested sires exacerbates this problem. In this series of experiments, both dietary and direct endocrine manipulations were used in growing bulls with the goal of positively affecting several physiological changes that precede puberty.

The first experiment was conducted in order to examine the effect of a high energy (HE) diet on prepubertal endocrinology, age at puberty, mature sperm production, and testicular characteristics of Holstein bulls. It was hypothesized that the HE diet would cause bulls to attain puberty sooner, and produce larger testes that produced more sperm. In order to test this hypothesis, Holstein bulls were either placed on a HE diet (HE, n = 9, targeted ADG 1.5 kg/d) or a control diet (CONT, n = 10, targeted ADG 0.75 kg/d) from 58 to 230 d of age. HE bulls experienced increased systemic LH concentrations at 125 d, increased testosterone concentrations from 181 to 210 d, and increased scrotal circumference from 147 to 367 d of age. Semen collections beginning at 241 d of age to assess puberty on a subset of bulls (HE, n = 8; control, n = 7) revealed no difference in the age at puberty between treatments (310 \pm 10 d), and mature sperm production did not differ between groups during thrice-weekly collections obtained in the four weeks preceding slaughter at 569 d of age. Testis weight (318.0 \pm 13.5 vs. 267.5 \pm 14.4 g), epididymal weight (31.6 \pm 1.1 vs. 28.0 \pm 1.2 g), and testis volume (305.0 \pm 11.9 vs. 244.9 \pm 12.9 cm³) were greater (P < 0.05) in the HE treatment at 579 \pm 4.7 d of age (post mortem), but seminiferous tubule diameter and area comprised of seminiferous tubule did not differ between treatments (252.23 \pm 2.44 µm; 72.1%). These data suggest that a HE diet initiated at 2 mo of age accelerated many aspects of sexual maturation and resulted in bulls with larger testes. However, no differences were noted in age at puberty or production of semen as adults using the approach employed in this experiment.

Two subsequent experiments were conducted to examine the effects of a slowrelease exogenous FSH treatment on the endocrinology and testicular development of prepubertal bulls. In the first experiment bulls were treated at 50 and 53 \pm 3 d of age with 0.75 ml containing either 30 mg NIH-FSH-P1 in 2% hyaluronic acid (FSH-HA, n = 5) or saline (control, n = 5). Blood was collected every 6 hours for 24 hours after treatment and every 12 hours thereafter. Bulls treated with pFSH had significantly greater (P < 0.05) concentrations of FSH 6 h after treatment on d50 and 53, and a tendency (P \leq 0.08) for greater FSH concentrations 12 h after treatment administration on both days. There were no differences in serum concentrations of FSH between treatments from 18 to 84 h after each treatment. In a second experiment, a new set of bulls (35 \pm 6.5 d age; FSH-HA, n = 11; control, n = 11) were given the same treatment as described above every 3.5 d from 35 to 91 d of age, with blood collected to measure testosterone and FSH before each treatment and BW and SC measured weekly. FSH concentrations increased compared to previous levels in the FSH-HA treatment bulls beginning at 70 d of age, and this increase was maintained and greater than control bulls through 91 d of age. All bulls were castrated at 93 d of age to acquire gross measurements and histological data, and to perform immunohistochemical staining to visualize Sertoli cells. Concentrations of intratesticular testosterone, BW, SC, testis weight and volume, percent of parenchyma comprised of seminiferous tubules, tubule diameter, or testosterone concentrations did not differ between treatments. However, number of Sertoli cells as evidence by GATA-4 nuclear staining was greater (P < 0.05) in the FSH-HA than control bulls (28.27 \pm 0.9 vs. 33.35 \pm 0.9 cells/round tubule). In summary, exogenous pFSH treatment enhanced endogenous FSH secretion beginning at 70 d of age and increased the number of Sertoli cells at 93 d of age. Therefore, exogenous FSH supplementation was able to alter the mechanisms regulating endogenous FSH secretion as well as augment Sertoli cell proliferation in young bulls.

These experiments employing nutritional and direct endocrine manipulation during the prepubertal period show promise in their ability to change the testis in terms of overall size and cellular composition. Such changes have potential in causing differences in sperm production when bulls reach maturity. However, in the nutritional study, the effect of larger testes as a result of the HE diet on mature sperm production or age at puberty may have been diluted due to a switch in diet upon the move to Select Sires, Inc. that put HE bulls on nutritional restriction. In the experiment where bulls were administered exogenous FSH, all bulls were castrated before the time when they would attain puberty, but changes induced by treatment were promising for increasing mature sperm production. A longer study would have to be conducted in order to examine these effects, however. Overall, these technologies represent useful ways in which cause changes early in the life of bulls that may positively affect their mature productivity.

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If you faint in the day of adversity, Your strength is small. Proverbs 24:10

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Publications

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Fields of Study

Major Field: Animal Sciences

Reproductive Physiology

Table of Contents

Abstractii
Acknowledgmentsvi
Vitaix
List of Tables xiv
List of Figures xvi
Chapter 1: A Review of the Literature
Introduction1
Reproductive and Economic Efficiency of Bulls used for AI
Traditional Use of Bulls for AI
The Use of Bulls for AI in the "Genomic Era" 11
The Events Leading to Puberty in the Bull
Defining Puberty in the Bull
Overview of Endocrine and Testicular Physiology in Mature Bulls
GnRH, LH, Leydig Cells, and Testosterone
FSH and Sertoli Cells

Inhibin and Activin24
Development of the Reproductive Axis in Prepubertal Bulls
GnRH
Luteinizing Hormone (LH), Testosterone, and Leydig Cells
Follicle-Stimulating Hormone, Sertoli Cells, Inhibin, & Activin
Attainment of Functional Spermatogenesis
Interventions to Hasten Puberty in Cattle
Useful Applications for Precocious Puberty in Bovine
Exogenous Gonadotropins 39
Immunological Manipulation of Endogenous FSH
Dietary Manipulation 44
Inducing Hyper- and Hypothyroidism 46
Statement of the Problem
Chapter 2: Effects of Dietary Energy on Sexual Maturation and Sperm Production in
Holstein Bulls
Introduction
Materials and Methods
Results
Discussion

		72
	Introduction	72
	Materials and Methods	74
	Results	77
	Discussion	78
Chapter 4: Ef	fect of Slow-Release pFSH from 5 – 13 Weeks of Age on Endocrinology	7
and Testicular	Histology of Prepubertal Bulls	85
	Introduction	85
	Materials and Methods	87
	Results	94
	Discussion	95
Chapter 5: Ge	eneral Discussion 1	104
Literature Cite	ed 1	107

Chapter 3: Development of a Slow-Release FSH Treatment in Prepubertal Bull Calves.

LIST OF TABLES

Table 1.1. Summarized results of four experiments looking at direct (FSH
administration) and indirect (FSH immunization) effects of FSH given to prepubertal
male animals41
Table 1.2. Effect of various neonatal hormonal manipulations in rats on final number of
Sertoli cells and testis mass
Table 2.1. Composite of experimental diets fed to Holstein bulls from 58 to 230 d of age
(% as fed). Diets were adjusted biweekly to be fed at $2.5 - 3\%$ of BW65
Table 2.2. Mean LH concentration and amplitude of LH pulses of Holstein bulls fed
either a high energy (HE) or a control (CONT) diet from 58 to 230 d of age66
Table 2.3. Testicular measurements, seminiferous tubule diamter, and percentage of
testicular parenchyma comprised of seminiferous tubules for Holstein bulls fed either a
high energy (HE) or control diet (CONT) from 58 to 230 d of age67
Table 3.1. Percent binding of validated bovine and known porcine FSH standards
analyzed via double-antibody RIA80

Table 4.1. Testicular measurements, histological evaluations, and intratesticulartestosterone concentration (ITT) of one testis of 93 day old bulls treated with either 30mg pFSH or saline every 3.5 days from 35 to 91 days of age......100

LIST OF FIGURES

Figure 1.1. Overview of the Hypothalamo-Pituitary-Gonadal Axis in Bulls26
Figure 1.2. The prepubertal changes in LH and testosterone concentration, and Leydig
cell numbers in bull calves
Figure 1.3. The prepubertal changes in FSH concentration, numbers of Sertoli cells, and
the age which inhibin production, spermatogenesis, and puberty initiates
Figure 2.1. The weights of bulls fed a high energy (HE) or control (CONT) diet68
Figure 2.2. Number of LH pulses detected in an 8 h sampling period from Holstein bulls
fed either a high concentrate (HE, ■) or control (♦) diet69
Figure 2.3. Average testosterone concentrations detected after pooling hourly blood
samples taken over an 8 h sampling period of Holstein bulls fed either a high concentrate
(HE, black bars) or control (CONT, open bars) diet70
Figure 2.4. Mean (±SE) of the scrotal circumference of Holstein bulls fed either a high
concentrate (HE, ■) or control (♦) diet

Figure 3.1. Pictoral representation of treatment administration and subsequent blood
collections during the Slow-Release FSH experiment. A blood sample was collected
immediately before each treatment (beginning when bulls were 50 \pm 6 d of age) and
every 6 hours thereafter for a 24 hour period. Twenty-four hours following treatment,
blood samples to assess FSH were collected every 12 hours
Figure 3.2. Percent binding for validated bovine (\blacklozenge) and porcine (\blacktriangle) FSH standards of
known concentrations in a double antibody RIA82
Figure 3.3. Parallelism for pFSH standards in a validated bovine FSH RIA83
Figure 3.4. Systemic FSH concentrations of bull calves after injection with either 30 mg
NIH-FSH-P1 equivalent in a 2% Hyaluronic Acid Solution (FSH-HA, ♦) or saline
(control, \blacksquare) at 50 and 53.5 ± 6.5 d of age
Figure 4.1. Average systemic testosterone levels of bulls treated with either 30 mg pFSH
(FSH-HA, ■) or Saline (control, ♦) every 3.5 days from 35 to 91 days of age101
Figure 4.2. Average systemic concentrations of FSH as measured by a bovine/ovine
double antibody RIA immediately before bulls were treated with either 30 mg pFSH
(FSH-HA, ♦) or Saline (control, ■) every 3.5 days from 35 to 91 days of age102
Figure 4.3. Immunolocalization of GATA-4 in nuclei of Sertoli cells (SC) in bull
testes

CHAPTER 1

REVIEW OF THE LITERATURE

INTRODUCTION

Animals must reproduce in order to ensure the continuation of their species. Oftentimes, animals possessing traits that enhance their odds of survival are more likely to pass on their genes to offspring; this is the basic principle of "survival of the fittest" (Spencer, 1964). In agriculture, humans more strictly control the reproduction of farm livestock because these animals are domesticated, and it is decided by the farmer which males and females should be mated. Selective breeding allows for the fastest gain of desirable traits in a species, and humans have used selective breeding since the beginning of the agricultural era to increase the prevalence of desirable characteristics within a given livestock species. For example, the continued phenotypic and genetic improvement of beef and dairy cattle is important for human food production because it allows for the maximization of production of beef and milk from each type of cattle, respectively. The use of selective or intensive breeding in cattle, especially within the last 50 to 60 years, has allowed for impressive increases in milk and meat production within the most common cattle species (Ruaw et al., 1998). Consider the change within the U.S. dairy industry in the last 50 years: in 1961, 17.4 million dairy cows produced 125.4 billion

pounds of milk (USDA, 1964). In the year 2011, the number of dairy cows had dropped to 9.2 billion cows, yet these animals produced a total of 196 billion pounds of milk (USDA, 2012). Stated another way, cows have gone from milking 7,223 pounds per lactation fifty years ago to now producing an average of 21,345 pounds per lactation, and this 295% increase in production represents a great increase in efficiency. Today, based on consumer demand, a growing population, and increasing production costs, farmers and those providing genetics to the cattle industries still aim for continual advancement and improvement of efficiency in cattle. The rate at which producers can cause genetic change in their herds is accelerated by the reduction of the generation interval. The two main factors affecting the ability to advance genetics in cattle are the ability to identify the genetics of young animals and the physical ability of the animal to provide genetic material in terms of oocytes and sperm.

Many advancements have been made regarding the first of these topics, the identification of genetically-superior animals. The use of genomics, or the sequencing of an animal's genes in an effort to predict their worth and future productivity, has become increasingly common and allows for the use of very young animals for breeding. The genetic worth of a bull can be calculated or determined using genomics even before the bull reaches sexual maturity (Schefers and Weigel, 2012).

However, in order to naturally pass along genes, an animal must have reached reproductive maturity, which is marked by the attainment of puberty (Blood and Studdert, 1999). Herein lies the second main challenge for animal breeders: the youngest animals theoretically (and factually with the use of genomics) possess the most desirable traits, yet they are unable to pass these genetic improvements onto subsequent generations until they attain mature reproductive function.

The attainment of puberty, and our understanding of it, is becoming increasingly important in the cattle industries as more technologies are becoming available which can determine an animal's genetic worth at a very young age. Accordingly, the focus of research described in this thesis is on nutritional and hormonal regulation of sexual maturation in bulls and the methodologies that have the potential to accelerate this process.

REPRODUCTIVE AND ECONOMIC EFFICIENCY OF BULLS USED FOR A.I. <u>Traditional Use of Bulls for AI</u>

<u>History of AI</u>

The implementation of artificial insemination (AI) has undoubtedly been one of the greatest advancements in the cattle industries and animal agriculture in general. In the most basic sense, AI is any process by which sperm enters the female reproductive tract by means other than sexual intercourse. Put in a more accurate physiological context, AI is performed when previously-collected sperm or semen is manually deposited in either the vagina or uterus with the intent to cause fertilization of an oocyte. The history and experiments culminating to a successful mammalian AI span centuries and are spread across many countries.

The advent of animal fertility research on the cellular level began in the Netherlands when Leeuwenhoek, who is considered one of the founding fathers of microbiology, first observed spermatozoa with the aid of his assistant as early as 1678 (Leeuwenhoek, 1683). Then, nearly one hundred years passed before the first successful AI in mammals occurred when Spallanzani (1784) successfully inseminated a dog which went on to whelp puppies in Italy. Despite these early fundamental breakthroughs related to reproduction and fertility, nearly a century passed before novel experiments related to livestock were reported.

Advancements in AI specifically in cattle began to occur at a brisk pace in the first half of the 1900s. Russian scientist Milovanov first pioneered the use of an artificial vagina (AV) to collect bull semen in 1938. According to a review by Foote (2001), this was a milestone for livestock breeding and represented vast improvements over the thencurrent practice of collecting sperm via an intravaginal sponge. One of the first largescale cattle breeding experiments took place in Denmark in 1936 and was carried out by the first AI stud cooperative in the world and led by a Danish scientist, Sørensen. At the end of the first year of this experiment, the researchers recorded a conception rate of 59% after the AI of 1,070 cows (Sørensen, 1940). Growth of AI in America began early in the 1940s and relied purely on the sale of fresh semen. The first American AI co-ops began in 1938 in New Jersey and New York. One of these companies, the New York Artificial Breeders Cooperative, Incorporated, is still operating today as Genex, Inc. (Foote, 2001). Many other AI companies formed after New Jersey Artificial Breeders Cooperative, Inc., relying on the need for semen at a local level due to the inability to transport fresh semen long distances. It was the ability to successfully freeze and extend semen that quickly caused an overall decrease in the number of AI companies beginning in the 1950s

(Salisbury et al, 1941; Polge et al, 1949; O'Dell and Almquist, 1957; Polge, 1968). The need to have small, local semen collection facilities with the ability to ship fresh semen had diminished, and many companies began to consolidate. Only 5 AI companies provide 90% of the semen to American cattle producers today (Funk, 2006).

Since their start, the AI cooperatives each desired to own a unique and marketable product. Therefore, as the size of the dominant AI companies grew each aspired to own bulls that were genetically elite, the most in demand, and consequently the most profitable. It was surmised by DeJarnette, et al. (2004) that "it is not the responsibility of the AI industry to dictate to the producer what type of cattle they should be breeding nor to narrowly define what types of genetic packages will be offered for sale to the industry." The responsibility of the AI industry is to simply supply genetics that are in demand, and this concept shapes the acquisition of bulls by the AI companies.

Traditional Acquisition and Rearing of Bulls

From the beginning of the commercial AI industry until the last decade, the genetic worth of a bull was determined initially by the animal's pedigree and individual physical characteristics. As the bull obtained daughters, their performance in terms of milk production (dairy) and growth rate, birthweight, etc. (beef) increased or decreased his desirability amongst producers accordingly. It must be noted that much of the following information is biased towards the acquisition and selection of dairy bulls since it is a more recent and less-common occurrence that American beef producers are relying heavily on the use of AI when compared to dairy producers. The aforementioned scenario

in which bulls are selected based on pedigree and progeny testing will be referred to as the 'traditional' system in this literature review.

The traditional system is still in use today, and although it is no longer the only source of evaluation for bulls, it still serves as the basis from which genomic evaluations gain their reliability. In the traditional system, the AI stud company purchases bulls from purebred producers who have current elite genetics. When an AI company chooses to acquire a bull from a particular breeder, a contract is usually created and signed in order to ensure a particular mating between a certain cow and predetermined bull. The contract also guarantees that the farmer will not sell the offspring to another stud company. There are various ways to plan, breed, and acquire offspring to fulfill AI contracts.

Beginning at the farm, most AI bulls are a result of embryo transfer (ET; Teepker and Keller, 1989). The use of multiple ovulations and embryo transfer (MOET) has been used frequently since the late 1980s to increase the reproductive rate, increase genetic selectivity, and decrease the generation interval of valuable cattle (Smith, 1988). When using MOET, the dam of the bull calf will be superovulated using a regimented hormone treatment that will cause her to grow multiple follicles. The time of ovulation and estrus is closely calculated or watched, and the cow is artificially inseminated to the sire of choice at the appropriate time. Collection of the embryos has been thoroughly researched and is most successfully performed when the embryos are 6-8 days-old (Seidel, 1981). Next, the embryos are evaluated or 'graded,' and those deemed viable will be introduced into one of the uterine horns of a 'recipient' cow that is the appropriate stage of her estrous cycle or that has had her estrous cycle manipulated such that she will be able to sustain a pregnancy. A second way to accomplish the same goal is to collect oocytes directly from a superovulated cow, perform *in vitro* fertilization (IVF), allow embryos to mature *in vitro* for a short period, and then implant the embryos into recipient cows. This process is called *in vitro* embryo production (IVP) and is a feasible alternative to the aforementioned MOET system (Hasler et al., 1995). The AI center obviously seeks a male from the resultant calves in either scenario, and the more to choose from, the better. After these processes, the AI center will allow the bulls to grow for some months to allow them the possibility of physically differentiating themselves from one another. Once the time to select an individual arises, the stud company will consider overall physical soundness, health, growth, and testis size when making their decision. Only after careful qualitative and quantitative assessment will the company be able to select the prime specimen out of several 'littermates' (Amann and DeJarnette, 2012).

Traditionally, the age at which bulls are moved to the AI company varies from 4 to 16 months of age. However, the historical average for dairy breeds tends to be before the animal is 12 months of age, and the average for beef breeds generally occurs when the animal is more than 12 months of age (Monke, 2006). Bulls from different farms do not enter the stud rearing-facilities as a uniform group due to differences in the manner in which they have been reared. This variation was an accepted fact within the AI industry for many years, and this problem is exacerbated when the use of a contract in is effect since bulls purchased in this manner are 'pre-paid,' and there is not large incentive to provide specialized care prior to transport. As a result, it is not uncommon for contracted bulls to be maintained on pasture or with diets that meet maintenance needs, but which do

not sustain optimal growth, before they are moved to collection facilities. The variability in growth resulting from acquiring bulls at approximately 12 months of age is contrary to literature stating a steady rate of growth from birth to 12 months of age is optimal. According to protocols on raising dairy bulls before semen production, large breed dairy bulls should weigh 400-450 pounds at 6 months of age, and they should preferably weight 800 to 900 pounds by 12 months of age (Monke, 2002). A gradual, consistent rate of growth, rather than rapid or fluctuating growth, is most desirable. Overall, it is recommended that dairy bulls grow approximately 2 to 2.5 pounds per day during their first year, and that the daily dry matter intake (DMI) be about 3% of body weight during this time in order to ensure that a bull does not undergo delayed puberty (Monke, 2002).

Under-nutrition early in life can postpone sexual maturation. Malnutrition in 0 – 12 month old bulls can lead to delayed or impaired androgenic function, which may have later impacts on testicular development and sperm production (Cupps, 1991). A latter scenario is represented as over-nutrition, which can have negative effects preceding puberty as well. Rapid growth from 6 to 12 months of age may result in excessive fat deposition in the body and neck of the scrotum that can negatively affect sperm production at later times in the bull's life (Monke, 2006). This process coincides well with a growth response that has been noted in over-fed dairy heifers; prepubertal heifers fed in nutritional excess can experience adverse effects of rapid weight gain if excess body fat deposition occurs. The developing mammary systems of these heifers experience a decrease in paracrine production of growth hormone and total parenchymal DNA and

8

RNA, as well as an increase in the number of adipocytes and a decrease in the number of epithelial cells (Capuco et al., 1995).

The assumption that all bulls entering the semen collection facilities are vastly different in their development from one another may be an exaggeration of the norm, but extremes in both directions can be observed, especially before AI companies were fully aware of the abovementioned effects that nutritional differences can have on the future productive life of bulls. Today, AI companies manage nutrition of their bulls more carefully because they are owned from a younger age, as will be described later in this review.

Collection of Semen

In a traditional system, the phases that must be completed before a bull becomes marketable, or even advertised, take several years and represent one of the largest costs to the AI industry. As previously mentioned, bulls arrive to collection facilities by twelve months of age. Once a bull arrives at the AI center, he is subjected to a brief quarantine, but the commencement of semen collection will occur as soon as possible. It typically takes several attempts to train a young bull to interact with a teaser animal and finally ejaculate in to an artificial vagina after mounting. It is important to note this represents an ideal scenario where the bull does not experience phimosis, subnormal testes and epididymal development, or a delay in puberty, all of which will delay or hinder semen production (Don Monke, personal communication).

The initial semen collections obtained when the bull is approximately 12 months old will be used to inseminate cows in different young sire testing herds. This process is necessary because little is known of the bull's genetic value outside of his pedigree. Each bull acquired by the AI company must undergo a process of 'progeny-testing' in order to more accurately determine his genetic transmitting abilities. Furthermore, data must be gathered when his daughters begin to generate milk production records. This is the only true way to measure a bull's genetic worth in the traditional system. The interval from after a bull's initial semen collections to the time when his daughters have begun their first lactation represents a lapse in the productive life of bulls in the traditional system. In terms of time, the cows bred to the bull will gestate for nine months, and the resultant daughters will not begin a lactation until they are approximately 2 years of age. During this 'sire in waiting' period, the bull will most likely spend 'down-time' waiting in a holding facility, most often not being collected.

Determining Bull Genetic Worth and Profitability in the Traditional System

To further examine the process of progeny testing, milking daughters of a sire can begin to be evaluated for their type and production, and these included values can increase the accuracy of the bull's predicted transmitting abilities (PTAs). This process of progeny testing results in the generation of a "proof" for the bull that will be updated periodically (3 or 4 times per year). Progeny testing must be performed on all sires, yet relatively few individuals compared to the total number of bulls that are acquired become popular amongst producers. To put sire popularity into context, forty-three Holstein sires have succeeded in producing and selling over 1 million units of semen as of February 2012 (Geiger, 2012a). Additionally, it is believed that six Holstein sires have produced more than 1.5 million units of semen during their productive lives, and recently, one bull has surpassed the world record in number of units of semen produced and sold when he produced his 2 millionth dose of semen in May of 2012 (Geiger, 2012b).

The abovementioned bulls are the exception in terms of popularity, and there are many other bulls used to generate profit for each AI company. The paradox of this process is that for every sire that becomes popular, there are several hundred others that enter as prospects but do not become as popular. Given that in the traditional system all bulls are maintained for an average of 55 months before their first proof, this is a huge investment for the AI company (Amann and DeJarnette, 2012). Economically, the cost of 'sires in waiting' is substantial. In terms of genetic improvement, the time sacrificed waiting for proofs on new sires constrains genetic progress by increasing the generation interval.

Regardless of the obvious inefficiencies within the traditional system, it has served as a working business model for the AI companies since the 1950s. However, with the emergence of genomics to more accurately predict genetic merit of young animals from the day they are a multi-celled embryo, it is recognized by both AI companies and producers that changes are imminent.

<u>The Use of Bulls for AI in the ''Genomic Era''</u>

The use of genomics is changing the industry by adding powerful tools to select bulls that will be used for AI. It is important to note that while genomics are increasing in popularity and reliability, they will never fully replace the proof system. Proofs supply direct examples of a sire's transmitting abilities through daughter type and production, and it is this information that gives genomic predictions their reliability. Both genomic and proof-based estimates of sire fertility are incorporated into one dynamic system, and genomics will probably never be able to function autonomously. However, genomics are useful because they shift the emphasis from bull and daughter phenotype towards a combination of genotypic and phenotypic traits, or in some instances, entirely towards the genetics of an animal. The niche market of cattle genetics will be undergoing a "paradigm shift," according to Amann and DeJarnette (2012). The genomic era of cattle reproduction shows no signs of slowing in its implementation or utility. First, it is necessary to illustrate the basics of genomics and genomic selection in order to understand its impact on the AI industry.

Basis of Genomics

It is possible to predict the genetic value of both plants and animals using genome-wide maps that mark useful traits (Humblot, 2010). Regarding cattle, the combined efforts of over 300 scientists in 25 countries resulted in the mapping of the *bos taurus* genome sequenced from a Hereford cow. The bovine genome contains approximately 22,000 genes, including 14,000 that are common amongst all mammalian species (The Bovine Genome Sequencing and Analysis Consortium, 2009). Each of these genes is encoded by a sequence of the four repeating base pairs that comprise DNA, and they are present on one of the 29 pairs of autosomal chromosomes that cattle possess.

The genetics of *bos taurus* cattle species, irrespective of breed, encompass very little genetic variety. However, it is the differences in the sequence of base pairs that make one animal unique from another. These differences arise at the point of fertilization

due to the randomness of which sperm and egg unite; each will already represent a random contribution of genetics belonging to the paternal or maternal side, respectively. The two genomes of the sperm and egg combine in such a way that the resultant offspring's genome possesses inherent differences from the sire and dam's genotypes. The randomness of the new organism's genome further stems from differences due to crossing over during initial fertilization, and these occurrences ensure the organism has a different genetic code from that of any other organism.

These abovementioned processes encompasses the large, endogenous variations between the genes of individuals of the same species, but even differences as small as a single base pair can have an effect on the phenotype of the animal. The difference in one base pair between genes serving the same function is referred to as a single nucleotide polymorphism (SNP). SNPs occur randomly throughout the genome, and on average there is a SNP present every 700 base pairs in *bos taurus* cattle (The Bovine HapMap Consortium, 2009). Inferences can be made based on large numbers of individuals that a difference in a particular SNP is associated with a certain phenotype. SNPs can be considered a marker for an allele, or variation of a gene, and by comparing enough resultant phenotypes it can be accurately predicted whether a specific gene is present or not in an animal. In essence, SNPs are the genetic markers used in the cattle industries to detect differences between individuals (Seidel, 2010).

The mathematical calculations used to compare individuals in this manner are enormous, but the technology and the large number of records make comparisons possible. As more animals are added to the genomic database, the accuracy of this type of data increases. Today's geneticists are confident enough to commercially offer SNP testing that concludes that if an individual lacks or possesses certain SNPs, then they are likely to have a given level of performance.

It is pertinent to briefly state the process by which the cattle industries are measuring and utilizing SNP data. Many sources identified SNPs within the bovine genome, and there are well over 2 million SNPs identified (Margulies et al., 2007; Matukumalli et al., 2009; The Bovine Genome Sequence and Analysis Consortium, 2009; Bovine HapMap Consortium, 2009). This has led to the development of the SNP genotyping microarray technology for cattle. The most common 'SNP chip' used for cattle is made by Illumina Inc., and is labeled the Illumina BovineSNP50 BeadChip (Illumina., San Diego, CA). The chip reads the entire genome and alerts of the presence of approximately 50,000 pre-selected SNPs, and the resulting SNP profiles effectively predict several phenotypic performance parameters of cattle (VanRaden et al., 2009). According to a recent report by VanRanden et al. (2009), over 5300 Holstein bulls and their progeny data have been inputted into one data set, making the genomic predictions for Holsteins using the Illumina BovineSNP50 BeadChip the most accurate to date.

Changing Demographics of Bulls in AI

The capacity to more accurately predict genetic merit of bulls with genomics has substantially impacted management and acquisition of sires by AI companies. The old paradigm for which progeny testing was the ultimate test of genetic merit is being modified by genotyping. In recent years many bulls have been selected initially on SNP data, with daughter proofs serving as a later addition to verify his genetic superiority (Amann and DeJarnette, 2012).

With genomics, an AI company will not choose bulls to enter their system that are 12 months of age, but rather will choose bulls at birth or even before by genotyping embryos and propagating those that contain a desirable genetic make-up (Humblot, 2010). Pregnancy and implantation rates are not affected by the embryonic genotyping procedure (Ponsart et al., 2008; Humblot, 2010), and genotyping embryos can further hasten the rate of genetic progress (Seidel, 2010). In the future, phenotype may simply be used to remove physically undesirable animals from a pool of potential AI sires.

The use of sires that are younger than ever before will affect the turnover rate of sires within the AI companies. In the genomic era it may be a rare occurrence to see bulls older than 5 years of age in collecting facilities. Young desirable sires will be marketed as soon as possible and will dominate the facilities and active lineups (Amann and DeJarnette, 2012). The use of genomically-selected bulls may quickly make the genetics of traditionally raised bulls obsolete, and the scenario could exist where bulls raised on a traditional system may have to compete with their genomically-selected sons, as their sons will not go through a 'sire in waiting' period and will possess current and more desirable genetics (Boichard, 2010).

As AI companies transition to marketing a larger proportion of their bulls based on genomics, their facilities and traditional housing methods will need be revamped to accommodate all ages of sires. It is now commonplace for AI companies to be in ownership of young calves that are not weaned, and these large numbers of immature animals will put stress on the infrastructure of housing. In the genomic era, all bulls would be housed in individual production stalls by 10 months of age, as this age represents the target for initiation of semen collection for young bulls today (DeJarnette, personal communication). Sires will remain in individual collection stalls for the remainder of their productive lives, which is in contrast to the traditional system where bulls assigned as 'sires in waiting' are not collected and may be group housed. Overall, there will be an increased number of young, extremely valuable young calves replacing the space originally needed for 'sires in waiting,' and there may be a decrease in space needed for mature, actively-producing sires because the turnover rate will be greatly accelerated due to genomics.

Operational Changes Within AI Centers

The reproductive efficiency of this changing young population of bulls will be different from older, more mature population of bulls of the past. Bulls are introduced to collection facilities beginning at 10 months of age, but the bull's scrotal circumference (SC) is probably the best estimate of his production potential. Bulls should have a SC of 30 to 32 centimeters before attempting semen collection (DeJarnette and Monke, personal correspondence). There are problems associated with semen collected from sires that are not yet fully mature. Semen from young animals may have too high a proportion of seminal fluid in their collections, and the total number of sperm cells they produce will always be lower than that of mature bulls (Monke, personal correspondence). Also, bulls that have recently attained puberty may fail to produce sperm of acceptable motility or enough sperm of acceptable quality to warrant packaging and cryopreservation (Amann

and DeJarnette, 2012). Unfortunately, it may take several years after the start of collections at 10 months of age for bulls for reach full production capacity (Monke, personal correspondence), and this will conflict with the fact that as much semen as possible is needed from a high-genomic sire as soon as he begins collections.

Lastly, there will be a transition and reallocation of funds within the industry in the genomic era. The largest and most apparent change will be in the acquisition costs of bulls. Bulls purchased based on pedigree information are a large investment to AI companies, but more so due to the large numbers of animals that are purchased, not necessarily because the acquisition cost of any one bull is exorbitant. Stated directly, the cost of each sire has increased significantly in recent years due to genomics. Even though eight AI companies provided funds to develop the Illumina BeadChip technology used to genomically test AI sires, breeders have quickly realized the worth of a genomicallysuperior bull and the acquisition costs of sires have increased a reported 4-5 times since the incorporation of genomics into the selection process. It is also becoming frequently common for breeders to choose to lease their bulls to AI companies rather than sell them outright (DeJarnette, personal correspondence). In anticipation of rising sire costs, several AI companies have begun owning and breeding females of high genetic merit with the goal of executing an internal breeding program and circumventing the rising costs.

Other notable financial changes that can be associated with genomics include the cost of labor for the AI companies due to the increasing need for more employees specializing in raising calves from birth. Also, collection schedules of greater intensity for popular bulls will translate into the need for more workers and facilities, or perhaps

the addition of extra days (i.e. weekends) for collection, processing, and freezing (Amann and DeJarnette, 2012). Intellectual property, such as the development of the Illumina BovineSNP50 BeadChip, had cost the major stud companies millions of dollars for use, and there may be similar technological advances in the future requiring the input of resources from stud companies and farmers alike (Seidel, 2010). Finally, the cost of a straw of semen for a producer may change in the future, but to what degree is uncertain. Farmers will probably pay a premium to utilize the most recent, in-vogue sires as they are released into the active lineups, but this is no different than what occurs in the traditional marketing scheme. Genomically superior sires' semen may sell for more than today's average semen price, but it has been argued that AI centers expect to house and maintain less bulls overall, perhaps evening out or controlling any drastic increases in semen price.

THE EVENTS LEADING TO PUBERTY IN THE BULL

Defining Puberty in the Bull

The attainment of puberty in bulls is marked using two criteria. The first marker is the ability of an animal to produce an ejaculate containing 5.0×10^7 sperm with 10% motility, and the second is the attainment of a scrotal circumference (SC) of 28 cm (Wolf et al., 1965; Lunstra, 1982). Often, the attainment of 28 cm SC precedes the animal's ability to produce the required amount of sperm (Wolf et al., 1965). Past studies quantifying puberty have used one or both of these definitions, but SC is the easiest and most readily obtained of these two parameters. The age at which puberty begins for conventionally-raised bulls varies by breed. For example, previous studies report puberty
at 326 d in Hereford bulls, 295 d in Angus bulls, and between 273 and 287 d of age for Holstein bulls (Lunstra et al., 1978, 1982).

In a practical sense, even bulls that meet the abovementioned physiological criteria may have some physical limitations that prevent their semen collection. For example, bulls are born with an intact penile frenulum, or a small fold of skin that secures the penis to the prepuce and prevents it from exiting the body cavity. It is believed that sufficient testosterone production in the time leading up to puberty causes the natural degeneration of the frenulum beginning around 4 months of age (Gill, 2003; Monke, personal correspondence). It was estimated from a large study of post-pubertal beef bulls of varying ages and breeds that approximately 3% of bulls exhibit physical abnormalities, including a persistent frenulum, that would make them fail a breeding soundness exam (BSE) even though they have attained all other physically aspects puberty (Kennedy et al., 2002). Such physical boundaries to successful semen collection will become increasingly important to the AI industry because of the necessity to collect bulls at younger ages than ever. Don Monke of Select Sires, Inc. has examined thousands of prepubertal bulls of varying breeds during their development. According to his records, less than 5% of bulls have a persistent frenulum at 12 months of age, but this proportion increases slightly at 11 months of age and more so at 10 months of age. This defect results in a condition termed phimosis (inability to extend the penis) which requires surgical intervention if the bull is to be collected (Monke, personal correspondence).

The two previously-described criteria for puberty serve as useful tools for researchers in terms of comparisons for experimental endpoints. While the ability to

ejaculate 5.0 x 10⁷ sperm with 10% motility may be the more functional of the two definitions, when resources, personnel, and facilities to house and collect developing bulls are lacking the criterion of SC still serves as a valuable tool for estimating bull fertility because it is known that testicular size, and therefore size of the scrotum, is highly correlated to the amount of semen a bull is able to produce (Almquist et al., 1976). Despite the evolving environment of the AI companies, the described two-parameter definition of puberty has not been altered since it originated in 1965, and it serves as a common physiological linkage between the majority of bovine puberty studies.

OVERVIEW OF ENDOCRINE AND TESTICULAR PHYSIOLOGY IN MATURE BULLS

An understanding of postpubertal male reproductive endocrinology and physiology aids in the understanding of the events occurring before puberty. The hypothalamo-pituitary-gonadal axis (HPG axis) controls the prepubertal maturation and the postpubertal function of the male reproductive tract and spermatogenesis.

GnRH, LH, Leydig Cells, and Testosterone

Starting with the hypothalamus, its major role in males is to secrete the decapeptide hormone GnRH (Figure 1.1). GnRH is synthesized within the arcuate nuclei of the hypothalamus and released in a pulsatile manner from specific GnRH-releasing neurons in mature males. These GnRH-releasing neuron's axons originate in the surge and tonic centers of the hypothalamus and release GnRH where they terminate upon blood vessels of the hypothalamo-hypophyseal portal system in the median eminence of

the hypothalamus. Once in the hypothalamo-hypophyseal portal system, GnRH's target receptors lie in close proximity, in the anterior pituitary, and secreted GnRH travels through the hypophyseal portal system to reach this tissue (Schwanzel-Fukuda and Pfaff, 1989). Upon release from the portal vasculature, GnRH reaches its cellular target tissue, the gonadotropes of the anterior pituitary. Here, the binding of GnRH to GnRH receptors (GnRH-R) in the anterior pituitary stimulates these cells to produce and release the hormones follicle-stimulating hormone (FSH) and luteinizing hormone (LH; Campbell et al., 2009).

The steps involving the release of LH from the anterior pituitary in response to GnRH are dynamic (Schanbacher and Echternkamp, 1978), and the time it takes for a GnRH pulse to elicit an LH pulse has been reported. Injection with 0.25 mg synthetic bovine GnRH to 13 months old bulls caused increased LH concentration in the periphery within 15 minutes of treatment. The main target tissue of LH in the reproductive axis is the testicular Leydig cells, which reside in the interstitial space of the testis outside of the seminiferous tubules. Mature Leydig cells function in producing testosterone upon LH binding to LH-R. Injection of GnRH as previously mentioned caused testosterone levels to increase above control levels from 60 to 120 minutes after the treatment (Malak and Thibier, 1982). Testosterone is then able to regulate hypothalamic hormone release as well as spermatogenesis in mature bulls most notably by feeding back negatively on the anterior pituitary and hypothalamus (Schanbacher, 1982). However, testosterone's major role in the adult involves the maintenance of spermatogenesis. Testosterone concentrations will begin to reach levels similar to those seen in adulthood around 6 months of age in bulls (Miyamoto et al., 1989), and concentrations within the testes will also increase drastically at this time. The concentration of testosterone within the testis is high due to binding with androgen binding protein produced by mature Sertoli cells (Dadoune and Demoulin, 1993). It is usual for the concentration of testosterone within in the rat testis to be between 50 to 100 times greater than that of the periphery, and at least 70 ng/ml of testosterone within the seminiferous tubules is required for spermatogenesis in the rat (Senger, 1997). These high concentrations are believed to be crucial for prevention of degradation of maturing spermatocytes (Walker and Cheng, 2005).

FSH and Sertoli Cells

GnRH controls another 'branch' of the reproductive axis which consists of the production of the hormone FSH and its target, the Sertoli cells, which are needed in order to facilitate spermatogenesis. In similar fashion to LH, FSH is produced by the anterior pituitary in response to GnRH binding GnRH-R, although most likely not in a pulsatile manner (Dunkel et al., 1992; Genazzani et al., 1994). FSH has target receptors on the Sertoli cells of the testis, and as previously mentioned, causes production of ABP by the Sertoli cells (Dadoune and Demoulin, 1993). FSH is also necessary for the general viability of Sertoli cells (Bagu et al., 2006).

In the adult, Sertoli cells line the seminiferous tubules and are responsible for supporting the maturation of spermatogonia to elongated spermatids. Specifically, Sertoli cells regulate the biochemical surroundings of germ cells because they enclose the cells, thus forming a distinct environment within the seminiferous tubules (Griswold, 1998). Sertoli cells aid in sperm production while maintaining high intra-tubule testosterone levels via the blood-testis barrier (Gilula et al., 1976). Tight junctions primarily function to form the blood-testis barrier which prevents immunologic destruction of developing germ cells. Furthermore, once tight junctions form, primary spermatogonia lose all external sources of nourishment and become solely dependent on their host Sertoli cells as they mature to elongated spermatids (Senger, 1997).

It is known that a mature Sertoli cell 'hosts' a fixed number of germ cells in bulls and other non-seasonally breeding mammals (Blanchard and Johnson, 1997; Leal et al., 2004). It was initially believed that a lack of Sertoli cell proliferation in the mature male was conserved across all species, but recent literature has challenged this concept. For example, Djungarian Hamsters have reduced Sertoli cell numbers when exposed to short day length. It is believed that the decrease in the number of viable Sertoli cells results from decreased gonadotropin production resulting from shorter daily photoperiod (Meachem et al., 2005). The Sertoli cells of these hamsters may be able to revert to an undifferentiated state during the nonbreeding season and exhibit characteristics common to both undifferentiated and mature Sertoli cells (Tarulli et al., 2006). Interestingly, exogenous FSH supplementation can restore breeding-season Sertoli number and cause resumption of spermatogenesis in hamsters that have been experimentally exposed to short day length (Lerchl et al., 1993). Stallions of all ages also experience changes in their numbers of Sertoli cells depending on the season (Johnson et al., 1991). However, in-depth studies of hormonal manipulation such as those performed in the hamster have not been completed in stallions.

While Sertoli cell numbers in some species do have the capacity to change after adulthood is reached, it is important to emphasize that this is not common across the majority of mammalian species, and in bulls the numbers of Sertoli cells within the testes exhibit no tendencies to differ with season (Curtis and Amann, 1981; Hochereau-de Reviers et al., 1987).

Inhibin and Activin

Inhibins and activins are dimeric glycoproteins that fit dynamically into the abovementioned processes, essentially working as brakes and accelerators to the system by controlling FSH production and secretion by the anterior pituitary (Vale et al., 1988). Both of these proteins are formed from combinations of the same dimers that are either designated as α -subunits or β -subunits. Furthermore, β -subunits have one of five varieties that are designated β_A through β_E , but only the β_A and β_B subunits have physiological effects in animals (Mason, 1987).

Inhibins are comprised of heterologous pairings of α - and β -subunits, with the two main types of inhibin being inhibin A and inhibin B, nomenclatured $\alpha\beta_A$ and $\alpha\beta_B$. Activins are homologous dimers of two β -subunits. The pairs of dimers form one of three forms of activin: Activin A ($\beta_A\beta_A$), activin AB ($\beta_A\beta_B$), and activin B ($\beta_B\beta_B$; Mason, 1987). Inhibin plays a negative regulatory role with respects to FSH production. It is produced by Sertoli cells upon the binding of FSH to the FSH-R located on the cell membranes and negatively feeds back on the gonadotrophs of the anterior pituitary to prevent further FSH production and release. In essence, circulating levels of inhibin relate inversely to circulating FSH in the male (Figure 1.1; de Kretser and Robertson, 1989). It was first observed that inhibin decreases the production of FSH from the anterior pituitary by injecting charcoal-extracted bovine follicular fluid (containing inhibin) into bulls aged 1-2 years of age and observing the decrease in circulating FSH (McGowan et al., 1988). Inhibin also positively correlates with the number of Sertoli cells present within the testes of rats, and this relationship hints at the possibility for the estimation of Sertoli cell number noninvasively via the systemic inhibin levels in other species (Sharpe et al., 1999). Additionally, since it is known Sertoli cell number in humans is correlated with mature sperm production, the level of systemic inhibin could possibly even be used to estimate the mature sperm production in other animals such as bulls (Johnson et al., 1984).

The role of activins in the mature male are less evident, but it is known that activin is produced and secreted by the adult Sertoli cells and has nourishing autocrine effects on the cells (Buzzard et al., 2004). However, the main source of activin is from outside the tubules, probably from peritubular myoid cells (Buzzard et al., 2003). The role of activin in the postnatal male will be discussed more thoroughly in later sections.

All aspects of the mature HPG-axis described must work in concert to allow the male to attain the endocrine milieu necessary to carry out functional spermatogenesis. Furthermore, there are several necessary cellular components, as well. This section has highlighted the working parts needed as machinery for spermatogenesis in mature bulls. The development of these systems, which is perhaps even more influential on this thesis' research, is discussed next.



Figure 1.1. A basic representation of the hypothalamic-pituitary-gonadal axis (HPG). Products of each organ applicable to male reproduction are indicated. Positive or synergistic effects are noted by dashed arrows. Negative feedback from the products of the testis (inhibin and testosterone) are indicated by curved, solid arrows.

DEVELOPMENT OF THE REPRODUCTIVE AXIS IN PREPUBERTAL BULLS <u>GnRH</u>

GnRH serves as an initial stimulus for the commencement of several prepubertal processes, and its release is therefore a crucial beginning step in the formation of a functioning endocrine system. There is a 'priming' that occurs in the hypothalamus and

pituitary early in the life of bulls (i.e. 6 - 10 weeks of age) that is a crucial first step towards puberty.

It was originally believed that there is a hormonal block to the production of GnRH in the postnatal bull (Amann et al., 1986), with circulating androstenedione or its metabolite, estradiol, being the likely sources (Shaw et al., 2010). However, androgen blocking of GnRH does not seem to occur early in life because castration (removal of androgen source) at 10 - 12 weeks of age does not immediately increase GnRH or LH discharge. Rather, castration results in a 4-8 week delay before an increase in LH secretion is observed (Bass et al., 1979; Wise et al., 1987). These early findings concluded that estradiol was the main hormonal block in the neonatal and early prepubertal stages in the bull. This fit appropriately, because while estradiol occurs in prepubertal males (McEwen, 1980; Dickson and Clark, 1981), as well as the adrenal glands (Henricks et al., 1988).

More recent research has revealed that there may indeed be GnRH secretion in the postnatal bull. There is not, however, concomitant increases in LH. According to Rodriguez and Wise (1989), GnRH pulses are detectable in bulls at 2 weeks of age, however, pulsatile LH is not detectable until 8 weeks of age. These authors cite a general lack in the capability of the pituitary gland to respond to hypothalamic stimuli (Rodriguez and Wise, 1989; Miller et al., 1996). These results refute earlier studies by Lacroix and Pelletier (1979) and Amann et al. (1986), which found no differences in GnRH concentrations in the median eminence of bulls before 18 weeks of age. However, the

27

study by Amann et al. (1986) does correspond with reports of the pituitary's inability to respond to GnRH, since the GnRH receptors (GnRH-R) within the anterior pituitary increase significantly (314%) between 6 to 10 wk of age, and the concentration of LH increases in the anterior pituitary during the same age interval by 67%. In conjunction with these changes, estradiol receptors (estradiol-R) in the hypothalamus decrease by nearly 70% during this time. Overall, it is hypothesized that these early changes allow for the necessary LH release in response to GnRH that initiates puberty (Amann et al., 1986). Studies seeking to link the GnRH and subsequent LH increase reveal that there is little-to-no discharge of LH from the anterior pituitary before 10 wk of age. However, when exogenous GnRH is given before 10 wk of age, the anterior pituitary is able to secrete LH (Rodriguez and Wise, 1989).

Overall, understanding of the events underlying postnatal to pubertal GnRH release have developed greatly over time, but all allude to the necessity of a priming stage in the prepubertal hypothalamus. More importantly, while the hypothalamus may function in producing GnRH early in the life of the bull, one of its most important target tissues, the anterior pituitary, needs several more weeks in order to gain responsiveness and the ability to produce LH.

Luteinizing Hormone (LH), Testosterone, and Leydig Cells

The prepubertal changes in LH are well researched and studies measuring the postnatal levels up to 25 weeks of age in the bull are fairly consistent in their findings. Most reports state that there is a transient increase in LH beginning at 6 weeks of age that continues until a decline at approximately 20 weeks of age (Amann et al., 1986; Evans et al., 1995; Madgwick et al., 2008). Others (Rawlings et al., 2008) have reported that the initial rise in LH occurs between 4 to 5 weeks of age, with a peak at 12 – 16 wk of age, and then a decrease at 25 wk of age. Most attribute this transient LH increase as the result of increased LH pulse frequency, which would be the direct result of increases in GnRH release (McCarthy et al., 1979; Amann, 1983; Rawlings and Evans, 1995). This early 'gonadotropin rise' coincides well with reported concentration of LH-receptor (LH-R) in the testes, which decrease in number from 17-25 weeks of age and then increase up to 56 wk of age. The cause of this nadir in LH-R may be an affect of fetal Leydig cells being replaced by adult, mature Leydig cells at around the 25 wk age mark (Bagu et al., 2006). As far as the latter decline in LH at approximately 20 wk of age, this is most likely caused by rising androgen levels after this age; LH that served to cause Leydig cell maturation experiences negative feedback from the androgen product of these same cells (Bagu et al., 2006).

The increase in LH secretion precedes, and is thought to be responsible for, a period of rapid testicular growth that begins at approximately 20 wk of age (Amann and Walker, 1983; Bagu et al., 2006). In support, suppression of the LH increase (10 - 14 wk of age) in postnatal bulls with Leuprolide acetate delays testicular development indefinitely (end of experiment at 50 wk of age) beginning at 20 wk of age due to low LH, FSH, and testosterone levels (Chandolia et al., 1997a). The pattern of LH secretion during the early gonadotropin rise in calfhood is necessary and important because it is considered one of the main factors in determining the age at which bulls reach puberty

(Brito et al., 2007). A visual and relative graphical representation of LH and testosterone secretion in the bull, as well as the early effects of these hormones on Leydig cell proliferation, can be seen in Figure 1.2.

In postnatal bulls the concentrations of testosterone are low before 12 wk of age, especially when compared to concentrations in bulls older than 12 wk old (Lacroix and Pelletier, 1979; Amann and Walker, 1983). Other complementary studies (Amann et al., 1986) cite that T levels are undetectable at birth, but increase significantly at 14 wk of age, and then again at 18 wk of age. Testosterone does not show marked increases until after the early gonadotropin rise in bulls, and this is consistent with what is seen in other species such as rams (Wilson and Lapwood, 1979) and rats (Kolho et al., 1987). To bring the initiation of testosterone production into temporal focus, the early rise in gonadotropins (FSH and LH) most likely causes the proliferation and final maturation of Sertoli and Leydig cells, respectively (Curtis and Amann, 1981), and these cells work concurrently to produce testosterone (Leydig) and maintain (ABP, Sertoli) the high concentrations needed for spermatogenesis to begin at approximately 20 wk of age (Amann and Walker, 1983).

The source of testosterone in males, the Leydig cells, was described as early as 1850 by Franz Leydig of Germany. It was initially concluded that they exclusively populate the testis interstitium, but their true function was not published until many years later. It was not until 1905 that Leydig cells were described as having endocrine function and as being responsible for male secondary sex characteristics (Bouin and Ancel) and not until 1958 that Leydig cells served as the main source of androgens in the mature male (Wattenburg). Today, it is known that there are two distinct populations of Leydig cells. Fetal Leydig cells are those that are established prenatally and are present immediately after the birth of the animal. Adult Leydig cells represent the type of cells present for the duration of the male's life after the fetal cells differentiate. Fetal and adult Leydig cells derive from mesenchymal stem cells prenatally and postnatally, respectively (Mendis-Handagama and Ariyaratne, 2001).

To summarize early development, Leydig cells first derive from the mesenchymal stem cells that have migrated to the testis interstitium. These mesenchymal stem cells differentiate into progenitor cells of the testis, and then they continue their development into fetal Leydig cells under the influence of thyroid hormone. Thyroid hormone is believed to be one of the main factors causing differentiation of mesenchymal cells into progenitor cells and then into fetal Leydig cells (Mendis-Handagama and Ariyaratne, 2001).

The interplay between Leydig cells, testosterone, and LH are dynamic during development. It is known that the concentration of LH-R on Leydig cells increase significantly around the time of birth in males, and levels are probably near their highest at, or soon after, birth (Purvis et al., 1997; Hardy et al., 1990; Bagu et al., 2006). Concentration of LH-Rs decrease from 13 to 21 wk of age as the number of undifferentiated progenitor and fetal Leydig cells undergo differentiation. However, while the number of LH-Rs is decreasing, their equilibrium association constant (K_a, the measure of how well a receptor binds target molecule) or receptor 'affinity' is maintained from birth through puberty. During this time, Leydig cells do not lose effectiveness in

31

binding the LH coming from the periphery as evidence of a stable K_a, and this is important to the testosterone production and rapid testicular growth observed starting at 28 wk of age (Bagu et al, 2006). After this time, Leydig cells have gained mature function and will serve as the main source of androgens in the mature male.



Figure 1.2. The prepubertal changes in LH and testosterone concentration in bull calves. LH levels begin to rise at 6 wk of age as the pituitary gains GnRH-R (Amann et al., 1986). Leydig cells begin to proliferate in response to the transient increase in LH (Curtis and Amann, 1981; Bagu et al., 2006), and their final maturational processes make it possible for them to produce testosterone at approximately 20 weeks of age (Curtis and Amann, 1981). The production of testosterone negatively feeds back on gonadotropin (LH) secretion, and a decrease in LH is seen around 20 wk of age (Bagu et al., 2006).

Follicle-Stimulating Hormone, Sertoli Cells, Inhibin, and Activin

Pituitary-derived FSH exerts its effects on developing germ cells via the FSH receptors (FSH-R) on Sertoli cells (Senger, 1997). Unlike the overall supportive and agreeing studies of prepubertal LH secretion in the bull, studies of prepubertal FSH secretion are more variable, with some reporting no significant increases after birth during the time preceding puberty (McCathy et al., 1979), while others report a transient increases in FSH beginning at 4 wk of age that lasts until 25 weeks of age, at which time levels decrease (Evans et al., 1993; Rawlings and Evans, 1995; Aravindakshan et al., 2000; Bagu et al., 2006). Other studies report a transient increase of a longer duration, such as Amann and Walker (1983), who report the increase at 4 weeks of age, but lasting until 32 wk of age.

When examining FSH signaling, the increasing levels beginning at 4 weeks of age reported by Bagu et al. (2006) may play a role in inducing the formation of FSH-R on Sertoli cells. In this study, the concentrations of FSH-R were stable from 5 to 17 wk of age, but levels began to fall at week 25, and this was attributed to the switch from indifferent supporting cells to Sertoli cells occurring at this time. FSH-R concentrations then increased at 29 wk of age and continued to increase until the last day of Bagu et al.'s experimental observation at 56 wk of age. The authors cite that this later increase in FSH-R concentration as puberty nears may be a function of Sertoli cells gaining maturity. The changing dynamics of Sertoli cells, FSH, and inhibin are highlighted in Figure 1.3. In the

same experiment (Bagu et al., 2006), FSH-R affinity for FSH (K_a) was consistent regardless of the systemic FSH levels during the prepubertal period. This unchanging FSH-R K_a coincides with the fact that Sertoli cells must remain sensitive to FSH during the early prepubertal period in order for functional spermatogenesis to ensue (Orth, 1984).

In the bull, the transition from undifferentiated supporting cells to Sertoli cells is rapid between 13 and 25 wk of age, and rapid testicular growth occurs after this time beginning approximately at 25 wk of age (Bagu et al., 2006). During this differentiation, Sertoli cells undergo distinct changes in morphology, including an increase in size, becoming more columnar in shape, and acquiring a tripartite nucleus consisting of three distinct visual parts (Sharpe et al., 2003). These nuclear parts include a large, predominant nucleolus and two satellite karyosomes. This unique shape to the nucleus is a distinguishing characteristic of mature Sertoli cells and allows for their visual identification in histological sections (Sharpe et al., 2003). This period of Sertoli cell proliferation and its connection to FSH concentrations has become a focus of research foremost because FSH has a positive stimulatory effect on the rate of Sertoli cell proliferation (Orth, 1984). The relevance of this occurrence is that prepubertal Sertoli cell proliferation can be tied to adult sperm production potential because the ability of Sertoli cells to divide and proliferate under the effects of FSH is lost once the cells have fully matured (Sharpe et al., 2003).

Inhibin and activin's actions in the developing bull are to regulate FSH production and action. Regarding the chronology of inhibin production in prepubertal bulls, inhibin is initially detectable at 8 wk of age (MacDonald et al., 1991). Furthermore, the level of inhibin secretion begins to relate inversely to FSH levels beginning at 10 wk of age. This is roughly the time that Sertoli cells gain the ability to reflexively produce inhibin in response to an increase in systemic FSH (MacDonald et al., 1991).

Activins, in contrast to the known narrow range of effects of inhibins, have more effects on the body during pre- and post-puberty than just the regulation of FSH release. For example, it is believed that activins play a large role in mammals from embryonic development through adulthood. Transgenic mice deficient in activin production have skeletal and facial abnormalities, defective FSH production, and compromised reproductive performance throughout their lives (Matzuk et al., 1995). In normal males, activins levels 'counteract' the actions of inhibin by stimulating basal and GnRH-induced secretion of FSH from the anterior pituitary (de Kretser and Phillips, 1998).

Males experience their highest levels of circulating activin after birth, and activin concentration is correlated to Sertoli cell proliferation at this time and may be the purpose behind high concentrations postnatally (Barakat et al., 2008). Activin's synergistic relationship to Sertoli cell proliferation decreases as the animal ages, however, and activin levels decrease at about the same time the transiently elevated gonadotropins begin to decline (Buzzard et al., 2004). The fact that the levels of activin and FSH decrease upon the cessation of Sertoli cell proliferation indicate that these may be the main factors regulating and fostering the postnatal increase in Sertoli cell numbers (Buzzard et al., 2004).



Figure 1.3. Pictorial representation of the prepubertal changes in FSH concentration and numbers of Sertoli cells in bulls. There is a transient increase in FSH in the postnatal bull (Amann and Walker, 1983; Bagu et al., 2006; Aravindakshan et al., 2000; Evans et al., 1993; Rawlings and Evans, 1995). This early rise in FSH causes the proliferation of Sertoli cells (Orth, 1984). As Sertoli cells proliferate and mature there is an inverse decrease in the number of undifferentiated (Undiff.) cells within the seminferous tubules of the testes (Bagu et al., 2006). The average age at which spermatogenesis in observed (32 wk of age; Chandolia et al., 1997a; Barth, 2004) is shown, as well as the literature-defined range at which puberty in the bulls of various breeds (Lunstra et al., 1978, 1982).

Attainment of Functional Spermatogenesis

The main hormonal constituents required for spermatogenesis and their prepubertal development have been highlighted in the previous sections. The combination of these endocrine changes and physiological development culminates in the bull's ability to produce and ejaculate viable sperm. The early stages of spermatogenesis begin once spermatogonia occupy the spaces along the basement membrane of the seminiferous tubules, and this can be visualized in testis histological sections beginning between 3 to 4 months of age in the bull (Chandolia et al., 1997a). Spermatocytes can be seen by 6 months of age, and elongated spermatids by 8 months of age (Chandolia et al., 1997a; Barth, 2004). Early ejaculations may contain sperm that have visual abnormalities if puberty has not been finalized since the testicular components necessary for sperm maturation (i.e. epididymis) may not be fully developed (Evans et al., 1995). These abnormalities may include the presence of proximal droplets or compromised motility, but there is a marked decrease in sperm abnormalities just prior to puberty (Evans et al., 1995). The decrease in visible sperm abnormalities coincides with the attainment in puberty as testosterone concentrations in the gonad are increasing and are known to be important for the maturation of sperm in the epididymis (Martig and Almquist, 1969).

The testes experience rapid growth beginning between 25 and 28 wk of age (Lunstra et al., 1978), and this is most attributable to the changes in individual functions and numbers of somatic and germ cells (Bagu, 2004). Overall, the composition of the testis is changing as the tubules begin to rapidly develop and fill with sperm cell-types of varying maturity. From 12 to 32 wk of age the percentage of testis comprised of

seminiferous tubules increases from 44 to 81% (Curtis and Amann, 1981), and the testes will continue to grow in size before they will be 90% of their final size by 24 months of age (96 wk of age; Coulter, 1986).

The accessory sex glands must develop in synchrony with the gonads, so the vesicular glands and prostate begin to increase in size rapidly after 34 weeks of age (Chandolia et al., 1997b). Select Sires, Inc. begins sperm collection attempts in bulls at 40 weeks of age, or at 28 to 30 cm scrotal circumference for some high genomic bulls. Few bulls will mount a teaser at this age, but as time progresses most will gain the ability to mount and ejaculate into an artificial vagina by 12 months of age, and it is estimated that 95% of bulls have gained the ability to mount and ejaculate sperm of acceptable quality by 12 - 13 months of age (personal correspondence, Mel DeJarnette). Sperm numbers per collection will also continue to increase during the initial year of collection, and most bulls will reach a peak of semen production around 48 months of age (personal correspondence, Don Monke).

INTERVENTIONS TO HASTEN PUBERTY IN CATTLE

<u>Useful Applications for Precocious Puberty in Bovine</u>

The time and money spent growing calves to maturity, regardless of breed, represents a loss to the industry. In dairy, replacement heifers must be grown to appropriate body size before breeding, with the eventual goal of calving them at approximately two years of age. In the beef industry, pre-slaughter animals must be fed and grown by either the cow-calf operator or the feedlot before reaching appropriate slaughter weights. Similarly, bulls owned by AI companies fail to provide income until they are able to produce semen as saleable product. To minimize these monetary losses, those who raise cattle aim to accelerate growth in various ways. For example, one of many tools used in the beef industry includes the use FDA-approved estradiol-based implants in cattle. All or most of these implants claim increased rate of weight gain and improved feed efficiency (Bagley et al., 1989).

More so than ever before, the industry demands semen from bulls at as early an age as possible. The following provides an overview for methods used in an attempt to manipulate and better understand the prepubertal endocrinology of the bull. Most treatments are administered in hopes of hastening puberty, increasing mature sperm production, or both.

Exogenous Gonadotropins

Manipulation of gonadotropin (FSH and LH) secretion through exogenous administration of GnRH has been tested to study mechanisms that regulate sexual maturation and to advance age at puberty. Madgwick et al. (2008) treated prepubertal bulls with 120 ng/kg GnRH twice daily from 4-8 wk of age. Bulls experienced an LH pulse after each injection, and the bulls that received GnRH had more rapid testicular growth from 22 to 44 wk of age, and age at puberty was expedited by 6 weeks relative to untreated bulls. The researchers attributed the reduction in age at puberty to the advancement of the transient increase in LH that is normally initiated at 6 weeks of age. Others, such as Chandolia et al. (1997c), who treated bulls with 200 ng LH-releasing hormone IV every 2 hours for 14 days from 4 to 6 weeks of age, have reported that increasing GnRH levels in the prepubertal bull attribute to increases in LH and FSH concentrations, and this can cause permanent positive effects on testicular function by increasing the numbers of Sertoli and germ cells within the testes (Chandolia et al., 1997c).

Follicle-stimulating hormone is the target of manipulation in prepubertal studies because of FSH's proliferative effects on Sertoli cell proliferation (Orth, 1984). One week of age is the youngest age at which FSH levels have been experimentally altered or even reported (Kaneko et al., 2001). In one of the prominent FSH manipulation studies, Bagu et al. (2004) treated bull calves from 4 to 8 wk of age with exogenous FSH (10 mg NIH-FSH-S1 equivalent every other day) that caused a transient and significant increase in systemic FSH concentrations. The exogenously administered FSH in this experiment hastened puberty, and histological evaluation of the testes at 56 wk of age revealed that FSH-treated bulls had greater numbers of Sertoli cells, elongated spermatids, and spermatocytes (Bagu et al., 2004).

A summary of studies in which FSH concentrations were manipulated in prepubertal males of a variety of species is provided in Table 1.1. In addition, Table 1.2 highlights the compiled results of three more experiments examining the up-regulation and down-regulation of FSH on the proliferation of Sertoli cells in the prepubertal rat. Overall, the promising results of the abovementioned studies have formed the foundation for the research described in this thesis. Table 1.1. Summarized results of four experiments looking at direct (FSH administration) and indirect (FSH immunization) effects of FSH given to prepubertal male animals.

Animals	Treatment	Treatment Period	Results	Authors
Bulls	4 mg FSH (10 mg NIH-FSH- S1 equivalent)	Every 2 d from 4- 8 wk of age	 Decreased age to reach 28 cm scrotal circumference More Sertoli and sperm cells per seminiferous tubule 	Bagu <i>et</i> <i>al.</i> , 2004
Bulls	5 mg FSH	2X daily (10 mg/day) for 10 d beginning at 4 mo of age Performed on summer group and winter group	 Increased testicular weight by 38% for summer group Increased levels of intratesticular testosterone synthesis in both seasons 	Meyers <i>et al.</i> , 1983
Rats	FSH immunization at 1 d of age (sacrificed at 3 d of age) FSH immunization 5 & 7 d of age (sacrificed 9 d of age) FSH immunization 14 and 16 d of age (sacrificed 18 d of age)	see 'treatment'	 Decreased Sertoli cell proliferation all groups Increased germ cell apoptosis all groups 	Meache m <i>et al.</i> , 2005
Boys (diagnosed with hypogonadotropic hypogonadism) (do not produce FSH)	r-hFSH (Gonal- f, Serono) in 3weekly s.c. doses starting from 1.5 IU/kg (range 180–450 IU/week)	2 mo-2.8 yrs preceding puberty. Puberty then induced by hCG (Profasi HP) (range from 500 IU per 2 weeks to 4000 IU per week, 1-3 times per week s.c.)	 Induction of prepubertal testicular growth Increase in inhibin- beta levels 6/7 boys able to ejaculate motile sperm at conclusion of treatment 	Raivio et al., 2007

Table 1.2. The effects of various hormonal FSH manipulations in rats. These illustrate that neonatal concentration of FSH are important in the rat because its suppression reduces the final number of Sertoli cells, whereas experimental increase of FSH by injection increases the number of Sertoli cells.

Effect of various neonatal hormonal manipulations in rats on final number of Sertoli cells							
and testis mass							
	Percentage compared to experimental control (100%)						
Neonatal Treatment	Number of Sertoli cells	Testis Mass	References				
GnRH antagonist (FSH suppression)	45-52%	46 %	Atanassova et al, 1999; Sharpe et al., 1999				
Treatment with recombinant FSH (FSH addition)	149%	124%	Meachem et al., 1996				

Immunological Manipulation of Endogenous FSH

A variety of immunological manipulations have been performed in rodent models and then adapted to domestic animal studies. One such comprehensive study in rats used immunization against GnRH to effectively shut down testes function in adulthood (McLachlan et al., 1995). The resultant male, in which FSH, LH, testosterone, and inhibin production are minimized, is an excellent model to study the independent effects of individual hormones on testes function. In GnRH-immunized male rate, administration of recombinant FSH for 7, 14, or 21 days began after the cessation of spermatogenesis. After 7 days of FSH treatment, testis weight increased by 43%, and there was an increase in the volume of the seminiferous tubules and a concomitant decrease in the proportion of the testis comprised of interstitium. Furthermore, 7 d of rFSH supplementation resulted in a resumption of spermatogenesis up to the stage of round spermatids as well as a restoration of serum inhibin concentration to normal, but it did not increase serum or testicular androgen levels. These changes were also observed, but did not differ from those seen after 7 d of rFSH, after 14 and 21 days of rFSH supplementation. Overall, this treatment showcases FSH's ability to maintain spermatogenesis up to the stage of round spermatids, yet this progression is telling because testosterone was absent in this system, and it was noted that testosterone is most likely the missing factor needed for normal spermatogenesis to occur (McLachlan et al., 1995).

The main target molecule that has been manipulated immunologically in bulls is inhibin. Early studies verified that immunizing bull calves against inhibin at 6 months of age increased FSH levels, but a link between inhibin immunizations early in life and mature FSH levels and sperm production were only theorized (Kaneko et al., 1993). Inhibin immunization studies in prepubertal bulls were performed by Kaneko et al. (2001), Bame et al. (1999), and Martin et al. (1991). Kaneko et al. (2001) immunoneutralized inhibin in four groups of bulls at either 7, 21, 60, or 120 d of age by injecting animals with inhibin antiserum on those respective days. Treatment with the inhibin antiserum resulted in increases in plasma FSH levels at all ages, but the FSH rise became greater as age of bull increased. Bame et al. (1999) immunized bulls against inhibin at 60 d of age, with boosters given on d 104, 124, 270, and 395. During 60 - 405d of age, the increase in scrotal circumference and serum concentrations of FSH were greater in immunized bulls. Lastly, Martin et al. (1991) immunized bulls against inhibin at 14 wk of age followed by boosters at 28, 30, and 34 wk of age. After the third booster at 34 wk of age, the immunized bulls had greater serum concentrations of FSH and testosterone (Martin et al., 1991), and it was revealed in subsequent analysis that immunized bulls had increased number of elongated spermatids per gram of tissue (Lunstra et al., 1993). Even though the use of inhibin immunizations represent one way in which to alter FSH levels, in the context of designing experiments presented in this thesis, more direct routes of increasing FSH were targeted through treating animals directly with FSH.

<u>Dietary Manipulation</u>

Prior to weaning, beef animals receive nutrition from the dam's milk and dairy replacement animals receive milk-replacer-based nutrition. Much of the research

regarding pre-weaning nutrition focuses on the attainment of puberty in suckling beef heifers. Early studies report that increased pre-weaning body weight (BW) gain results in earlier onset of puberty in beef heifers (Wiltbank et al., 1966), and other studies have sought to compare pre-weaning gain *post hoc* by examining weaning weights versus ages at puberty in beef heifers. These latter studies conclusively report that increased weight at weaning translates into earlier onset to puberty in beef heifers (Arije and Wiltbank, 1971; Greer et al., 1983; Roberts et al., 2007). Much of the other literature on manipulating preand post-weaning nutrition in dairy animals is related to mammary development in replacement dairy heifers. For an in depth review, see articles by Sejrsen and Purup (1997) or Brown et al. (2005).

Using diet to regulate puberty essentially tests the ability to change the known and necessary endocrinology associated with the initiation of puberty. Studies such as those by Gasser et al. (2006a, b, c, d) demonstrated that early weaning and utilization of a high-concentrate diet can advance puberty and result in multiple estrous cycles before the onset of the first breeding season, which results in a greater probability for early conception and optimal lifetime productivity. In bulls, particularly those utilized in AI, studies manipulating prepubertal nutrition are less common. It has been demonstrated that limiting nutrient intake during sexual maturation delays puberty in bulls (Flipse and Almquist, 1961; Pruitt et al., 1986). Furthermore, limiting nutrient intake in bulls prior to puberty results in smaller testes (VanDemark and Mauger, 1964), decreased SC (Pruitt et al., 1986), lower intratesticular testosterone concentrations (Mann et al., 1967), and lower sperm numbers in ejaculates of lower volume (VanDemark et al., 1964). Attempts to

examine the effects of high-energy diet on attainment of puberty and the HPG-axis revealed little in Brahman bulls (Nolan et al., 1990). High-energy diets prior to puberty (1 kg/d ADG versus 0.10 and 0.25 kg/d ADG) in Brahman led to earlier puberty, and this was most apparent in increased SC in 'high gain' bulls. However, there were no apparent changes in the secretion of GnRH or LH between the groups, albeit testosterone concentrations were greater in high-energy groups.

Overall, the effects to nutrition, particularly those using a high-energy diets to induce increased prepubertal ADG, are known to hasten the age at puberty. Yet the endocrine functions underlying these changes are not well understood in the bull. Apropos this lack in understanding, more experiments such as the one conducted and presented in this thesis are needed to document the endocrine changes associated with dietary changes and the attainment of puberty in bulls.

Inducing Hyper- and Hypothyroidism

The thyroid hormones (T3 and T4) are important not only for prenatal gonadal development, but also a milieu of other developmental processes. Thyroid hormone's role in the attainment of puberty has been studies most extensively in rodents. Inactivating the thyroid in neonatal mice results in an extended time of Sertoli cell proliferation, enlargement of the testes, and an increase in spermatogenic capacity (Joyce et al., 1993). While Sertoli cell proliferation ceased on d 15 in these control mice, the Sertoli cells in treated mice continued to proliferate until d 25. Inactivation of the thyroid allowed Sertoli

cells a longer window of time for proliferation, and there was a subsequent increase in their numbers as a result (Joyce et al., 1993).

Other in vitro studies involving T3 levels in rat testicular cells have shown that both FSH and T3 induce androgen receptor expression in immature Sertoli cells (Arambepola et al., 1998). Induction of androgen receptor is one of the physiological occurrences during maturation of the testes (Arambepola et al., 1998). Additionally, inducing neonatal hypothyroidism in the rat has been reported to cause increases of 82-157% in the final number of Sertoli cells (Hess et al., 1993). Contrastingly, the induction of neonatal hypothyroidism can cause a 50% reduction in the final number of adult Sertoli cells in the rat (van Haaster et al., 1993).

In larger species, hypo- and hyperthyroidism has been studied in boars more so than bulls. This is most likely because boars are unique in that FSH concentrations are not associated with Sertoli cell proliferation during fetal and neonatal development (McCoard et al., 2003), and the increase in neonatal FSH is not related to mature testis size (Ford et al., 2001). However, in similar fashion to rodents, neonatal boars experiencing transient hyperthyroidism had a decline in proliferation and an earlier age at puberty, thereby resulting in lower numbers of Sertoli cells overall (McCoard et al., 2003). Whether or not induced hypothyroidism in neonatal boars causes increases in Sertoli cell proliferation in unknown. The effects of hyper and hypothyroidism have not been extensively evaluated in neonatal bulls. This may be because of the apparent effectiveness of manipulating the hormonal milieu via immunizations and direct GnRH and gonadotropin treatment postnatally, such as the treatments summarized above.

STATEMENT OF THE PROBLEM

Numerous treatments to manipulate the reproductive physiology and endocrinology of modern cattle have been implemented in production settings. Furthermore, within the last few decades the processes involved in collecting, freezing, storing, and distributing semen from AI sires to the beef and dairy industries has been vastly advanced. However, the efficiency of these advancements have come under pressure from new methods of sire selection in cattle. The industry is undergoing a 'genomic revolution,' and improvements in reproductive technologies have led to a more efficient and user-friendly food production system overall.

A recent problem and limiting factor to genetic progress in the cattle industries that has not been easily overcome is the fact that the most popular, and oftentimes most genetically superior, sires cannot produce enough semen at an early enough age to meet the demand of farmers. This is attributable to the growing trend of using genomically superior sires exacerbates the problem. Genomic evaluations can determine the genetic potential of a bull at a very young age, and in the near future it will not be uncommon to genotype cattle embryos in order to collect this information (Humblot et al., 2010). This process has been perfected and is used frequently with human embryos before implantation in the mother in order to determine sex and to test for genetic disorders (Baruch et al., 2008). Similar principles will be used more frequently during the genotyping of cattle embryos.

An increase in semen supply, especially early in the productive life of a genomically prominent sire, would allow greater accessibility to superior genetics, and

48

this would prevent farmers from resorting to breeding their females to sires of lesser genetic merit due to semen shortages. Therefore, a reliable system for increasing and maximizing semen production in bulls would increase profits for AI companies and provide farmers with greater access to genetically superior sires. Accordingly, the focus of this thesis research is to examine both dietary and hormonal manipulations that may affect puberty and gonadal development in bulls. It is known that several treatments early in life have the potential to positively affect sperm production in the mature bull. For example, treatment with exogenous FSH preceding puberty, during the short period of Sertoli cell proliferation, can cause increases in the number of final, mature Sertoli cells (Berndtson et al., 1987, Bagu et al., 2004). However, the time at which FSH administration is *most* effective in impacting Sertoli cell proliferation, as well as the modes of action, are not well delineated. The need for more studies examining the division of Sertoli cells in the prepubertal bull, as well as the factors that affect their maturation, was suggested by Amann and Schanbacher in 1983, and this need still exists.

The studies within this thesis were designed with the objective of resolving these current gaps in knowledge through the direct testing of the effect of diet or FSH given during a specific window of time preceding puberty. Specifically, the first goal is to gain understanding of the effect of a high energy diet on bull endocrine profiles, age at puberty, mature semen production, and testis morphology. Secondly, we seek to gain understanding of the effect of prepubertal FSH administration on the numbers of Sertoli cells within the testes at the conclusion of a succinct treatment period while also examining the bulls' testicular growth and morphometry.

49

CHAPTER 2

EFFECTS OF DIETARY ENERGY ON SEXUAL MATURATION AND SPERM PRODUCTION IN HOLSTEIN BULLS

INTRODUCTION

Puberty in bulls has been defined as both the ability of an animal to produce an ejaculate containing $5.0 \ge 10^7$ sperm with 10% motility and the attainment of a scrotal circumference (SC) greater than 28 cm (Wolf et al., 1965; Lunstra, 1982). Typically, a bull attains a SC of ≥ 28 cm before the defined threshold of sperm production (Wolf et al., 1965). Nutritional manipulation during the prepubertal period that seeks to vary the age at puberty has been the focus of many investigations. In general, increased nutrient intake hastens puberty and, although the specific metabolic signals remain unclear, it is obvious that the endocrine mechanisms that control puberty are advanced with increased nutrition. We have demonstrated that puberty in heifers can be induced at ages substantially less than the typical range (precocious puberty) with targeted nutritional programs, and this reduction in age at puberty is preceded by precocious activation of the endocrine mechanisms that are known to regulate age at puberty (Gasser et al., 2006a,b,c,d). A primary finding from this series of studies indicated that increased LH pulse frequency

was experienced at younger ages and with greater intensity in heifers fed a high energy diet (Gasser et al., 2006a). The initial increase in LH secretion necessary for ovarian maturation (for a review, see Day and Anderson, 1998) occurred at a younger age and was characterized by a greater frequency of LH pulse (Gasser et al., 2006a). Bulls also experience an initial transient increase in gonadotropin secretion between 6 and 20 weeks of age (Amann et al., 1986), and this initiates maturation of the cellular components of the testes (Curtis and Amann, 1981). The resultant increase in concentrations of testosterone by the partially matured testes feeds back negatively on LH and FSH secretion, thereby leading to cessation of the transient rise in gonadotropin levels (Miyamoto et al., 1989).

Age at puberty in bulls has been linked to the age and magnitude of this transient increase in gonadotropins. For example, Evans et al. (1995) reported that greater concentrations of LH were present in bulls that reached puberty at younger ages. Bulls fed high energy diets starting at 10 wk of age had larger testes, greater SC, and greater total daily sperm production compared to bulls fed control rations (Brito et al., 2007). Furthermore, bulls fed to 70% of recommended energy levels from birth to 208 wk of age had lower sperm output per ejaculate at 80 wk of age compared to bulls fed 130% of the recommended values during the same period (Flipse and Almquist, 1961).

Based on a review of the literature, we hypothesized that feeding a high energy diet beginning at 8 wk of age would advance and increase the prepubertal increase in LH, which would lead to advanced testicular maturation and age at puberty. The objective of the present experiment was therefore to evaluate the effect of a high energy diet initiated early in life on the initial transient increase in LH secretion, testosterone concentrations, SC, age at puberty, mature sperm production, and characteristics of the mature testes in Holstein bulls.

MATERIALS AND METHODS

Animals and handling were conducted in accordance with procedures approved by The Ohio State University Agricultural Animal Care and Use Committee (#2010AG0007).

Animals and Treatments

Nineteen purebred Holstein bulls purchased from a commercial dairy were raised at this dairy to 50 d of age on an intensive, replacement heifer feeding program. Bulls received milk replacer twice daily (1.9 L/feeding) and had *ad libitum* access to a commercial dairy starter grain and hay beginning at 14 d of age. Calves were weaned on the same day at 50 ± 0.3 d of age and transported to The OSU Beef Center. After a 7 d acclimation period, bulls were randomized by age, BW (74.2 ± 2.2 kg), and sire to receive a diet formulated to support an ADG of 0.75 kg/d (CONT, n = 10), or to a high energy diet (HE, n =9) formulated for an ADG of 1.5 kg/d. Diets were isonitrogenous but differed in energy content (Table 2.1). Bulls were group fed these diets from 58 ± 0.3 to 230 ± 0.3 d of age (Stage 1 of the experiment). Diets were initially fed at 2.5% of body weight, but intake within dietary treatment was adjusted (between 2.5 to 3.0% of BW) to support targeted weight gains after bulls were weighed biweekly. At 230 ± 0.3 d of age, bulls were transported to Select Sires, Inc. in Plain City, Ohio (Stage 2 of the experiment) and remained there until slaughter at 569 ± 4.7 d of age. At Select Sires, Inc., all bulls were fed the company's standard bull ration consisting of a corn silage and hay TMR formulated to provide 1.5 Mcal/kg NEm and 0.9 Mcal/kg NEg fed at 2.4% of mature BW.

Of the original nineteen bulls, four were not moved to Select Sires, Inc. due to health testing issues. These four bulls were removed from the experiment at this point in time, resulting in 15 bulls (HE, n = 8; CONT, n = 7) to be assessed for age at puberty and semen production at Select Sires. Data for all 19 bulls are included for variables collected only during Stage 1 (LH and testosterone), whereas for traits that included both Stage 1 and Stage 2 (SC and BW), or were exclusive to Stage 2, data are presented for the 15 bulls transferred to Select Sires.

Body Weight and Scrotal Circumference Measurements

Body weights were recorded every 14 days during Stage 1 from 58 to 230 d of age, and on 265, 309, 330, and 360 d of age during Stage 2. Scrotal circumference was measured monthly at the widest point of the scrotum while grasping at the neck of the scrotum and securing the testes from 58 to 376 d of age using a flexible cloth scrotal tape by a single operator.

Blood Sample Collection

Serial blood samples were collected at 10 minute intervals for 8 h at 69, 97, 125, 156, 181, and 210 d of age on a randomly selected subset of six bulls from each treatment. The same subset of six bulls was sampled at each age indicated. Samples were collected via indwelling jugular catheters, stored for 48 hours at 4°C, and then centrifuged at 7,735 x g for 20 minutes. Serum was harvested and frozen at -20°C until

analyzed for circulating concentration of luteinizing hormone (LH). During the serial blood collection, blood samples for testosterone analyses were collected hourly in EDTA vacutainer tubes (Becton Dickinson and Company, New Jersey, USA). These samples were collected on ice and centrifuged within 4 hours of collection at 7,735 x g for 20 minutes. Harvested plasma was frozen at -20°C until analysis for testosterone concentration.

Hormone Analysis

Concentrations of LH were determined in duplicate with a double antibody radioimmunoassay previously validated in our laboratory (Anderson et al., 1996). Average intra-assay CV was 7.4% and inter-assay CVs for a pool containing 1.6 ng/ml LH was 13.34% and the CV for a pool containing 5.5 ng/ml LH was 15.89%. Sensitivity of the LH assay was 0.19 ng/ml. For LH analysis, an 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 8 hours of sampling (Goodman and Karsch, 1980).

For determination of testosterone concentrations, the hourly plasma samples collected during each serial collection were pooled to obtain an average testosterone concentration over an 8 h period. Samples were analyzed in duplicate using a commercial radioimmunoassay kit (Coat-a-Count, Siemens Medical Solutions Diagnostic, Los
Angeles, CA) as described previously (Burke et al., 2005). All samples were analyzed in a single assay with an intra-assay CV of 1.98%.

Puberty Assessment and Mature Sperm Production

Bulls (HE, n = 8; CONT, n = 7) were allowed an adaptation period of ten days to acclimate to housing and feed after transfer at 230 d of age to Select Sires, Inc. Beginning at 240 d of age, trained technicians at Select Sires attempted to collect semen from each bull every 14 days using a teaser animal and artificial vagina (AV) method. When collection was successful, semen was evaluated for percent of motile sperm and concentration to determine total sperm numbers. The age at which a bull ejaculated >50 million spermatozoa with at least 10% motile sperm was defined as the age at puberty for that bull. Bulls were removed from semen collection upon attainment of puberty, with the last bull attaining puberty at 396 d of age.

From 541 to 569 d of age, mature semen production was assessed via thrice weekly semen collections using industry standard procedures (teaser animals, false mounts, and collection of two consecutive ejaculates each day with an AV). Mature sperm production was determined using collections from the final six days of collection to allow for adaptation of the bulls to the collection process and to minimize variation due to potential differences in epididymal storage capacities between bulls.

Testicular Size and Histology

Bulls were slaughtered at 569 ± 4.7 d of age and intact testes were collected. The scrotum and testes were removed by incising the scrotal skin and severing the spermatic cord proximal to the pampiniform plexus. Testes were maintained on ice until the scrotal

tissue and parietal tunica vaginalis could be removed. The cauda epididymis and the pampiniform plexus were removed leaving only testis and tunica albuginea. Once separated, each testis and its respective epididymis were weighed. Volume of each testis was determined by submersion in a graduated cylinder filled with saline. Three sections representing the proximal, middle, and distal third of each testis were collected for histological analysis. Sections were fixed in 10% formalin for 12 hours and then placed in 70% ethanol. Following fixation the tissue was imbedded in paraffin for microtome sectioning by standard methods. Histological sections were stained with hematoxylin and eosin. Seminiferous tubule diameters were measured using an ocular micrometer (Zeiss, Fisher Scientific, Pittsburgh, PA, USA). Seminiferous tubule diameter was measured for ten randomly selected round tubule cross sections per histological section from each testis. The percentage of the testis comprised of tubules was determined by capturing five frames from each section of testis using a camera-equipped microscope at the same magnification (SPOT Insight, SPOT Imaging Solutions, Sterling Heights, MI, USA). For each frame, the tubules were traced within that given area to generate a percentage using ImageJ software (U.S. National Institutes of Health, Bethesda, Maryland, USA).

Statistical Analyses

Mean LH concentration, LH pulse frequency, amplitude of LH pulses, and mean testosterone concentrations are presented for the 12 bulls for which serial blood collection was performed. For all other variables, data were analyzed for the 15 bulls that were transferred to Select Sires, Inc. for Stage 2 of the experiment. These variables include ADG, BW, SC, mature sperm production, age at puberty, and post mortem testicular measurements.

The effect of treatment, age, and their interaction on BW, SC, mean LH concentration, LH pulse frequency, amplitude of LH pulses, and testosterone concentration were analyzed by ANOVA using the MIXED procedure of SAS (version 9.2, 2002) with repeated measures (age) analysis included in the model.

Age at puberty, ADG in Stage 1 and 2, post mortem testicular measurements, and mature sperm production were analyzed with a PROC GLM procedure of SAS. When analyzing testicular measurements, side and section of testis were initially included in the model, but these variables were not significant sources of variation and were removed from the final model. Results are reported in terms of mean \pm SE.

RESULTS

During Stage 1 ADG was 1.51 ± 0.14 kg/d for bulls in the HE and 1.00 ± 0.15 kg/d for bulls in the CONT treatment (P < 0.01). Body weight (Trt*Age, P<0.01) was greater (P < 0.05) in the HE than CONT treatment at 146 d of age and this difference was maintained through the remainder of Stage 1 (Figure 2.1). During Stage 2, from 230 to 360 d of age, ADG was 0.87 ± 0.16 kg/d for the HE and 1.16 ± 0.1 kg/d for the CONT treatment. Despite the substantial shift in ADG in Stage 2, BW remained greater (P < 0.05) in the HE than CONT treatments to 360 d of age (Figure 2.1).

Mean LH concentration and amplitude of the pulses differed between HE and CONT treatments (Table 2.2). Mean LH (Trt*Age, P < 0.0001) in the HE treatment was greater (P < 0.01) than in the CONT treatment at 125 d of age, however, at 181 and 210 d of age, CONT bulls had greater (P < 0.05) mean LH than HE bulls. Amplitude of LH pulses (Trt*Age, P < 0.05) was greater (P < 0.05) in the CONT than HE treatment at 125, 181, and 210 d of age, and had a tendency to be greater at 97 and 156 d of age.

A treatment by age interaction was detected for LH pulse frequency (Trt*Age, P < 0.01; Figure 2.2). Within the CONT treatment, frequency of LH pulses increased (P < 0.05) from 69 to 97 d of age and then declined (P < 0.05) and remained constant thereafter. In the HE treatment, a similar increase (P < 0.05) was observed from 69 to 97 d of age. However, this increase was sustained through 125 d of age, and then declined (P < 0.05) thereafter and remained unchanged. Between treatments, frequency of LH pulses was greater (P < 0.0005) in the HE than CONT treatment at 125 d of age and tended (P < 0.10) to be greater at 181 and 210 d of age. Pulse frequency did not differ between treatments at other ages.

Testosterone concentration was greater (P < 0.05) in the HE than CONT treatment at 181 and 210 d of age (Trt*Age, P = 0.02; Figure 2.3). Within the HE treatment, testosterone concentrations increased (P < 0.05) at each age compared to the previous age except for a tendency (P = 0.08) to increase from 181 to 210 d of age. Within the CONT treatment, testosterone concentration increased (P < 0.05) at each age measured from the prior age until 156 d of age, after which time they remained unchanged.

Scrotal circumference increased over time in both treatments albeit at a different rate (Trt*Age, P = 0.002). At 146 d of age and thereafter to 360 d of age, SC was greater (P < 0.05) in the HE than CONT treatment (Figure 2.4).

Age at puberty did not differ between the HE (323.3 ± 11.5 d of age) and CONT (301.9 ± 13.0 d of age) bulls. Mature daily sperm production also did not differ between the HE (6.5 ± 0.7 billion sperm cells/d) and CONT (6.1 ± 0.8 billion sperm cells/d) treatment. At slaughter (569 d of age) testis weight, epididymal weight, and testis volume were greater in the HE than CONT treatment (Table 2.3). Additionally, percentage of the testis comprised of seminiferous tubules and average diameter of seminiferous tubules did not differ between the HE and CONT treatments (Table 2.3).

DISCUSSION

Feeding a high energy diet from 58 to 230 d of age in Holstein bulls increased ADG by approximately 0.5 kg/d and significantly influenced several key aspects of sexual maturation during this time. The initial effect was the augmentation of the initial increase in secretion of LH. While timing of this initial increase in LH frequency did not appear to differ between diets, the magnitude of this increase at 125 d of age was approximately 3-fold greater in the bulls receiving the high energy diet. Increased pulsatile LH secretion in bulls fed the high energy diet was followed by enhanced testicular growth and peripheral testosterone concentrations relative to bulls on the control diet. Given these differences in development of the testes in prepubertal bulls, it was surprising that no difference in age at puberty was detected.

It is speculated that the transfer of bulls to a different location and to a different diet at the initiation of this puberty evaluation may have influenced the experimental outcome. The ADG of bulls on the HE diet decreased at this time, and they may have essentially been placed on nutritional restriction, which is known to negatively impact the attainment of puberty in bulls (Flipse and Almquist, 1961). As adults, it was clear that the enhanced testicular size noted during the prepubertal period was a permanent effect of the high energy diet, as the proportional difference in either testis weight (19%) or volume (24%) at 569 d of age was actually greater than that noted in SC (12%) at 230 d of age. Feeding of the high energy diet did not influence seminiferous tubule diameter nor the proportion of the testis comprised of seminiferous tubules. Hence, the failure to detect a difference in daily sperm production between mature bulls fed diets differing in energy during the prepubertal period was perplexing. In the present experiment, the advancement in measures of sexual maturation as a result of the high energy diet was not reflected in age at puberty or sperm production adults.

Low concentrations and the lack of pulsatile LH secretion from birth to 8 weeks of age (56 d of age) in bull calves (Amann, 1983) may be due to steroid-independent suppression of the hypothalamo-pituitary axis (Wise et al., 1987). The initial increase in LH secretion is likely preceded by increased hypothalamic GnRH secretion (Rodriguez and Wise, 1989) and timing the LH increase can be hastened by administration of exogenous GnRH pulses (Rodriguez and Wise, 1991). Consistently, pulsatile LH secretion was present at 69 d of age in the present experiment regardless of dietary treatment and increased further by 97 d of age. As previously reported (Amann, 1983), pulsatile LH secretion declined following the initial increase in the present study, presumably due to the initiation of testicular steroidogenesis reflected in Figure 2.3. The suppression of the initial increase in LH has been reported to be associated with the development of testicular Leydig cells (Amann, 1983). The impact of the high energy diet on pulsatile LH secretion was two-fold in the present study. First, the high energy diet extended the increase in pulsatile LH secretion from 97 to 125 d of age, an interval during which LH pulse frequency declined in the bulls fed the control diet. Secondly, the frequency of LH pulses at 125 d of age was slightly less than 3-fold greater in the high energy treatment than detected at the same age with the control diet. Furthermore, bulls in the high energy treatment tended to have elevated LH pulse frequency through 210 d of age. This augmentation of pulsatile LH secretion was accompanied by greater SC (by 147 d of age) and testosterone concentration (by 181 d of age) in bulls fed the high energy diet. Similar impacts of feeding diets varying in energy content on testosterone in prepubertal bulls have been previously reported (Brito et al., 2007). Feeding a high energy diet from 58 to 230 d of age clearly accelerated and/or augmented the endocrine regulation of LH secretion, testicular growth, and testosterone production during Stage 1 of the present experiment.

The effects of a high energy diet initiated early in life in bull calves on gonadotropin secretion and gonadal development in the present study were consistent with impacts observed in prepubertal heifers (Gasser et al., 2006 a, b, c). In heifers, these effects translated into a reduction in age at puberty by approximately 85 d (Gasser et al., 2006 a, b, c, d). In the present study, earlier puberty was not observed in the bulls fed the high energy versus the control diet, and this was surprising due to the negative association previously reported between age at puberty and SC (Siddiqui et al., 2008) and testosterone (Secchiari et al., 1976) concentrations in bulls. Furthermore, it was demonstrated (Bratton et al., 1959) that Holstein bulls fed from 7 to 560 d of age at

160%, 100% or 70% of dietary requirements attained puberty at 300, 340, and 399 d of age, respectively. In our previous work with heifers (Gasser et al., 2006 a, b, c, d) and the report of Bratton et al. (1959) in bulls, animals on the high energy dietary treatment remained on this diet through attainment of puberty. Contrastingly, in the present study all bulls were placed on the same standard diet at the collection facility at the initiation of the puberty evaluation period at 230 d of age. In the high energy treatment, ADG was decreased by 42% in Stage 2 (0.87 kg/d) relative to Stage 1 (1.51 kg/d). Conversely, in the control treatment, ADG was increased by 16% in Stage 2 (1.16 kg/d) versus Stage 1 (1.0 kg/d). Hence, bulls in the HE treatment were placed on a dietary restriction relative to the growth they experienced during Stage 1 at the initiation of the puberty assessment period, whereas bulls in the control treatment were exposed to an increase in dietary energy at the initiation of this period. The acute effects of changes in dietary energy on age at puberty have not been directly studied in bulls, but studies examining the effect of acute dietary restriction by fasting prepubertal heifers report that fasting for 48 hours reduces the mean frequency of LH pulses, which are known to be correlated to the onset puberty in both males and females (Amstalden et al., 2000). Although fasting represents an extreme example of lessened energy intake compared to that which the HE bulls experienced in this experiment, it is unknown if this diet switch delayed puberty in the HE bulls and/or advanced puberty in the control treatment. It is possible that differences in rate of sexual maturation that appeared evident in Stage 1 were masked during and after Stage 2, and further research is necessary to determine if continuation of the high

energy diet through the prepubertal period would result in hastened puberty or enhanced sperm production.

No differences in mature sperm production were detected, even though bulls in the high energy treatment had larger testes with a similar density of seminiferous tubules. Logically, this suggests that bulls in the high energy treatment possessed more surface area of seminiferous tubule from which spermatogenesis could occur. However, further examination is needed to conclude whether HE and control bulls had equivalent numbers of cell types (i.e. Sertoli) related to sperm production. It is also possible, and probably more likely, that the collection regimen used (twice daily ejaculates three times per week) did not accurately measure the epididymal reserves and testicular production of the bulls, since larger testes and epididymides should have the ability to produce and store more sperm cells. A future study using the histological sections could use a marker of Sertoli cells, such as GATA-4, that may shed insight into the cell types present and responsible for sperm production (McCoard et al., 2001), therefore explaining the surprising lack of difference in mature sperm production.

Overall, these data suggest that the high energy diet affected the reproductive axis of these bulls, probably by enhancing the production and secretion of GnRH around 125 d of age (Rodriguez and Wise, 1991). This is deduced based on the increase in LH pulse frequency and decrease in LH pulse amplitude at this time, as well as the subsequent rise and hastening of the production of testosterone concentration in the time following the LH increase. A high energy diet also caused an increase in SC during treatment (147 d of age) that was maintained for the duration of the study. However, because these promising changes failed to result in a reduced age at puberty, further research is needed examining how to best take advantage of the impacts of a HE diet on endocrine and physiological endpoints in bulls. In the postmortem findings, it was determined that the HE diet resulted in greater epididymal and testicular size, but treatment bulls did not produce more sperm or have any detectable differences in testicular composition. Therefore, it may be advantageous or necessary to find an approach to better measure sperm production in the bulls of this study. This may include a more intensive collection that better represents a bull's ability to store sperm. Lastly, more histological data, including the number of sperm per testis as well as the density of Sertoli cells, may provide insight into the discrepancy of the larger testes of HE treatment bulls' inability to produce more sperm.

Table 2.1. Composition of experimental diets fed to Holstein bulls from 58 ± 0.3 to 230	
\pm 0.3 d of age (% as fed). Diets were adjusted biweekly so that bulls were being fed at 2.1	5
– 3% of BW.	

Ingredient	HE Diet	Control
Whole Shelled Corn (%)	60	30
13% CP Alfalfa Pellets (%)	10	30
Pelleted Soybean Hulls (%)	10	30
Supplement (%)	20	10
Crude Protein (%)	14.1	14.1
NEm (Mcal/kg)	2.2	1.70
NEg (Mcal/kg)	1.37	1.09
TARGET ADG (kg/d)	1.5	0.75

Table 2.2. Mean LH concentration and amplitude of LH pulses (Trt*Age, P < 0.001) from Holstein bulls fed either a high energy (HE) or a control (CONT) diet (HE group, n = 6; CONT group, n = 6).

		Age (days)					
	Trt	69	97	125	156	181	210
Mean LH	HE	1.38 ± 1.28	1.88 ± 1.00	1.83 ± 0.86^a	1.49 ± 1.16	1.07 ± 0.77^{a}	1.00 ± 0.55^a
(ng/ml)	CONT	1.27 ± 1.22	1.92 ± 1.39	$1.5\pm1.28^{\text{b}}$	1.66 ±1.39	$1.43\pm1.37^{\text{b}}$	1.34 ± 1.13^{b}
Amplitude of LH	HE	3.58 ± 1.14	$2.18 \pm 1.23^{\circ}$	1.63 ± 0.94^{a}	$2.32\pm1.85^{\rm c}$	1.75 ± 1.24^{a}	1.21 ± 0.92^a
pulses (ng/ml)	CONT	3.16 ± 1.47	3.22 ± 2.25^{d}	3.35 ± 2.27^{b}	3.60 ± 2.15^{d}	3.57 ± 2.00^{b}	3.00 ± 1.56^{b}

 ab values within column and characteristic differ, $P\!\!<\!\!0.05$

 $^{\rm cd}$ values within column and characteristic differ, P<0.10

Table 2.3. Testicular measurements, seminiferous tubule diameter, and percentage of testicular parenchyma comprised of seminiferous tubules (mean \pm SE; 569 d of age) for Holstein bulls fed either a high energy (HE) or control (CONT) diet from 58 to 230 \pm 0.3 d of age (HE, n = 8; CONT, n = 7).

Variable	CONT	HE	Significance
Testis weight (g)	267.5 ± 14.4	318.0 ± 13.5	P < 0.05
Epididymal weight (g)	28.0 ± 1.2	31.6 ± 1.1	P < 0.05
Testis volume (cm ³)	244.9 ± 12.9	305.0 ± 11.9	P < 0.01
Seminiferous Tubule Diameter (µm)	252.8 ± 7.5	251.7 ± 7.0	P = 0.95
Parenchyma Tubule Percentage (%)	71.8 %	72.3 %	P = 0.86



Figure 2.1. Body weight of Holstein bulls fed either a high concentrate (HE, n = 8; dashed line) or a control (CONT, n = 7; solid line) diet from 58 to 230 d of age. Body weight of bulls fed a HE diet was greater (P < 0.05) than bulls fed the CONT diet from 146 d of age until the end of the evaluation at 380 d of age (treatment*age, P < 0.001). The asterisk (146 d of age) denotes the first age at which weights were greater (P < 0.05) in the HE than CONT treatment. The diamond (230 d of age) denotes the timing of the shift in diets between Stage 1 and 2.



Figure 2.2. Number of LH pulses (Trt*Age, p < 0.01) detected in an 8 h sampling period from Holstein bulls fed either a high energy (HE, n = 6; dashed line) or control diet (CONT, n = 6; solid line).

* Treatments differ, (P < 0.05)

** Treatments tend to differ, (P < 0.10)

^{ab} values within treatment differ, (P < 0.05)



Figure 2.3. Average testosterone concentration (Trt*Age; P = 0.02) detected after pooling hourly blood samples taken over an 8 hour sampling period from Holstein bulls fed either a high concentrate (HE, n = 6; black bars), or a control diet CONT, n = 6; open bars). * Treatments differ (P < 0.05)



Figure 2.4. Mean (\pm SE) of the scrotal circumference measurements from Holstein bulls fed either a high energy (HE, dashed line, n = 8) or a control diet (CONT, solid line, n =7). Values differed (P < 0.05) from 146 d of age until the end of the measurement period at 360 d of age (treatment*age, P = 0.002) * Treatments differ (P < 0.05)

CHAPTER 3

DEVELOPMENT OF A SLOW-RELEASE FSH TREATMENT IN PREPUBERTAL BULLS

INTRODUCTION

There is a transient increase in the concentrations of circulating gonadotropins in the bull calf from approximately 6 to 20 weeks of age (Amann et al., 1986), with the initial increase occurring anywhere from 4-6 weeks of age (Amann et al., 1983; Bagu et al., 2006). In particular, the increase in FSH is responsible for the proliferation of Sertoli cells within the testis (Orth, 1984). The proliferation of Sertoli cells is transient, occurring rapidly between 13 and 25 weeks of age (Bagu et al., 2006). After indifferent supporting precursor Sertoli cells have reached maturity at around 25 weeks of age in the bull they lose the ability to divide and proliferate (Sharpe et al., 2003). The maturation of Sertoli cells and the formation of the blood-testis barrier marks the finalization of the processes needed for spermatogenesis to initiate (Gilula et al., 1976). Accordingly, Sertoli cell proliferation before puberty is of interest because their final numbers correlate to the total sperm production of the adult animal (Berndtson et al., 1987). Since each Sertoli cell can only 'host' a fixed number of germ cells, the potential exists for a bull with more Sertoli cells per testis to produce more sperm per unit time than a bull possessing lesser numbers of these cells (Orth, 1984). This relationship has driven investigation of methods to manipulate Sertoli cell numbers for the purpose of increasing sperm production.

Methods to manipulate prepubertal Sertoli cell numbers include immunization against inhibin to indirectly increase FSH concentrations (Kaneko et al., 2001). However, the immunological response mounted by individuals to such immunizations is variable, and so is the concomitant rise in FSH and adult sperm production. The development of a method to directly and efficiently increase systemic FSH levels in the prepubertal bull would provide a means to circumvent challenges associated with inhibin immunization. In this regard, Bagu et al. (2004) administered the equivalent of 10 mg NIH-FSH-S1 every other day to 1 month old bull calves. This dose increased FSH concentrations to approximately 3-times the systemic levels of FSH at the start of treatment when bull calves were 1 month of age, with a peak observed 105 minutes after treatment. However, due to the relatively short half-life of FSH in the body, systemic levels had returned to normal within 24 hours post-treatment. Similarly, when bulls were again treated with the same dose at 2 months of age, systemic FSH levels increased within 30 minutes and peaked 120 minutes later, but they returned to control levels within 9 hours after treatment. These authors reported that this regimen resulted in earlier attainment of puberty as evidence by the hastened attainment of a SC \ge 28 cm, as well as more Sertoli cells, elongated spermatids, and spermatocytes in histological sections when the bulls were 56 weeks of age (Bagu et al., 2004). Due to the short duration of the increase in FSH resulting from this treatment, and the necessity of handling the bulls every other day

during treatment, we report in this chapter the development of a method to increase the half-life of FSH to extend the period of elevated FSH with each administration and to reduce the number of animal handlings.

The rationale for the proposed approach is based upon demonstration that mixing FSH with either 2% (Bó and Mapletoft, 2012) or 1% (Tribulo et al., 2012) hyaluronic acid (HA) could be used to reduce the number of FSH treatments for superovulation of beef cattle from eight treatments at 12 h intervals to a single dose or to a double dose given at 48 h intervals. In the present experiment the FSH (Folltropin-V; Bioniche Animal Health, Ontario, Canada) used was pituitary-derived porcine FSH suspended in a solution containing 2% HA as a vehicle as has previously been used for superovulation in cattle (Bó et al., 1991). The use of HA is thought to extend the release of a drug at its site of absorption by acting as a mucoadhesive (Surini et al., 2003). The dosage was chosen to slightly exceed the weekly dose used by Bagu et al. (2004) and was to be administered twice weekly (every 3.5 d). The objective of this experiment was to evaluate the effect of suspending FSH in HA on systemic FSH concentrations in 7 week old bull calves. A secondary objective was to determine the cross-reactivity of porcine FSH within our current ovine/bovine FSH radioimmunoassay.

MATERIALS AND METHODS

Animals and handling were conducted in accordance with procedures approved by The Ohio State University Agricultural Animal Care and Use Committee. *Animals, Treatments, and Treatment Formulations* Ten Angus-cross bull calves (50 ± 6.5 d of age; 92.3 ± 7.6 kg BW) born at The OSU Beef and Sheep Center from multiparous cows were used. Bulls were allowed *ad libitum* access to pasture, water, and suckling of dams for the duration of the experiment. Calves were randomly allocated based upon birth date and sire to receive two injections of either 0.75 ml of 30 mg NIH-FSH-P1 equivalent (Folltropin-V) in a 2% hyaluronic acid solution (FSH-HA, n = 5) or an equivalent volume of saline (control, n = 5) spaced by 3.5 d beginning at 50 ± 6.5 d of age. The HA was obtained from Lifecore Biomedical (Chaska, MN, USA) as research grade dried sodium hyaluronate with molecular weight ranging from 601 - 850 KDa.

The FSH dose used was based upon the treatment delivered by Bagu et al. (2004) where bulls received 10 mg NIH-FSH-S1 equivalent every other day for a total of 35 mg NIH-FSH-S1 equivalent weekly from 4 – 8 weeks of age. This treatment was successful at transiently increasing systemic FSH concentrations for 24 hours (at 4 wk age) or approximately 8 hours (at 8 wk age), so it was considered a minimum dosage for use in our bull calves that were approximately 7 weeks old. The 35 mg/wk NIH-FSH-S1 used in Bagu et al. (2004) is equivalent to approximately 46 mg/wk NIH-FSH-P1 based upon NIH-FSH-P1's relative biopotency of 0.76 to that of NIH-FSH-S1 (Reichert and Wilhelmi, 1973). Briefly, the FSH-HA treatment was formulated by mixing Folltropin-V to 40 mg/ml in a 2% HA solution and then delivering 0.75 ml every 3.5 days to achieve the administration of 30 mg per injection (60 mg NIH-FSH-P1 weekly). Injections were given intramuscularly in the neck while being sure to deliver the entire bolus of the injection in one area. Side of the neck of the injection was noted and switched every other

treatment day to ensure previously-placed boluses were not disturbed. Control animals received 0.75 ml saline on each treatment day.

Blood Sample Collection

Blood samples to assess FSH were collected immediately before each treatment and every 6 hours thereafter for a 24 hour period. After 24 hours, blood samples to assess FSH were collected every 12 hours (Figure 3.1). Samples were collected from the jugular vein using a 1 inch, 18 gauge needle and syringe, placed into vacutainers without EDTA (Becton Dickinson and Company, New Jersey, USA), and then placed on ice until storage at 4°C. Samples were allowed to clot for 48 hours at 4°C and then centrifuged at 7,735 x g for 20 minutes and serum was harvested and frozen at -20°C until analyzed for concentration of FSH.

Radioimmunoassay for Hormone Analysis

Concentration of FSH was determined in duplicate for all samples with a double antibody radioimmunoassay previously validated (Burke et al., 2003) for use in measuring bovine FSH concentrations. All samples were analyzed using the same iodinated FSH in one assay. Average intra-assay CV was 3.2% and CV for duplicates of the low pool (2.65 ng/ml) was 4.05% and CV for duplicates of the high pool (4.04 ng/ml) was 5.35 %. Sensitivity of the assay was 0.13 ng/ml.

Solutions containing 0.5, 1, 2, 5, 10, and 20 ng/ml of pFSH (A.F. Parlow, National Hormone & Peptide Program, Torrance, CA, USA) were prepared to measure cross reactivity of porcine FSH with the bovine radioimmunoassay. These concentrations are the same as those used in the standard curve for the validated (Burke et al., 2003) assay that are prepared with ovine FSH (LER 1972-A2, L.E. Reichert).

Statistical Analysis

FSH concentrations between FSH-HA and control treatments were analyzed by ANOVA using the MIXED procedure of SAS (version 9.2, 2002). Repeated measures analysis was included in the model and the initial FSH concentration of each bull was included as a covariate in the model.

RESULTS

Cross Reactivity of Bovine and Porcine FSH

The percent binding of the current and validated ovine/bovine FSH standards and the equivalent concentrations of porcine FSH can be seen in Table 3.1. A graph comparing the percent binding of standards across the range tested is also provided (Figure 3.2). The average percent-binding of the equivalent concentrations of porcine FSH did not differ from the percent-binding of the ovine standards by more than 11.0%. Standard curves generated using the two sources of FSH (Figure 3.3) result in a slope of -0.73 for porcine FSH and -0.97 for the ovine standards. The validated bovine FSH assay provides a relatively accurate assessment of peripheral concentrations of FSH achieved in the present experiment from both endogenous bovine and/or exogenous porcine FSH.

FSH Profiles

Peripheral FSH concentrations (Trt*Hour, P = 0.0002) were greater (P < 0.05) in the FSH-HA than control treatment 6 hours after each of the two respective treatments and tended to be greater ($P \le 0.08$) 12 hours after each treatment was administered. Concentrations of FSH did not differ between treatments from 18 to 72 hours after treatments on d 0 and d 3.5 (Figure 3.4).

DISCUSSION

The foremost objective to develop a method to increase systemic FSH concentrations in bull calves was successful in that pFSH in hyaluronic acid preparation (FSH-HA) transiently increased circulating serum concentrations of FSH for 6 to 12 hours, after which it appeared that concentrations gradually declined to basal concentrations (Figure 3.4). Additionally, the second objective of determining whether a validated bovine FSH double-antibody RIA accurately measured porcine FSH in the serum of bull calves was addressed. The greatest difference in percent binding amongst bovine and porcine standards in the assay standard curve (0.5 - 20 ng/ml) was 7.4%, and within the range of standards where most sample concentrations fell (1 - 5 ng/ml), respective bovine and porcine standard percent binding did not differ by more than 5.1%. Additionally, the standard curves of the bovine and porcine standards demonstrated reasonable parallelism. Hence, the bovine FSH RIA used was deemed suitable and accurate at determining the systemic concentrations of FSH in bulls administered exogenous porcine-derived FSH.

When comparing increases of systemic FSH in this experiment to Bagu et al. (2004), results were similar in that treatment caused a transient increase in FSH. Bagu et al. (2004) reported treatment (10 mg NIH-FSH-S1 equivalent every other day) caused a peak in FSH levels 105 minutes after injection, with an elevation in FSH lasting nearly 24

hours in four week old bulls. However, a more valid comparison to animals in this experiment (50 \pm 6.5 and 53.5 d age) is when Bagu treated 8 wk old (57 \pm 6 d) bull calves with the aforementioned treatment and reported a peak in FSH concentration 120 minutes after treatment administration that remained elevated (P < 0.05) over controls for 8.25 hours. In comparison, our treatment (30 mg NIH-FSH-P1 equivalent in 2% HA) significantly increased (P < 0.05) FSH levels six hours after treatment, which is considered a peak due to our less-frequent blood sampling, and caused a tendency (P \leq 0.08) for increased FSH levels 12 h after treatment. In this aspect, our treatment represents a slight advantage to that used by Bagu et al. (2004) in terms of the length of time that FSH remained elevated in the bulls' circulation. Furthermore, treatment with 30 mg NIH-FSH-P1 in 2% HA was considered successful enough in transiently elevating FSH levels that an identical treatment regimen was implemented for use in a subsequent, longer-term experiment (Chapter 4. Effect of Slow-Release pFSH from 5-13 Weeks of Age on Endocrinology and Testicular Histology of Prepubertal Bull Calves). Table 3.1. Comparisons of percent binding between bovine FSH standards and matching porcine FSH standards via double-antibody RIA (Burke et al., 2003) to determine the ability of the RIA to detect porcine FSH in the range of physiological concentrations seen *in vivo* (0.5 - 20 ng/ml). A percent binding of 1 (100%) represents the total amount of binding capable in the assay. Lesser binding is based on the B₀ of the assay minus the non-specific binding (NSB), and divided by the Total cpm (amount of labeled hormone added to each sample). Within a respective standard, percent-binding between sources did not differ by more than 7.4%.

Assay Standards	percent binding (1 = 100%)			
(ng/ml)	Validated Ovine/Bovine Assay Standards	Newly-Made Porcine Standards		
0.5	0.817	0.744		
1	0.749	0.698		
2	0.549	0.551		
5	0.364	0.365		
10	0.221	0.262		
20	0.115	0.189		



Figure 3.1. A graphic representation of the timing of treatment administration and subsequent blood collections during the experiment, with each arrow representing a blood sample collection. Blood samples were collected immediately before each treatment (when bulls were 50 and 53.5 ± 6.5 d of age) and every 6 hours thereafter for a 24 hour period. After 24 hours following treatment, blood samples to assess FSH were collected every 12 hours. Approximately 5 ml of blood was collected from the jugular vein and placed into 5 ml-vacutainers without EDTA, allowed to clot for 48 hours at 4°C, and then centrifuged at 7,735 x g for 20 minutes in order to harvest serum to analyze FSH concentrations.



Figure 3.2. Percent binding for validated bovine and porcine FSH standards of known concentrations (0.5, 1, 2, 5, 10, and 20 ng/ml). Within the measured range of 0.5 - 20 ng/ml, no sample differed from its respective standards for comparison by more than 11%. Within the range of FSH concentrations observed in this experiment, it was determined that our lab's double-antibody RIA was successful in measuring FSH concentrations of bulls injected with porcine-derived FSH (Folltropin-V).



Figure. 3.3. Parallelism for porcine FSH standards (A.F. Parlow, National Hormone & Peptide Program, Torrance, CA, USA) in a validated bovine FSH radioimmunoassay (Burke et al., 2003). Linear equations including the slope of the line for each curve are located next to their respective legend.



Figure 3.4. Systemic FSH concentrations in bull calves after injection of 0.75 ml containing either 30 mg NIH-FSH-P1 in 2% hyaluronic acid (FSH-HA) or saline (control) at 50 ± 6.5 d of age and 53.5 ± 6.5 d of age (arrows). Blood was collected every 6 hours following treatment for 24 h, and at 12 h intervals until d 3.5. A Trt*Hour interaction (P = 0.0002) was detected. Concentrations of FSH differed 6 h after treatments (* P < 0.05) and tended (** P ≤ 0.08) to be greater in the FSH-HA than control treatment 12 hours after treatment administration. Concentrations of FSH did not differ between treatments from 18 to 72 h.

CHAPTER 4

EFFECT OF SLOW-RELEASE pFSH FROM 5-13 WEEKS OF AGE ON ENDOCRINOLOGY AND TESTICULAR HISTOLOGY OF PREPUBERTAL BULLS

INTRODUCTION

The shift from traditional progeny testing of AI bulls to a system placing emphasis on genomics is changing the chronology involved in bull selection (Amann and DeJarnette, 2012). Proofs generated from daughter production records are becoming secondary to genomic estimates of genetic worth which can be acquired as early as when the bull is an embryo (Humblot et al., 2010). The market for genomically-tested bulls is growing because the reliability of this system increases as more SNPs and animals are added to the databases. For example, when genomic predictions are added to predictions based on parent averages alone, the reliability in predicting a bull's ability to transmit certain traits increases from 29.6 to 61.1% (Van Raden et al., 2013).

Due to genomics, there is a desire within the AI industry to market bulls as early as possible. However, age at puberty has a primary impact on the age when sufficient semen can be produced and marketed. Puberty in bulls has been defined using several parameters, the first being the ability of an animal to produce an ejaculate containing 5.0×10^7 sperm with 10% motility, and the second being the attainment of a 28 cm scrotal circumference (SC; Wolf et al., 1965; Lunstra, 1982). The market demand for a genomically superior bull is greatest when he first attains puberty because his genomics represent the 'best' and will allow the most rapid genetic progress. Thus, it is not only desirable to decrease the age at which a bull reaches puberty, but also to increase the amount of semen the bull produces at this time.

Aspects of testicular physiology, such as the number of Sertoli cells contained within the testes, are pertinent to this topic because the number of Sertoli cells is correlated with the sperm production and testis size of in mature bulls (Berndtson et al., 1987). A unique opportunity lies in the early development of these cells since Sertoli cells divide in response to the stimulatory effects of prepubertal FSH (Orth et al., 1984). Furthermore, Sertoli cells divide until they have reached a mature status at a time closely preceding puberty (Sharpe et al., 2003; Gilula et al., 1976). Therefore, this time of plasticity in Sertoli cell proliferation represents a possible window when mature sperm production could be experimentally manipulated during sexual maturation. Past experiments have sought to hasten puberty and enhance testicular physiology by maximizing the transient increase in FSH from 6 to 20 wk of age (Amann et al., 1986) with the use of exogenous FSH administration. Bagu et al. (2004) treated bulls with exogenous FSH from 4 - 8 weeks of age. The administration of 10 mg NIH-FSH-S1 equivalent in this experiment, given every other day to total 35 mg weekly, caused an increase in the number of Sertoli cells within the seminiferous tubules at 56 wk of age

86

and hastened puberty by approximately 5 weeks compared to control animals. Based on the changes reported in this study, a preliminary experiment was conducted (Chapter 3. Development of a Slow-Release FSH Treatment in Prepubertal Bulls) to develop a timerelease pFSH treatment that could in turn be used to examine the effect of exogenous FSH on testicular development and endocrine profiles of prepubertal bulls. Based on the previous finding that an extended-release FSH could transiently increase systemic FSH concentrations, it was hypothesized that administration of this treatment from 5 to 13 wk of age would cause an increase in the number of Sertoli cells per testis as well as an increase in the percent of the testis comprised of tubules at the end of the treatment period. Therefore, the objective of this experiment was to test the effect of this exogenous slow-release FSH treatment from 5 - 13 weeks of age on the endocrinology and testicular histology of bulls at the conclusion of treatment.

MATERIALS AND METHODS

Animals and handling were conducted in accordance with procedures approved by The Ohio State University Agricultural Animal Care and Use Committee.

Animals and Treatments

Twenty-two Angus-cross bull calves born at The OSU Beef Center from multiparous cows were allowed *ad libitum* access to pasture, water, and suckling of dams for the duration of the experiment. At 35 ± 2.0 d of age calves were randomly allocated into two treatment groups based on birth date and pedigree. Treatments consisted of IM injection with 0.75 ml containing either 30 mg NIH-FSH-P1 (Folltropin-V; Bioniche Animal Health, Ontario, Canada) in a 2% hyaluronic acid solution (FSH-HA, n = 11) or saline (control, n = 11) every 3.5 d until 91 d of age. Hyaluronic acid was obtained from Lifecore Biomedical (Chaska, MN) as research grade dried sodium hyaluronate with molecular weight ranging from 601 - 850 KDa. Injections (IM) were given in a single area in the neck. The side of the neck that the treatment was administered was alternated to ensure previously placed boluses were not disturbed. Treatment formulation specifics can be seen in 'Materials and Methods' of Chapter 3.

Blood Sample Collection

Blood samples to assess FSH concentrations were collected immediately before treatment every 3.5 days from 35 to 91 ± 2.0 d of age. Approximately 5 ml of blood was collected from the jugular vein using a one-inch, 18 gauge needle and syringe, placed into vacutainers without EDTA (Becton Dickinson and Company, New Jersey, USA), and placed on ice until storage at 4°C. Samples were allowed to clot for 48 hours at 4°C and then centrifuged at 7,735 x g for 20 minutes after which serum was harvested and frozen at -20°C until analyzed for concentration of FSH.

Blood samples to assess testosterone were collected in the same manner and at the same times as FSH samples. Samples were placed into K2-EDTA vacutainer tubes (Becton Dickinson and Company, New Jersey, USA) and stored on ice until centrifuged at 7,735 x g for 30 minutes until plasma was harvested and samples frozen at -20°C until analyzed for concentration of systemic testosterone.

Hormone Analyses

Concentration of FSH was determined in duplicate for all samples with a validated double-antibody radioimmunoassay (RIA; Burke et al., 2003). Briefly, purified ovine FSH (LER1976-A2) was iodinated using the chloramine-T method, primary antibody was rabbit anti-ovine FSH (JAD #17-7.6.9), and secondary antibody was Donkey anti-rabbit IgG and Normal Rabbit Serum. Average intra-assay CV was 4.56%. Inter-assay CV for the low pool (2.69 ng/ml) was 4.22% and for the high pool (4.26 ng/ml) was 2.63%. Average sensitivity of the assays was 0.32 ng/ml. Concentrations of testosterone in plasma were analyzed in duplicate using a commercial

RIA kit (Coat-a-Count, Siemens Medical Solutions Diagnostics, Los Angeles, CA) validated by Burke et al. (2005). The inter-assay CV for the low pool (.01 ng/ml) was 1.67%, medium pool (0.37 ng/ml) was 18.55%, and high pool (0.81 ng/ml) was 14.08%. The average intra-assay CV was 13.0% and the average sensitivity was 0.03 ng/ml.

Body Weight and Scrotal Circumference

Body weight (BW) of the animals was recorded every 7 days from 31 to $91 \pm 2 d$ of age as bulls using a cattle squeeze chute equipped with a scale. SC was taken every 7 d before respective treatment administration. Briefly, with the bull properly restrained, the scrotum was grasped at the neck with one hand and the testicles were pulled gently into the bottom of the scrotum. The cloth scrotal tape was placed around the site of the greatest circumference, snugged, and the measurement recorded.

Castration and Testicular Measurements

The bulls were castrated according to the OSU Beef and Sheep Center Standard Operating Protocol at 93 ± 2 d of age d of age and testes were collected. Local anesthetic (Lidocaine HCl 2%, 5 mL per testis; Pro Labs Ltd., MO, USA) was injected into the scrotal neck after bulls were restrained in a squeeze chute. A single opening was cut into the bottom of the scrotum and each testicle pulled out of the scrotum and away from the body cavity. Testicles were pulled downward, exposing the spermatic cord while pushing back the connective tissue. The cord was cut above the testicle using an emasculator with crimper, and bulls were treated with spray-on antibacterial medication and insect repellant in the area post-operatively.

Immediately following castration, testes were randomly designated for either dissection or histological evaluation and snap freezing by alternating between right or left in bulls within the same treatment. For testis dissection, the epididymis and the pampiniform plexus were removed, leaving only the testis and tunica albuginea, which was weighed. Testis volume was measured by submersion in a graduated cylinder filled with saline. In the other testicle, sections approximately 5 mm thick representing the proximal, middle, and distal third of each testis were collected for histological analysis. Sections were fixed in 10% formalin for 12 hours and then placed in 70% ethanol. Following fixation the tissue was imbedded in paraffin for microtome sectioning. A section adjacent to each section that was fixed (3 per testis) was collected and immediately placed in a labeled cryovial and snap frozen in liquid nitrogen. After freezing, samples were stored at -86°C until analyzed for intratesticular testosterone concentration.

Testicular Histology
The sections were stained using a standard hematoxylin and eosin stain for measurement of seminiferous tubule diameters using an ocular micrometer (Zeiss, Fisher Scientific, Pittsburgh, PA, USA) and determination of the percentage of the testis comprised of seminiferous tubules. Seminiferous tubule diameter was measured for ten randomly selected round tubules per section from each testis. The percentage of testis comprised of tubules was determined by capturing five frames from each section using SPOT 5.0 software (SPOT Imaging Solutions, Sterling Heights, MI) on a cameraequipped microscope at the same magnification (400X). For each frame, the tubules were traced within the given image frame using ImageJ (U.S. National Institutes of Health, Bethesda, Maryland, USA) to generate a percentage of the parenchyma comprised of tubules based on pixel ratios.

Testicular Immunohistochemistry

Localization of GATA-Binding Protein 4 (GATA-4)

Tissue sections were subjected to immunohistochemistry for localization of the transcription factor GATA-4 as a marker of Sertoli cell nuclei (McCoard et al., 2001). An affinity purified goat polyclonal antibody (C-20, Santa Cruz Biotechnology, Santa Cruz, CA) developed against a peptide mapping at the carboxy-terminus of GATA-4 of mouse origin was used at a 1:100 dilution. The epitope is highly conserved between several species (mice, human, pigs, cattle) and the antibody has been used in cattle (Jimenez-Severiano et al., 2005). Secondary antibody was a Rabbit-Anti-goat, and slides were counterstained with hematoxylin, dehydrated, cleared, and mounted. All procedures were

performed by The OSU Comparative Pathology and Mouse Phenotyping Shared Resource.

Validation of GATA-4 Staining as a Marker of Prepubertal Bovine Sertoli Cell Nuclei

Additional microtome sections taken from both the experimental prepubertal bulls and 18-month-old Holstein bulls were collected, fixed, and subjected to the immunohistochemistry procedures described above but with a preliminary incubation with GATA-4 Blocking Peptide (Santa Cruz Biotechnology, Santa Cruz, CA) used in 10X excess of the primary antibody. Negative controls were created by incubating with buffer solution and no primary antibody to check for nonspecific binding of the secondary antibody. Examination of stained sections from 18-month-old bulls confirmed the GATA-4 stain (C-20) was selectively staining Sertoli cell nuclei but not germ cell nuclei or spermatozoa based on cell location and morphology.

Counting Sertoli Cells per Seminiferous Tubule

After GATA-4 immunohistochemistry, each section (3 per bull) was examined at 400X magnification, and six round seminiferous tubules were randomly selected. The number of stained Sertoli cell nuclei present in a circular monolayer within the round tubule was recorded.

Intratesticular Testosterone Concentration

Frozen (-86°C) pieces of testicular parenchyma (0.4 to 0.8 grams per bull) originating from the upper third of the testis were placed in 3 ml cold PBS and allowed to thaw. Tissue was homogenized at 10,000 rpm for three minutes using a hand-held tissue homogenizer (Omni GLH-01, Omni International, Kennesaw, GA, USA), and samples

were centrifuged at 7,735 x g for 3 hours at 4°C. Supernatant was collected and each respective sample adjusted for differences in original weight of tissue by adding cold PBS to bring the dilution of all samples to be equivalent to that of the least concentrated sample. Next, an aliquot (0.6 ml) from each sample was used for extraction with diethyl ether. Intratesticular testosterone (ITT) concentrations were measured from extracted samples using a commercial testosterone RIA kit (Burke et al., 2005; Coat-a-Count, Siemens Medical Solutions Diagnostics, Los Angeles, CA). All samples were run in duplicate within the same assay, and average intra-assay CV was 4.9%. Sensitivity of the assay was 10.6 pg/ml.

Statistical Analyses

Bull weight, SC, FSH and testosterone concentrations were analyzed by ANOVA using the MIXED procedure of SAS (version 9.2, 2002) with repeated measures analysis included in the model. The initial concentration of FSH was used as a covariate for analysis of FSH concentrations throughout the experiment. Intratesticular testosterone concentrations (ITT), testis weight and volume, epididymal weight, seminiferous tubule diameter, percent of testis comprised of tubules, and number of Sertoli cells per tubule were analyzed using PROC GLM procedure of SAS. All results are presented in terms of means \pm SE. The correlations between average age of the bulls, weight, ITT, testis weight, testis volume, number of Sertoli cells per round seminiferous tubule cross section, and all FSH and testosterone concentrations (35 to 91 d of age) were analyzed within treatment using PROC CORR of SAS.

RESULTS

Weight and SC at 35 (89.9 \pm 4.1 kg; 14.2 \pm 0.64 cm) and 91 (151.0 \pm 6.8 kg; 16.3 \pm 0.74 cm) d of age did not differ between treatments. Peripheral testosterone concentrations (Figure 4.1) increased with age (P < 0.001) but did not differ between treatments (Trt*Age, P = 0.282). Concentrations of FSH did not differ between treatments from 35 to 67.5 days of age but increased (P < 0.05) in the FSH-HA treatment at 70 days of age (Trt*Age, P = 0.049; Figure 4.2) and remained elevated over previous concentrations through 91 d of age. In the control treatment, concentrations of FSH did not change during the experiment. Accordingly, concentrations of FSH from 70 to 91 d of age were greater (P < 0.05) in the FSH-HA than control treatment.

Post mortem testis weight, volume, and intratesticular testosterone concentrations did not differ between treatments (Table 4.1). Histological evaluation revealed no differences in the percent of parenchyma comprised of seminiferous tubules or diameter of seminiferous tubules between treatments. Validation of the GATA-4 staining of Sertoli cells can be visualized in Figure 4.3. Bulls in the FSH-HA treatment had greater (P < 0.05) numbers of Sertoli cells per seminiferous tubule cross section than control bulls (Table 4.1).

Expected and unexpected relationships of dependent variable were revealed by correlation analysis for age, weight, ITT, testis weight, testis volume, number of Sertoli cells, and peripheral FSH and testosterone concentrations. Age at castration was correlated (P < 0.05) with testis weight (R = 0.68) and volume (R = 0.73). Regarding correlations between testicular morphometry and endocrinology, the number of Sertoli

cells was correlated with systemic concentrations of testosterone at 11 wk of age (R = 0.66, P = 0.03) and tended to be related to concentrations of testosterone at 10 (R = 0.53, P = 0.09) and 13 weeks of age (R = 0.57, P = 0.07). Consistently, testosterone concentrations averaged over the duration of the experiment tended (P < 0.08) to be correlated with number of Sertoli cells (R = 0.55).

DISCUSSION

The most notable effect of FSH-HA on the endocrinology of bulls was the increase in systemic FSH concentrations beginning at 70 d of age, whereas no differences in systemic or intratesticular testosterone concentrations were detected. Neither SC, testis size, diameter of seminiferous tubules, nor percent of the testis comprised of tubules were influenced by FSH-HA treatment. However, consistent with the experimental hypothesis, number of Sertoli cells per tubule were increased in the FSH-HA bulls. This suggests that there are more Sertoli cells per testis in the FSH-HA treatment because there were no differences in testis size between treatments.

Previous reports of changes in FSH secretion in prepubertal bulls have been variable. While an initial report suggested no substantial changes in peripheral FSH during sexual maturation (McCarthy et al., 1979), others report a transient increase that begins at 4 to 6 wk of age that returns to basal concentrations at 25 to 32 wk of age (Amann and Walker, 1983; Amann et al., 1986; Evans et al., 1993; Rawlings and Evans, 1995; Aravindakshan et al., 2000; Bagu et al., 2006). The first observation of FSH concentrations in the present experiment (5 wk of age) occurred during or after the initial increase in FSH that has been reported by others, and the final observation (13 wk) was at an age much younger than when FSH has been reported to return to basal concentrations. Therefore, the static concentrations of FSH in the control treatment in the present experiment are generally consistent with previous reports. The increased FSH concentrations observed in the FSH-HA treatment may represent further increases over the already elevated basal levels present during the transient gonadotropin increase. Whether FSH-HA bulls would have experienced a decrease in FSH levels at 25 to 32 wk of age remains to be determined.

The mechanisms underlying the increase in FSH concentrations in FSH-HA bulls at 10 wk of age are more difficult to discern. In Chapter 3 of this thesis, 'Development of a Slow-Release FSH Treatment in Prepubertal Bulls,' more intensive blood sample collection demonstrated clearly that exogenously administered FSH (FSH-HA treatment) is cleared from circulation within 24 to 36 h after administration. Therefore, since samples in the present experiment were collected 3.5 d after the previous FSH treatment, we are confident that increased FSH at 10 wk of age and thereafter in this treatment are the result of increased endogenous secretion and not residual pFSH remaining from the previous injection. It is unlikely that the FSH-HA treatment directly affected pituitary secretion of FSH because the presence of FSH-R in the pituitary has not been defined in any species. It is more likely that the exogenous pFSH affected the hypothalamopituitary-gonadal axis at the gonadal level. One possibility is that the FSH-HA treatment hastened or increased production of activin by Sertoli cells (Buzzard et al., 2004) or peritubular myoid cells (Buzzard et al., 2003) in the testes of FSH-HA bulls. It has been reported that activin concentration is correlated to Sertoli cell proliferation (Barakat et al., 2008) and a greater number of Sertoli cells were detected in the FSH-HA bulls. Because activin counteracts the actions of inhibin and causes basal (pituitary-derived) and GnRH-induced secretion of FSH from the anterior pituitary (de Kretser and Phillips, 1998) quantification of changes in activin in response to a similar treatment is of interest.

One of the most relevant findings that agreed with the experimental hypothesis was the increase in the number of Sertoli cells in the FSH-HA bulls. It can be inferred that the increase in Sertoli cells per tubule cross section in the FSH-HA treatment would translate to larger numbers of Sertoli cells on a per-testis-basis since there were no differences in weight or volume of testes, or proportion of the testes comprised of seminiferous tubules between treatments. This increase in Sertoli cells can likely be attributed to the exogenous pFSH and perhaps the increase in endogenous FSH concentrations noted in the last few weeks of the study.

This linkage is supported by past evidence in bovine (Bagu et al., 2004) and other species (Orth et al., 1984) that FSH has a synergistic and positive effect on the proliferation of Sertoli cells in prepubertal males. The increase in the number of Sertoli cells (approximately 5 Sertoli cells per tubule monolayer) in response to FSH-HA is promising because of the positive correlation between the number of Sertoli cells within the adult testis and the total sperm output in mature animals (Berndtson et al., 1987). Sertoli cells can only host and nourish a fixed number of germ cells (Blanchard and Johnson, 1997; Leal et al., 2004), which is supportive of the aforementioned correlation reported by Berndtson et al. (1987). Therefore, if this enhancement in the number of

prepubertal Sertoli cells was sustained in the bulls until adulthood, this represents a potential means to increase sperm production once bulls reach maturity. Future longer-term experiments are needed to examine this possibility.

Intratesticular testosterone was utilized as a measure of testicular maturity in this experiment. A bull must have functioning mature Leydig cells in order to produce testosterone (Boockfor et al., 1983), so the level of Leydig cell maturation was targeted as a marker of development in this experiment. However, the pattern of ITT showed no obvious trends between or within treatments. ITT was not correlated to age (R = -0.13, P = 0.73) or any parameter of endocrine or testicular development measured. It is presumed that the chronology involved in testicular maturation, such as Leydig cell maturation and production of testosterone, was not well represented using ITT at 93 d of age. In contrast to intratesticular concentrations, systemic concentrations of testosterone had several important correlations to other experimental variables. The number of Sertoli cells per tubule cross section had a tendency to correlate to systemic concentrations of testosterone (averaged by week of age) when the bulls were 10 and 13 weeks of age, with 13 weeks of age being the last week of treatment. Also, systemic testosterone had a significant positive correlation at 11 weeks of age to number of Sertoli cells per tubule at the time of castration. In this sense, systemic testosterone levels seem to be more useful or reflective to prepubertal testis development than the actual levels of testosterone within the testes.

Overall, the changes observed in the bulls after FSH-HA treatment in this experiment are noteworthy, because if preserved to puberty, they could translate to increased sperm production in mature animals. This has the greatest applicability for genomically-valuable bulls, which have great demand for their genetics as soon as puberty is attained. Accordingly, follow-up studies examining the effect of this FSH-HA treatment during sexual maturation on age at puberty and sperm production as mature bulls are necessary. Furthermore, understanding of the mechanisms by which exogenous FSH alters endogenous FSH secretion, Sertoli cell proliferation, and other aspects of the maturation process in bulls is warranted. Table 4.1. Post mortem testicular characteristics of 93 ± 6.5 d old bulls treated with either 30 mg pFSH (FSH-HA, n = 11) or saline (control, n = 11) every 3.5 days from 35 to 91 days of age. Results are reported as mean \pm SE.

	Control	FSH-HA	Р
Testis Volume (mL)	221.7 ± 1.7	222.9 ± 3.1	0.78
Testis Weight (g)	23.43 ± 2.0	24.14 ± 2.4	0.87
Seminiferous tubule diameter (µm)	78.6 ± 2.1	80.2 ± 2.1	0.6
Percent of testicular parenchyma comprised of seminiferous tubules	51.0%	51.9%	0.18
Sertoli cells per round tubule cross- section	28.27 ± 0.9	33.35 ± 0.9	0.0008
Intratesticular Testosterone (ng/g)	806.1 ± 100.8	738.9 ± 96.1	0.63



Figure 4.1. Peripheral testosterone concentrations of bulls treated with either 30 mg pFSH (FSH-HA, n = 11) or saline (control, n = 11) every 3.5 d from 35 to 91 d of age. Concentrations were not different at any age between treatments (Trt*Age, P = 0.282), but did increase for all animals as they aged (P < 0.001).



Figure 4.2. Systemic concentrations of FSH immediately before bulls received either 30 mg pFSH (FSH, n = 11) or saline (Control, n = 11) every 3.5 d from 35 to 91 d of age. Concentrations of FSH (Trt*Age, P = 0.049) were increased (P < 0.05) over prior concentrations in the FSH-HA treatment from 70 to 91 d of age, and were greater (P < 0.05) than control concentrations from 70 to 91 d of age. * P < 0.05



Figure 4.3. Immunolocalization of GATA-4 in nuclei of Sertoli cells (SC) in bull testes. All slides were counterstained with hematoxylin in order to visualize all cell nuclei present. Both (**B**) Prepubertal and (**D**) postpubertal testis with GATA-4 staining showcasing Sertoli cell nuclei (SC) stained reddish brown within the seminiferous tubules. Germ cells (GC) remained unstained and are noted in both (**B**) prepubertal and (**D**) postpubertal images. Leydig cell nuclei (interstitium) were stained in both prepubertal and postpubertal animals. Panels (**C**) and (**D**) depict sections in which GATA-4 blocking peptide was used to confirm the specificity of the primary antibody in prepubertal and postpubertal bulls, respectively. The appearance in Panels C and D was identical to sections stained only with hematoxylin, indicating the specificity of GATA-4 immunostaining procedures to identify Sertoli cell nuclei. Magnification bar on each micrograph represents 100 μm.

CHAPTER 5

GENERAL DISCUSSION

An overall goal of this thesis research was to examine the efficacy of technologies that may advance puberty and enhance sperm production in bulls. The benefits of such technologies are twofold. Foremost, advancements in either of these objectives represents monetary benefits to both AI companies and cattle producers. Successful implementation of these technologies means AI companies will have more saleable product from a bull at an earlier time, and producers may be less limited in the product they wish to use. However, a second and possibly more valuable benefit results from the genetic advancements the industry may experience by increasing use of young, genomicallysuperior bulls. Increasing the availability of the best genetics from young bulls will allow for expedited improvement of genetic traits that are useful in today's cattle. With these needs and goals in mind, the two experiments in this thesis were performed utilizing two different methods of accomplishing the endpoints listed above.

The first of these experiments focused on dietary manipulation, but it may have fallen short of final expectations. The use of a high energy diet was effective in changing testicular size, but there is no doubt an opportunity was missed in terms of the age at which these bulls experienced puberty as well as their potential mature sperm production. The consolidation of bulls to one standard diet upon the move to Select Sires, Inc. most likely caused a decreased plane of nutrition for the 'High Energy' diet bulls, and we may have missed or diluted significant results because of the interruption in growth (ADG) these bulls experienced. However, this experiment does show the potential usefulness in feeding a high energy ration to growing bulls. The increase in testicular size alone indicates that bulls have the potential for increased mature sperm production (albeit not detected in our study), and this may compensate for any additional input costs in raising animals on this diet.

The second of the experiments utilized exogenous administration of pFSH to prepubertal bulls and showed promise in its ability to affect sperm production positively (increase in number of Sertoli cells per testis at 93 d of age) had the bulls been left intact to maturity. This study was novel because it verified the effectiveness of a 'time-release' treatment formulated from porcine-pituitary-derived FSH that caused an increase in circulating FSH concentrations; this specific form of treatment has not been used for this purpose in prepubertal bulls. Besides the potential usefulness of the treatment, this study essentially yielded more questions than it provided answers. The mechanisms underlying an increase in endogenous FSH production in FSH-HA treated bulls needs to be elucidated. Whether this increase is due to an enhanced development of the gonad (activin production) or another explanation, more experiments would need to be conducted to be certain. Also, we have interest in knowing how FSH-HA bulls would have performed in maturity based on the changes seen at 93 d of age. It is unknown how the reported endocrine and physiological changes would have affected the age at puberty and the mature sperm production of FSH-HA bulls, but a subsequent, longer-term experiment would have to be conducted in order to examine this.

The endocrine events of the prepubertal bull have been mapped out thoroughly in the past, and in my opinion, there will be few or very occasional novel findings that can result from further study. However, there is usefulness for novel research focusing on ways to *manipulate* these autonomous prepubertal endocrine changes in order to benefit cattle production. As previously stated, in the case of bulls possessing superior genetics, room for improvement still exists in terms of successful increased production of their genetics in a timely manner. Until drastic improvements are made to the commercial production of sperm (i.e. *in vitro* spermatogenesis), there should be a continued goal of introducing technologies to facilitate earlier acquisition of sperm from genetically superior sires and of maximizing their production while they are young and have influence on the industry.

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