# ENHANCING SPERM PRODUCTION IN THE BULL

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of MUHAMMAD SALMAN WAQAS find it satisfactory and recommend that it be accepted.

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Abstract

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Spermatogenesis is a complex process highly regulated mainly by somatic cells of testes, the Sertoli cells. The number of Sertoli cells for a male is fixed between birth and puberty, and does not increase after puberty except in the horse. Prepubertal Sertoli cells highly express receptors for thyroid hormone and the expression significantly decreases after puberty. Studies in rodents showed that the size of the Sertoli cell population is set via prepubertal signaling from thyroid hormones. Spermatogenic capacity of all mammals is strongly correlated to the number of Sertoli cells in the testes (R=0.83 for the bull). Male spermatogenic capacity is a critical component of cattle production and the majority of genetic gain is made via selective utilization of gametes from desirable sires. Thus, strategies that enhance sperm production increase the availability of elite genetics for use in improving production characteristics of the populations on a worldwide scale. Here, we devised a strategy to increase Sertoli cell number in bulls via induction of a transient hypothyroidic state just prior to and extending beyond the period of Sertoli cell proliferation that we found to normally cease between 4.5 and 5 months of age. We treated Angus calves (n=3 vs n=3 age matches controls) with the drug Methimazole®, 2mg/kg body weight twice a day, from 4-6 months of age to transiently induce hypothyroidism. Analyses

for serum levels of Thyroxine and Triidothyronine demonstrated effectiveness at inducing transient hypothyroidism and reversion to euthyroidism by approximately 9 months of age. In adulthood, these bulls produced a significantly greater number of sperm, up to 282%, compared to age matched controls and their testes contained nearly two times more Sertoli cells. Importantly, sperm morphology; fresh and post-thaw sperm motility, fertilizing ability, and viability were found to be no different for treated bulls compared to untreated control bulls. This strategy of transient induction of hypothyroidism during a defined period of pre-pubertal development in bulls could prove to be an efficacious approach for enhancing daily sperm production in genetically desirable sires that will, in turn, provide an avenue for improving the efficiency of commercial cattle production.

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**DEDICATION**

To my family back home, whom I always missed!

# CHAPTER ONE: INTRODUCTION

Traditionally, the bull has been truly called half of the herd. The bull contributes to the genetic and production potential of the herd more extensively and perpetually than does the cow. If the replacement heifers are maintained, the bull affects the production potential of the herd for about 25 years [1]. Spread of male germplasm through artificial insemination is the major tool for genetic improvement in cattle production. In United States, adaptation of artificial insemination led to a 4.5 fold increase in milk production per average dairy cow from 1940 to 2009[2].

## 1.1 Rationale

Sexed semen enables rapid herd expansion by supplying replacement heifers for dairy and increases profit through male calves for beef production[3]. One of the criterion for a bull to be enrolled in sex sorting program is that it must be able to produce ejaculate(s) with at least six billion spermatozoa for each run of semen sexing process[3]. Low daily sperm production (DSP) in some adult bulls makes them unsuitable for supplying this many spermatozoa for semen sexing process even if they possess elite production potential. Moreover, all younger bulls cannot produce six billion sperm cells for each run of semen sexing process because they have lower DSP. Therefore, bulls are enrolled in sex sorting programs at or after two years of age. As the bull attains puberty at about one year of age, this late enrollment at or after two years of age delays genetic improvement[3]. An increase in DSP may eliminate this problem of no enrollment to semen sexing program of a bull with lower DSP or late enrollment due to lower DSP in younger bulls. This in turn will enhance the speed of genetic improvement through sex sorting program. Moreover, the cost of bull maintenance and daily sperm production are major factors affecting the cost of sexed semen and of artificial insemination[4,5]. The cost of semen production can be reduced by

obtaining more spermatozoa per bull with fixed cost of maintenance per bull i.e., by increasing daily sperm production per bull. Increased DSP in the bull is advantageous for rapid genetic improvement in cattle production, for decreasing cost of semen production in AI and semen sexing programs, for obtaining more spermatogonial stem cells (SSCs) for SSCs transplantation[6] and therefore is of interest to livestock industries.

Several methods including unilateral castration, immunization against inhibin and immunization against estradiol have been used to increase the testicular size and daily sperm production in the bull, however none of these have gained commercial success [6]. Daily sperm production in the bull is positively correlated (R= +0.83) to the number of total SC per testes and becomes fixed once proliferation of SC stops[7]. A potential method to increase DSP in the bull is by increasing Sertoli cell number per testis leading to an increased DSP.

### 1.2 Hypothesis

Prepubertally induced transient hypothyroidism in the bull calves will increase adult Sertoli cell number and the spermatogenic capacity in adulthood without significantly affecting the semen quality.

### 1.3 Objectives

1. To determine the age when Sertoli cell (SC) proliferation stops in the bull.
2. To determine if induced transient hypothyroidism (ITH) expands the time window of testicular SC proliferation and hence increases their number.
3. To examine if ITH-induced increased SC number subsequently increases sperm production after puberty.

4. To evaluate the spermiogram, freezability and in vitro fertilization capacity of spermatozoa from ITH-treated bulls.
5. To investigate the effect of ITH on weight gain of the bull.

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# CHAPTER TWO: SPERMATOGENESIS, SERTOLI CELL AND THYROID HORMONE: A REVIEW

## 2.1 Spermatogenesis

Spermatogenesis is a highly proliferative and regulated process of multiple germ cell divisions to increase their number and differentiation to spermatozoa in the seminiferous tubules (ST) of testes. Testes produce spermatozoa through their exocrine function and, steroids mainly by Leydig cells through endocrine function. Spermatogenesis can be divided into spermatocytogenesis, meiosis and spermiogenesis. During spermatocytogenesis, germs cells divide by several mitoses to increase the yield of spermatogenesis, renew spermatogonial stems cells, produce more undifferentiated spermatogonia and finally produce primary spermatocytes. During meiosis, recombination of genetic material happens, homologous chromosomes move apart and chromosome number is reduced by half to yield haploid round spermatids. Spermiogenesis involves differentiation of haploid round spermatids into mature haploid elongated spermatozoa without mitosis or meiosis [1]. The process starts at puberty and human beings produce 10,000 spermatozoa with each heartbeat. This enormous spermatogenesis is maintained by spermatogonial stem cells (SSCs) at the basement of ST [2]. Alkaline phosphatase positive epiblast cells of ectoderm are the embryonic origin of SSCs. About 100 such cells, which had been lineage-restricted from day 7.2 post mating, develop to primordial germ cells (PGCs) in the mouse embryo [3]. The PGCs multiply and reach genital ridge which is prominent by day 11.5 post coitum [4]. About 10,000 unipotent PGCs are found in each mice testis. In gonadal ridge, PGCs harbor the seminiferous cords and become mitotically arrested at day 13.5 post coitum, hereafter termed as gonocytes or prospermatogonia. Prospermatogonia, morphologically different from PGCs, undergo extensive epigenetic reprogramming for their

later function as male gametes [5]. Once prospermatogonia reach basement peripartum, they are morphologically and biochemically different from prospermatogonia and are termed as $A_{undifferentiated}$ spermatogonia. $A_{undifferentiated}$ spermatogonia proliferate but remain connected as $A_{single}$, $A_{paired}$, and $A_{aligned}$ by intercellular bridges. $A_{aligned}$ are syncytia of 4,8 and 16 undifferentiated spermatogonia. Currently, no method distinguishes among different types of undifferentiated spermatogonia [6]. Syncytia of $A_{undifferentiated}$ spermatogonia can break to yield SSCs. $A_{undifferentiated}$ spermatogonia irreversibly differentiate to $A_1$ spermatogonia under the repeated stimulation of retinoic acid. In rodents, chain identity is maintained upon this transition and it has been estimated that 64% of $A_{paired}$, 94% of $A_{aligned4}$ and 100% of $A_{aligned8\text{-}16}$ transition to the differentiating A1 state following a retinoic acid pulse [7]. A1 spermatogonia undergo 6 synchronized mitoses through $A_2$, $A_3$, $A_4$, intermediate (In) and B spermatogonia to form primary spermatocyte. [2]. Intercellular bridges play a role in cells to cell communication and synchronization of these mitoses. In the classical Huckins and Oakberg model, SSCs are contained within $A_{single}$ population [8,9]. All undifferentiated spermatogonia other than SSCs are called progenitors. $A_{single}$ undergo symmetric divisions forming syncytia of undifferentiating spermatogonia, or asymmetric division forming an SSCs and a progenitor $A_{single}$ thus maintaining steady-state spermatogenesis [2]. The total number of $A_{single}$ in adult mouse testes is estimated at 35,000 and each adult mice testes has about 3,000 SSCs which is about 0.01% of total testis cells [10]. While, SSCs are 0.03% of the entire testicular cell population. Furthermore, $33x10^4$ undifferentiated spermatogonia and $280x10^4$ differentiating spermatogonia populate each testis in the mouse [7]. SSCs, as well as epiblast and PGCs from days 6-16.5 post coitum embryo can regenerate spermatogenesis upon transplantation to appropriate donor testes [11].

SSCs capable of self-renewal and generating progenitor cells through symmetrical and asymmetrical divisions reside in a specific microenvironment within ST termed as germline stem cell niche [12]. Sertoli cells in communication with other somatic cells of the testes secrete factors to maintain microenvironment of the niche. Within the niche, SSCs are located towards areas of ST closer to interstitial vasculature [13].

Development of preleptotene spermatocyte from type B spermatogonia marks cells detachment and migration away from the basement membrane. Preleptotene spermatocytes cross the tight Sertoli cell junction and reach the adluminal portion of ST [1]. Primary spermatocytes first divide mitotically and then form short-lived haploid secondary spermatocytes through meiosis I. During prophase of meiosis preleptotene spermatocytes differentiate into leptotene, zygotene, pachytene and diplotene, respectively. Equational meiosis II of secondary spermatocytes forms haploid spermatids. Round spermatids mature into spermatozoa through various steps of spermiogenesis. Spermatozoa are releases into the lumen of ST through the process of spermiation.

### 2.2 The cycle of the seminiferous epithelium

The cycle of the seminiferous epithelium is histological observation at a specific point of ST in time [1]. The notion of the cycle of the seminiferous epithelium was established towards the end of $19^{th}$ century [14]. Several generations of germ cells are present along ST because spermatogenesis is a continuous process and subsequent spermatogenesis does not pause to wait for completion of the first generation to spermatozoa and release in the lumen of ST [15]. Along the section of ST, the development of germs cells is influenced by neighboring cells resulting in specific germ cell associations following one another in time at a given section of ST in a

perfectly organized pattern, termed the cycle of the seminiferous epithelium. The cycle results from the fact that: 1) at specific points of ST new spermatogonia enter spermatogenesis at constant time intervals; 2) once the cells are engaged in spermatogenesis, their rate of differentiation to next cell type is always a constant and; 3) each step of spermatogenesis has a constant and fixed time duration. The specific germ cell associations at a given point of ST during the cycle of the seminiferous epithelium are termed as stages or less commonly as phases of the cycle.

### 2.3 The spermatogenic wave

The spermatogenic wave is "a sequence of segments showing the complete series of cell associations corresponding to the stages of the cycle of the seminiferous epithelium". One or more modulations, however, can be excluded from the sequence of the segments considered." While the cycle of the seminiferous epithelium refers to the temporal arrangement of the germ cells at given point of ST, the spermatogenic wave refers to the spatial arrangement of germ cells along ST [16]. However, the wave is not in space what the cycle is in time, "the wave is not a dynamic process but is a static way to describe in the spatial distribution of the associations along the tubule [1]. Spermatogenesis progresses along ST in the waveform, the wave is formed by the fact that specific germ associations, i.e., stages, start again and again at a specific distance. The regular order of the wave emergence along ST follows numerical order of the stages of the cycle of the seminiferous epithelium [17,18]. Different waves start as we move along ST and at a specific section of ST, cells from different waves are seen together in a cross-section of ST according to the stage of the cycle of the seminiferous epithelium. The waves move along the ST in an inward spiral fashion with $A_{undifferentiated}$ at the outermost border of the wave and

spermatozoa at the inner border of the wave. One spermatogenic wave starts with initiation of spermatogenesis, i.e., from the conversion of type $A_{undifferentiated}$ to type $A_1$ differentiating spermatogonia under the repeated actions of retinoic acid and ends with the formation of spermatozoa [19].

### 2.4 Spermatogenesis in the bull

In the bull, the cycle of the seminiferous epithelium is divided into eight stages and spermiation is taken as a reference point [20,21]. In bulls, the duration of the seminiferous epithelium cycle is 13.5 days. The total duration of spermatogenesis is 4.5 times the duration of the cycle that is 61 days. The durations of spermatocytogenesis, meiosis and spermiogenesis are 21, 23 and 17 days, respectively [1].

### 2.5 The efficiency of spermatogenesis in the bull

Histologically, spermatogenesis is evaluated qualitatively by the appearance of ST and quantitatively by differential cell count in the ST. Daily sperm production per gram of decapsulated testes is an appropriate measure of the efficiency of spermatogenesis and is used for species comparison [22]. The efficiency of spermatogenesis in terms of daily sperm production (millions) per gram of testes is: 4 to 6 for the man, 12 for the bull, 16 to 19 for the stallion, 21 for the ram, 23 for the rhesus monkey, 20 to 24 for the rat, 24 for the hamster and 25 for the rabbit [22–24]. Daily sperm production is affected by longer duration of spermatogenesis, the longer cycle of the seminiferous epithelium, density of germ cells in ST and germ cell degeneration during spermatogenesis [24]. The reason for the lower efficiency of spermatogenesis in the bull is not understood [1]. In the bull, germ cell degeneration during the transition from $A_1$- $A_4$, and at intermediate spermatogonia stage accounts for 30% losses in daily sperm production. Moreover,

degeneration at $B_1$ and during the transition from $B_1$ to $B_2$ caused another 30% loss. However, there is no loss during meiosis and spermiogenesis in the bull. In the absence of this degeneration, the bull would have a daily sperm production of around $30 \times 10^6$/gram of testes [1,17].

### 2.6 Sertoli cells and Spermatogenesis

Sertoli cell (SC) plays an indispensable role in the regulation of spermatogenesis, establishing the rate of spermatogenesis and in development and movement of germ cells. [25,26]. SC is one the most complex and dynamic cells in biology [27].

The cell was first described by and hence named after Enrico Sertoli [28]. SC are large irregular shaped columnar cells extending from base of ST to the apex of ST and occupy 17-19% volume of the tubule [26]. The large surface area of SC allows for interaction with an enormous number of germ cells as SC: germ cell ratio in adult rat is about 1:50 [29]. SCs are the most important somatic cells of testes and possess high plasticity synchronized with the cyclic evolution of germ cells. The cells change their structure during their development and according to the cycle of the seminiferous epithelium. At any life point, SCs of type A and type B are seen in ST. Type A SCs have cytoplasmic crypts for attachment of mature spermatids ready for release into the ST lumen. In type B SC such cytoplasmic crypts are less prominent or absent [30]. SC nucleus is multi-lobed and the cytoplasm is rich in the endoplasmic reticulum, glycoproteins and cytoplasmic droplets; while the cytoskeleton varies with the cycle of the seminiferous epithelium [31].

### 2.7 Functions of Sertoli cell

#### *2.7.1 Structural support to spermatogenesis*

SCs in association with peritubular myoid cells secrete basement membrane of ST [32]. The cytoskeleton of SC participates in organizing and shaping ST [33]. Major components of SC cytoskeleton are actin, intermediate filaments, and microtubules and each has a unique distribution pattern according to different stages of the cycle of the seminiferous epithelium [34]. The functions of SC cytoskeleton include: 1) maintaining SC shape; 2) positioning and transporting organelles within the cell; 3) forming and stabilizing SC membrane at sites of cell-cell and cell-extracellular matrix contact; 4) positioning, anchoring, and aiding in the movement of developing germ cells, and 5) involvement in the release of mature spermatids from the ST during spermiation [26].

*2.7.2 Blood-testis barrier*

At puberty, neighboring SCs form tight junction forming an impermeable blood-testis barrier [32]. The barrier is a modified occluding junction located in basal third of seminiferous epithelium[35]. Blood-testis barrier divided the seminiferous epithelium in basal and adluminal compartments [36]. The basal part houses spermatogonia, preleptotene, and leptotene spermatocytes while further advanced meiotic spermatocytes and spermatids reside in adluminal part [26]. Blood-testis barrier protects germ cells in the adluminal part of ST from direct contact with blood protecting the cells from toxic, mutagenic and autoimmune reactions. The barrier maintains specific concentrations of androgen-binding protein, inhibin, activin, and enzyme inhibitors within the adluminal compartment of ST. It controls transport of molecules and wastes to and from the adluminal compartment. [37,38]. Blood-testis barrier also functions as an immunological barrier to protect novel proteins on spermatocytes and spermatids from the autoimmune reaction and inhibits immunoglobulins and lymphocytes from entering the

adluminal part [27]. SC and germ cells produce interferons, interferons induced proteins, interleukins, and cytokines to maintain an antiviral defense system [39].

*2.7.3 Germ cell translocation*

As germ cells lack necessary architecture for migration, SCs are responsible for germ cell migration across the seminiferous epithelium. SC assist in the translocation of preleptotene spermatocyte from basal to adluminal compartment of ST across the blood-testis barrier [26]. Specialized adhesion junctions, termed ectoplasmic specializations, of SC attach to spermatid head and translocate spermatids up and down across seminiferous epithelium. Ectoplasmic specializations consist of regions of SC plasma membrane adherent to the spermatid head, a submembrane layer of tightly packed SC actin filaments and an attached cistern of SC endoplasmic reticulum. Motor proteins of SC endoplasmic reticulum translocate ectoplasmic specializations and hence spermatids are also translocated [40].

*2.7.4 Spermiation and phagocytosis*

During spermiation, mature spermatids are released from SC into ST lumen before their subsequent passage to the epididymis. For spermiation, mature spermatids are oriented towards the lumen of ST and subsequently released into the lumen by spermiation machinery over several days. Spermiation involves remodeling of the spermatid head and cytoplasm, removal of specialized adhesion structures between SC and mature spermatids and the final expulsion of mature spermatid heads from the SC crypts [41]. After spermiation, SC phagocytize degenerated residuals bodies of released spermatids. Moreover, SCs phagocytose the germ cell degenerated during spermatogenesis [42]. Moreover, SCs are capable of pinocytosis in the adluminal compartment and receptor-mediated endocytosis in the basal compartment of ST [43].

*2.7.5 Secretory function*

SC secrete different types of proteases, protease inhibitors, hormones, energy substrates, growth factors, paracrine factors, and extracellular matrix components. Proteases and protease inhibitors from SC participate in ST maintenance, repair, growth, remodeling and restructuring ST. Proteases inhibitors are required for germ cell translocation across the blood-testis barrier and spermiation [44]. Moreover, protease inhibitors are involved in assembly and disassembly of cellular junctions in ST [45]. SCs secrete adluminal fluid and maintain a specific environment for differentiation of germ cells towards the lumen of ST [26]. FSH regulated functions of SC include the transfer of testosterone and glucose, and germ cell nurturing by the provision of lactate and pyruvate. Under the influence of FSH and androgens, SCs secrete proteins necessary for germ cells and interstitial cells of the testes and secrete androgen-binding proteins to ensure bioavailability of androgens. [46,47]. Inhibin and activins from SC act on hypothalamic-pituitary-gonadal axis and also on Leydig cells [48]. The iron carrier protein, transferrin, is produced by SC and is its concentration in seminal plasma are correlated with fertility and spermatogenic capacity in the bull [49]. SCs co-cultured with germ cells stimulate DNA and RNA synthesis in the germ cells [50]. Seminiferous growth factor is involved in spermatogonial proliferation, nerve growth factor is required for DNA synthesis in preleptotene spermatocytes, and insulin-like growth factor-1 helps in germ cell differentiation [51]. The germ cells residing in the adluminal compartment cannot receive nutrients directly from blood due to the blood-testis barrier. The spermatocytes and spermatids in the adluminal compartment are nursed by amino acids, carbohydrates, lipids, vitamins, and metal ions from SCs [26].

**2.8 Sertoli cell junctions**

SCs make and break junctions among themselves and with different types of germ cells. Major junctions formed by SC are tight junctions, anchoring junctions, ectoplasmic specialization, tubulobulbar complexes and communication junctions. Tight junctions are present between adjacent SC at the level of blood-testis barrier and function as a semipermeable barrier- the barrier function. Tight junctions divide SC into basal and adluminal compartment preventing mixing of molecules in the two compartments- the fence function. Anchoring junctions include adherens junctions also known as zonula adherens), focal contacts or adhesions, desmosomes and hemidesmosomes. All these anchoring junctions are biochemically and structurally different from one another and mainly function to connect adjacent cells to one another or extracellular matrix through cytoskeleton thus maintaining tissue integrity. Anchoring junctions also function in signal transduction to regulate cell proliferation, differentiation, and translocation. Ectoplasmic specialization is a modified form of adherent junctions and mainly functions in spermiation and maintenance of other junctions present among adjacent SC and SC-germ cells. Tubulobulbar complexes, also a modified form of zonula adherens, are present between adjacent SC at the level of tight junctions and between SC-mature spermatids ready for spermiation. Focal contacts are the actin-based junction between testicular cells and extracellular matrix and play adhesive function at ectoplasmic specialization. Desmosomes are intermediate filament based junctions found between SC-SC and SC-germ cell. Hemidesmosomes are intermediate filaments based junctions found only between SC and basal lamina; these have an adhesive function. Communication junctions are either chemical synapses or gap junctions. Gap junctions are intercellular channels formed by connexons and transduce signals between SC and germ cells and thus participate in germ cell translocation. Gap junctions are present between SC-SC, SC-germ cell and between two Leydig cells [26].

### 2.9 Sertoli cell and spermatogenic capacity

In the bull, daily sperm production is correlated with total Sertoli cell number (R = +0.83) and Sertoli cells per gram (R = +0.47) but is not correlated with the number of germ cells supported per Sertoli cell. Testicular parenchyma weight is also correlated with the total Sertoli cell number (R = +0.61). Total Sertoli cells number is the foremost determinant of the variation in DSP among bulls ($R^2$ = 68.2%) [52,53]. However, total SC numbers and the numbers of germ cells per SC are not correlated with the quality of either fresh or frozen bull semen [54]. Moreover, SC number in the horse ($R^2$ = 68) and human ($R^2$ = 39) is also correlated with daily sperm production. The association between SC and DSP may be based upon the association between SC and the number of $A_1$ spermatogonia ($R^2$ = 55 for horse) [25].

In the bull majority of SC proliferation happens during fetal life in utero. SC number increases by five times from birth until puberty, and no further increase in SC number occurs after puberty. Once the mitosis in SC stops, the cells differentiate to mature SC cell and continue their adult function for the reproductive life of the bull [55]. However, stallion being a seasonal breeder has no permanent SC numbers after puberty and SC number and volume of SC nuclei per testes increases during breeding season for a 4-20 years old stallion. The size of the horse SC does not differ concerning the breeding season [25]. A concurrent increase in the number of A spermatogonia accompanies increased SC number during the breeding season [56].

### 2.10 Sertoli cells, age and spermatogenesis

When a bull can produce an ejaculate with 50 million spermatozoa with at least 10% motility, he is pubertal [57]. Age at puberty varies from 38 to 48 weeks of age with an average age of 42 weeks [58]. After maturity, a bull can give two useable ejaculates daily; however commercially,

bulls are collected thrice a week for a balance between management and number of artificial insemination doses. The aging-related decrease in sperm production has not been studied in the bull as the breeding bulls are not maintained for old age. One of the reasons for the age-related decline in spermatogenesis is decreased Sertoli cell number in old age [1].

### 2.11 Thyroid hormone

Thyroid hormones (TH) 3,5,3′-triiodothyronine ($T_3$) and thyroxine ($T_4$) are iodinated hormones synthesized by the thyroid gland in response to thyroid stimulating hormone. The mammalian hypothalamic-pituitary-thyroid axis is controlled centrally by neurons located within the parvocellular region of the paraventricular nucleus of the hypothalamus. These neurons synthesize and release thyrotropin releasing hormone into the median eminence. Thyrotropin releasing hormone stimulates the secretion of thyroid stimulating hormone from adenohypophysis. Thyroid stimulating hormone travels through the blood to thyroid gland where it stimulates the synthesis and release of TH. Thyroid stimulating hormone acts on thyroid follicle through its receptors on the basolateral membrane of the follicles leading to sodium-iodide symporter facilitated uptakes of iodide by the thyroid follicles [59,60].

TH regulates its own secretion through negative feedback on hypothalamic-pituitary-thyroid axis. TH inhibits transcription of the genes transcribing both thyrotropin releasing hormone in hypothalamic neuron and thyroid stimulating hormone in pituitary thyrotrophs. It also inhibits posttranslational modification and release of thyroid stimulating hormone from pituitary thyrotrophs. To govern negative feedback T3 and TH receptor complex bind to the response element in the promoter of genes of thyrotropin releasing hormone and thyroid stimulating hormone leading to repression of the genes while in the absence of T3 the transcription is activated. Recent research demonstrates that thyrotropin releasing hormone has a major role in

regulating thyroid stimulating hormone from adenohypophysis. The stimuli for thyrotropin releasing hormone to regulate thyroid stimulating hormone from adenohypophysis is circulating levels of TH and the cellular environment around thyrotropin releasing hormone neurons [59].

### *2.11.1 Thyroid hormone deiodinases*

About 85% of TH secreted by thyroid gland is prohormone T4 which serves as a reservoir for TH in the blood [60]. Less bioactive T4 is converted to more bioactive T3 by 5-monodeiodination in the liver, kidney, and skeletal muscle. The cells of body lacking 5-monodeiodination, for example cardiomyocytes, need uptake of T3 and not of T4 for normal functioning [61]. Different body tissues regulate the circulatory levels of TH through three iodothyronine deiodinases on the tissue's need basis: type I, type II, and type III [62]. The major conversion of T4 to T3 is through outer-ring deiodination by type I deiodinase. Type II deiodinase is involved in local T4 to T3 conversion with body tissues through outer ring deiodination. Deiodinase type II is needed for adaptive thermogenesis as it involved in TH regulation of metabolism by local T4 to T3 conversion. It is expressed in the hypothalamus, white fat, brown adipose tissue, and skeletal muscle. These tissues along, liver and pancreas are the sites where TH acts to regulate metabolism [63]. Hyperthyroidism stimulated increased metabolism is characterized by increased resting energy expenditure, weight loss, reduced cholesterol levels, increased lipolysis, and gluconeogenesis. Though euthyroidism stimulates both lipogenesis and lipolysis, however, the net effect of hyperthyroidism is fat loss. Whereas, hypothyroidism induced decreased metabolism is associated with reduced resting energy expenditure, weight gain, increased cholesterol levels, reduced lipolysis, and reduced gluconeogenesis (Gregory and Brent, 2008; Mullur et al., 2014). Type III deiodinase is present at

blood-brain and feto-maternal interface and inactivates TH to inactive iodothyronines by inner ring deiodination. D1 recycles iodine from inactive iodothyronines like reverse T3 [60].

### 2.12 Thyroid binding proteins

TH are transferred from thyroid gland to target tissues, by blood, bound to transfer proteins. The major binding protein is thyroid binding globulin while transthyretin and albumin are minor transporters in human beings. Only less than 1% of TH is present in unbound or free form in the blood. Transthyretin is minor transporter in human beings but major transporter in rodents. Moreover, transthyretin transfers TH from blood to brain across the blood-brain barrier and also across the blood-placental barrier [64].

### 2.13 Thyroid hormone receptors and functions

Functions of TH are highly conserved across evolution [60]. THs and their metabolites are found in both animal and plant kingdom. If TH is involved in cellular maturation and development in mammals, it also regulates metamorphosis and lung maturation in amphibians. Even in some non-vertebrates lacking TH, exogenous TH is involved in starting metamorphosis [60]. TH can affect gene expression in virtually every vertebrate tissue [62].

The actions of TH on cells are through its receptors within the nucleus, on the plasma membrane, inside the cytoplasm, and at the mitochondrion. Single polypeptide chains intranuclear thyroid hormone receptor (TNRs) belong to nuclear steroid receptor superfamily [65]. Unlike other steroid receptors, TNRs regulate transcription both in the absence and in the presence of ligand. In the absence of TH, TNRs are bound to corepressor which inhibits transcription; while in the presence of TH the corepressor is released and coactivator is recruited resulting in the initiation of transcription [60]. Biologically TNRs are T3 inducible transcription factors [66]; TNRs are

the products of two different genes, alpha and beta, locates on two different chromosomes. The alpha gene encodes for alpha 1 and the beta gene encodes for beta 1, beta 2, and beta three isoforms of TNRs [67]. Once TH is within nucleus bound to its TNRs, the complex occupies TH response element in the promoter regions of positively regulated genes leading to modulation of cellular transcription [68]. This regulation is exerted by any of: direct action of T3, type of thyroid hormone response elements located on the promoters of T-3 target genes, different TNRs isoforms in different tissues, and different nuclear corepressors and coactivators proteins in different cells. If no T3 is available, corepressor suppresses the transcription while in the presence of T3 coactivator activates T3 regulated transcriptional activity. Mutation in TNRs genes leads to the insensitivity of cells to T3, attention deficit, dyslexia, goiter, dwarfism, reduced weight, tachycardia, hearing loss, hyperactivity disorder, decreased IQ, thyroid cancer, pituitary tumors, and metabolic abnormalities [69]. Physiological actions of TH mediated through TNRs are tissue development and differentiation, maintenance of mental and psychological health status, bone remodeling, regulation of cellular metabolism, regulation of cell structure and modulation of membrane transport [70].

Cellular effects of T3 not mediated by TNRs but mediated through the receptors, not always homologous to TNRs, at the plasma membrane in cytoplasm or mitochondrion are called non-genomic actions of TH [70]. Although, T4 is prohormone to T3 however for its receptors at plasma membrane T4 acts directly as an agonist without any conversion to T3. Though the presence of TH in the cytoplasm may be attributed to transient presence during transport from the plasma membrane to the cell nucleus, intracytoplasmic proteins functioning as receptors for TH are known establishing some actions of TH through cytoplasmic receptors [71]. Examples of such nongenomic actions are: prolonged half-life of mRNA [72], increased Ca-ATPase activity

in presence of high fat diet [73], increased 2-deoxyglucose uptake by thymocytes [74], slowing of the inactivation of Na current in cardiac muscles [75], modulation of epidermal growth factor receptor activity in tumor cells [76], increased activity of the plasma membrane sodium potassium ATPase in alveolar cells, regulation of endocytosis by plasma membrane, regulation of sodium-proton exchange antiporter, proangiogenic effects and induction of cellular proliferation of certain neoplasia like gliomas [68].

Mitochondrial actions of TH account for heat production, ATP generation, mitochondriogenesis and oxygen consumption. Actions of TH on mitochondrion can be through non-genomic TRs on mitochondria or through TNRs to act on mitochondrial DNA resulting only in the modulation of mitochondrial transcription factors [77]. T4 and reverse T3 are important for nervous system development as they affect cellular migration in neurons and glial cells to nurture developing neuronal projections, for example Purkinje cell dendrites, helping in normal synaptogenesis. Such actions of TH on cytoskeleton are mediated by alpha 1 TNRs isoform [78]. Hypothyroidism reduces neuronal growth and differentiation in the cerebral cortex, hippocampus, and cerebellum [64].

### 2.14 Thyroid hormone in reproductive physiology

The first report describing the involvement of TH in reproduction dates to 1905. It was reported that a classic case of 9 years old female was accompanied by precocious puberty characterized by the start of menstruation at five years of age, coarse hairs in axilla and pubis, and breast development. Treatment with TH led to the cessation of menstruation, the absence of hairs from axilla and pubis, smaller breast and smaller breast nipples [79]. The role of TH in female reproductive function has been established since then. Conversely, before the early 1990s, the

male reproductive function was considered non-responsive to any TH action until the research in 1990s showed Sertoli cell as novel TH responsive cells [80]. TH is involved in maturation of Sertoli cells, Leydig cells, and of some stages of germ cells. Leydig cells are formed from mesenchymal cells through mesenchymal precursor cells, progenitor cells, newly formed adult Leydig cells, immature Leydig cells, and mature Leydig cells. TH hormone plays a role in the multiplication of mesenchymal precursor cells and newly formed Leydig cells, initiates differentiation of mesenchymal precursor cells to progenitor cells and enhances this differentiation both in neonatal and ethane dimethane sulfonatetreated adult rats [81]. TH regulates the duration of Sertoli cell proliferation hence establishing their adult number in testes which ultimately affects the spermatogenic capacity of testes. TNRα1 is highly expressed in testes within the nuclei of proliferating Sertoli cells and is responsible for actions of TH on testes. Its expression peaks during multiplication of Sertoli cells and decreases gradually as the Sertoli cell division stops between days 15–20 postnatally. TNRα1 is also expressed in germ cells from intermediate spermatogonia to mid-cycle pachytene spermatocytes and in Leydig cells. mRNA for TNRα2, TNRα3, and TNRβ1 were found in testes however the corresponding protein could not be detected [82]. The effects of hyperthyroidism and hypothyroidism both in female and male gonadal functioning are well established [83].

### *2.14.1 The effect of thyrotoxicosis and hypothyroidism on the female reproduction*

Both in vivo and in vitro studies have shown the influence of TH on estrogenic effects in female reproductive physiology [84]. In rodents, $T_3$ affects estrous cycle regulation, behavior, pregnancy maintenance, fetal growth, lactation and estrogen-mediated mating behavior in ovariectomized mice [84].

Pregnancy alters levels of TH in rodents. In pregnant rats, $T_4$ and $T_3$ decrease in all plasma, liver, kidney, lung, heart, and skeletal muscle as normal physiological phenomenon helping in energy conservation. This strategy is an adaptive advantage for the dam and the conceptus [88]. Puberty, characterized by early vaginal opening and onset of estrous cycles, is enhanced in female mice receiving lower dose TH. The ovaries exhibit multiple follicles and corpora lutea. [89]. However, puberty, first estrus and the vaginal opening are delayed in female mice neonatally with higher doses of TH. The changes were thought to result from excessive exogenous TH during a "critical" neonatal period of neural development controlling pituitary-gonadal function [90]. The treatment, for the first five postnatal days, was followed by a period of hypothyroidism. Hence, it is controversial whether this delayed puberty resulted from TH administration or from resulting hypothyroidism [83]. Administration of higher $T_4$ doses to adult female rats results in a delayed return to estrus, prolonged diestrus and fewer follicles on ovaries [91]. The treatment results in reduced serum LH level, normal serum FSH levels and normal pituitary levels of gonadotropins. The treatment did not affect serum LH level in male rats [92].

FSH synergizes with TH in the process of porcine granulosa cell maturation. Granulosa cells from smaller follicles have higher numbers of TNRs than the larger follicles. The authors concluded that TH through its TNRs in immature granulosa synergizes with FSH for granulosa cell differentiation, during early follicular maturation [93]. TNRs are present in rat uterus [94] and administration of TH to female rats increased uterine metabolism at the proestrus stage of the estrus cycle. However, estradiol administration induced uterine metabolism in spayed females was reduced. Ovaries were not affected by the treatment. The administration for six days before breeding increased ovulation rate, implantation rate and litter size [95]. Moreover, TH administration to rats results in decreased in vivo retention and in vitro uptake of estradiol-

17β by the uterus. Thyroidectomy results in increases uterine in vivo retention and in vitro uptake of estradiol-17β [96]. Hypothyroidism inhibits and delays exogenous estradiol stimulated uterine cell division in endometrium and myometrium in female rats [97].

On the other hand, hypothyroidism in fetal life results in smaller ovaries in female rats [98]. de Rooij's group [99] studied the effect of prepubertally induced hypothyroidism with 6-propyl-2-thiouracil (PTU) from birth up to day 40 postpartum in drinking water of pups and dam. The data were collected from day 12-40 postnatal. The PTU induced hypothyroidism resulted in increased serum TSH levels indicative of hypothyroidism, lower body weight, lower ovarian weights and normal serum FSH and inhibin levels. At day 40 postnatal, the treated rat had delayed vaginal opening and sexual maturation, inadequately developed vagina and uterus and smaller ovaries. The ovaries had more secondary follicles, lesser antral follicles, smaller non-atretic antral follicles, more atretic follicles and no corpora lutea. BrdU-labelling index of the granulosa cells did not differ from the control however, the diameters of antral follicles were smaller than the control. The authors concluded that abnormal folliculogenesis was due to hypothyroidism which hampers the differentiation and not the proliferation of granulosa. Involvement of TH in the differentiation of porcine granulosa cells is already known [93].

The induced hypothyroidism tempts significant changes in the steroid synthesis by corpora lutea and growing follicles from ovaries [100]. In this study, the effect of hypothyroidism on pituitary-ovarian axis was examined using $^{131}$I-radiothyroidectomized adult female rats. Within two weeks of induction of hypothyroidism, the female had prolonged and irregular estrus cycles. Prolongation of diestrus in hyperthyroidic adult rat had been reported earlier [91]. From day 7-18 after the induction of hypothyroidism, about half of the treated female showed no vaginal

proestrus, no preovulatory LH surge and had higher plasma progesterone level. The other half of the treated female rats, showed vaginal estrus, had higher plasma progesterone level except two days before and after vaginal proestrus. During 4-5 weeks after induction of hypothyroidism, preovulatory LH surge was significantly greater, peri-estrus progesterone dynamics were not affected and ovulation rate was lesser in the treated females. The majority of hypothyroidic rats had lower plasma estradiol level. Injection of GnRH to this group resulted in a relatively smaller increase in LH; no increase in estradiol and progesterone and no ovulation induction. In summary, induction of hypothyroidism resulted in prolonged estrus cycle, reduced ovulation rate, greater preovulatory LH surge, lower estradiol, higher progesterone, poor response to exogenous GnRH in terms of LH surge, induction of ovulation and plasma levels of progesterone and estradiol. The authors speculated that the altered steroid metabolism by hypothyroidism could be the cause of these changes. Besides hypothalamic-pituitary-ovarian axis, hypothyroidism also affects hypothalamic-pituitary-adrenal axis. Hypothyroidism in adult female rats, induced by 4-Methyl-2-Thiouracil in drinking water, resulted in lower adrenal weight, smaller ovaries and decreased plasma corticosterone throughout the estrous cycle. During diestrus and proestrus, uterine weight, plasma levels of estradiol and LH were reduced while of prolactin and progesterone increased in the treated female rats. The treated rats had a larger increase in plasma level of ACTH and a smaller increase in the level of corticosterone than the control rats [101].

PTU induced prepubertal hypothyroidism resulted in the transient ovarian follicular cyst, which stayed on ovaries 2-6 post hCG treatment. Plasma levels of progesterone and testosterone were also increased [102]. Thyroidectomy during gestation led to lower uterine acid phosphatase, lower alkaline phosphatase and increased glutamic oxaloacetic acid and glutamic pyruvic acid

transaminases. Concentrations of these enzymes did not differ significantly from the control rats. Uterine and cervical levels of glycogen, ascorbic acid and cholesterol were not affected by thyroidectomy. [103]. Uterine acid phosphatase and alkaline phosphatase are important for implantation, embryo growth, uterine carbohydrate metabolism and glycogen deposition [104].

Mature but not immature hypothyroidic female mice are infertile due to the failure of follicular development and conception. Exogenous thyroxine administration can cure such infertility due to hypothyroidism. Hypothyroidism in immature female mice does not affect gonadotrophins induced ovulation rate or in vitro oocyte maturation. However, mature hypothyroidic female mice had fewer ovulations and corpora lutea after gonadotrophins treatment, lower progesterone, prolonged diestrus and did not get pregnant. Thyroxine treatment of hypothyroidic females before mating resulted in ovulation, well-developed corpora lutea, the establishment of pregnancy and improved progesterone level. Subsequent thyroxine treatment after mating was not necessary to obtain these improvements [105]. Propylthiouracil induced hypothyroidism in sheep, leads to enlarged polycystic ovaries, ovarian stromal hyperplasia and thickened ovarian tunica albuginea. Endometrial hyperplasia, mammary alveolar duct proliferation, and myometrial hypertrophy in hypothyroidic sheep may be due to observed prolonged estrus [106].

*2.14.1.1 Role in the breeding season*

In ewes, LH levels in thyroidectomized and control ewes have been throughout the breeding season. No difference in LH was observed between the groups until the end of breeding season. At the end of breeding season, thyroidectomized ewes had more frequent LH pulses than thyroid-intact ewes both in the presence and absence of estradiol. It shows that TH is required for the transition to anestrus possibly by suppressing neuroendocrine mechanisms of LH production.

However, melatonin and prolactin were not affected by thyroidectomy negating any effect of TH on these hormones [85]. The role of thyroid hormone in the physiological cessation of the breeding season is mediated via the GnRH neurosecretory system [86]. In birds, thyroidectomy is associated with continuation of reproducibility beyond breeding season. Contrarily, administration of TH to thyroidectomized bids results in a normal duration of the breeding season. The need of TH for photorefractoriness was suggested to be permissive but not actively involved [87].

*2.14.2 The effect of thyrotoxicosis and hypothyroidism on the male reproduction*

The widespread animal studies targeting TH manipulation have resulted in decreased sexual activity and fertility. Though the underlying mechanism varies among species and studies contradict finding among them [83].

TH alteration adversely affects dog and cat fertility, pregnancy and neonatal health. Euthyroidism results in optimal reproduction however significant reproductive insult are observed only under specific thyroid dysfunction in dog and cat. In many cases treatment of thyroid dysfunction does not reestablish normal reproductive function; some underlying factor but not direct TH abnormality is the cause of reproductive dysfunction [107]. In prepubertal mice, low dose TH administration leads to earlier puberty. And, high dose TH administration results in a decreased weight of seminal vesicles and testes in mice and rabbit [83]. Hyperthyroidism affects testicular metabolism especially intra-testicular lipid metabolism; thyroxine-treated testes have reduced ability to consume oxygen [108], and contain decreased concentrations of total lipids, total glyceride, glycerols, total cholesterol and total phospholipids. However, after withdrawal of one-month thyroxine treatment all lipids returned to their normal

euthyroid levels [109]. Hyperthyroidism affects hypothalamic-pituitary-testicular axis in male rats either by direct pituitary suppression or through accelerated FSH metabolism. Thyroxine treated rats had lower serum FSH but normal serum testosterone and estradiol. In vitro, testes from thyroxine-treated rats produced more testosterone showing that hyperthyroidism may stimulate intra-testicular 17β-hydroxysteroid dehydrogenase [110]. Eight Weeks of thyroxine treatment to mature Merino rams did not affect spermatogenesis or daily sperm production. Sperm motility was reduced possibly due to the altered androgen-dependent maturation of spermatozoa in the epididymis as plasma testosterone was reduced. Conversely, plasma testosterone concentration from internal spermatic vein was higher than from peripheral blood plasma suggesting possible increased metabolism of testosterone under hyperthyroidism despite normal testosterone production. The treatment resulted in higher serum prolactin level and low basal LH level however basal FSH level was not affected [111]. Hypothyroidism in adult rams affects hormonal synthesis both from pituitary and testes however no direct effect on spermatogenesis was noticed. Methylthiouracil treated hypothyroidic rams had lower sperm motility suggesting altered androgen-dependent maturation of spermatozoa in the epididymis and lower serum testosterone suggesting TH requirement of Leydig cells. Basal plasma testosterone and LH concentrations, and increase in plasma testosterone after hCG and GnRH administration were reduced [111]. PTU induced Hypothyroidism in adult rats led to decreased TH profile, but no significant changes were seen in testes weight, spermatogenesis, serum testosterone level, basal lamina or in the lumen of seminiferous tubules [112]. These two studies suggest no effect of hypothyroidism on Sertoli cell number and, no direct or indirect, thru Sertoli cell, effect of hypothyroidism on spermatogenesis in adult animals. However, thyroxine treatment of infertile rdw mutant hypothyroidic rats resulted in partially improved sexual behavior, increased serum

TH levels, increased weight of testes and epididymides, decreased cytoplasmic droplets in spermatozoa and birth of live offspring from epidydimal spermatozoa [113].

### 2.15 Induced transient hypothyroidism in rodents

Effect of TH on body organs including liver, kidney and skeletal muscles is by indirect on the tissue metabolism [114]. However, TH alterations do not affect testicular metabolism [114] or testicular function [115] in the adult animal. Adult testes do not respond to TH by increasing oxygen consumption and mitochondrial alpha-glycerophosphate dehydrogenase and possess meager TH receptors [116]. Thus, adult testes are not affected by hypothyroidism or hyperthyroidism. Conversely, adverse effect of TH manipulation precisely of hypothyroidism on prepubertal ovarian [79] or testicular [117] function is known since long. Maqsood, (1952) reported that hypothyroidism inhibited testicular development and delayed the onset of spermatogenesis. Though these effects of hypothyroidism on testicular function could be attributed to effects of the deficiency on testicular metabolism or gonadotropins; however, effects of TH on the testicular function as direct actions of TH on testicular parenchyma were verified during the late 1980s [118]. Though, adult testes lack significant TH receptors demonstration of TH receptors in neonatal testes suggested a possible role of TH in testicular function during prepubertal development [118]. TNRs [119] and associated mRNA [120] is highly expressed in neonatal rat testes predominantly in Sertoli cells and the expression is highest until few days after birth. The levels of both TNRs and associated mRNA decreases significantly by day 15-20 postnatally with the meager level in adult testes [116,119–121]. The expression of TNRs is strongly correlated to the proliferation dynamics of Sertoli cells. Though the rat Sertoli cells proliferate maximum during late fetal life from day $16^{th}$ of gestation to

onwards and the proliferation is the highest two days before birth [122]. However, the Sertoli cells continue to divide postnatally until day 16 thus coinciding with the time window when TNRs expression is also maximum for postnatal life. TNRs are mainly expressed in Sertoli cells and reported to be absent in Leydig cells and other interstitial cells by [121]. This correlation between Sertoli cell proliferation and TNRs expression along with the absence of TNRs from other Testicular somatic cells suggests a role of TH in testicular proliferation. TH regulates TNRs and the associated mRNA in the rat testes and is responsible for the postnatal decline in TNRs expression [123]. Postnatal hypothyroidism inhibits Sertoli cell maturation [124], delays seminiferous tubular lumen formation by inhibiting pro-luminal fluid secretion from Sertoli cells and increases germ cell degeneration [125]. TH also affects secretion of androgen binding proteins [126] and IGF-1 [127], glucose transport [128], protein synthesis and other metabolic processes of developing Sertoli cell [124]. Thus, TH is required for proliferation, capacity building to support complete spermatogenesis, development of secretory function, differentiation and maturation of Sertoli cells [118].

Hypothyroidism adversely affects Sertoli cell development and hence testicular function, and thyroxine treatment reverses these adverse effects. However, if hypothyroidism is induced transiently, the animal reverts to euthyroidism and the induction helps to boost testicular function. When hypothyroidism was induced transiently from birth to day 25 postnatally in the rat by feeding 0.1% propylthiouracil (PTU) through dam's drinking water, the cessation of the treatment resulted in euthyroidism. This PTU inducted transient hypothyroidism (PTU-ITH) resulted in decreased body weight, which rapidly increased after cessation of PTU but always remained 15-25% lower than the control. PTU-ITH led to 40% increase in testes weight at 90 days of age and 80% increase at 160 days of age and almost double DNA content indicating

cellular hyperplasia in the treated testes. Moreover, 83% increases in daily sperm production at 90 days of age, 140% at 160 days of age, 25% increases in the efficiency of spermatogenesis regarding daily sperm production per gram of testes were observed. The treated rat had no sperm motility problem and sired normal litter [115,129].

Plasma $T_4$ concentration in PTU treated rats decreases by days five after treatment and remains low throughout the treatment. The decrease in plasma $T_3$ is lesser than the decrease in plasma $T_4$. Contrarily plasma level of thyroid stimulating hormone starts increasing by days five of the treatment, increase by 10-14-fold at days 25 of the treatment, and returns to normal by days 45-50 of the age of rats. After cessation of the hypothyroidic treatment, plasma TH levels return to normal by days 45-50 of the age of rats [130].

The induction of the hypothyroidism with 0.1% PTU in dam's drinking water suppressed weight gain. The body weight of the treated rat was 15-15-25% lower than the control rats at 160 days of age. PTU-ITH using 0.006% PTU had similar effects in terms of hypothyroidism, testicular weight and sperm production, however, the body weight was reduced by 16%, maternal water consumption was improved and the rats returned to euthyroidism faster than with 0.1% PTU. Hence, 0.006% PTU is optimum dose for the rats to get desirable side effects and decrease side effects. ITH using 0.025% methimazole results in a lesser severe decrease in TH than by 0.1 or 0.006% PTU. And about 100% increase in daily sperm production, 18% increase in testes weight and 11% decrease in body weight by days 18. 0.025% methimazole is lesser potent than 0.1 or 0.006% PTU for the desired effects of ITH [131]. If PTU-ITH is accompanied by TH supplementation [129], the increase in testes weight or daily sperm production is not observed. Moreover, the observation that methimazole also increases testes weight and sperm production

[131] confirms that these effects of ITH are due to hypothyroidism and not a direct pharmacological effect of the hpothyroidic agent [118]. In PTU-ITH rats, plasma levels of FSH and LH were decreased during and after the treatment and remained low throughout the life of the rats. While pubertal testosterone peak is delayed by 15 days, testosterone then increases rapidly from days 50 of the age and reaches the normal adult level for all adulthood [130]. Decreased FSH levels with paradoxical rise in testosterone; and first decreased then normal testosterone, together with paradoxical increase in testes size and daily sperm production; suggest that the observed effects of PTU-ITH are direct effect of hypothyroidism independent of pituitary gonadotropins or testosterone [118,129,130].

PTU treatment from birth to days 9, 17 or 25 increases testicular weight and daily sperm production proportional to the treatment. Starting the treatment at day 4 of age also produced the desired effects. However, if PTU treatment is started at days 16 or 24 of life, increase in testes weight or daily sperm production is not observed [132]. The first week of neonatal is a critical period for the desired effects of PTU-ITH and PTU-ITH induced later does not increase testes weight or daily sperm production [132,133]. PTU-ITH extends the period of Sertoli cell proliferation by slowing down the inhibitory process of their proliferation. In the rat, this window of Sertoli cell proliferation is extended to day 35 of life as compared to day 16 of life in untreated control rats [134,135]. Sertoli cell number in adult rats increases by 84% on $36^{th}$ day of life [135] and by 157% on $90^{th}$ day of life [136]. During the treatment phase, ITH results in germ cell degeneration, delay in seminiferous tubular lumen formation and smaller diameter of the seminiferous tubules. However, once the treatment is stopped these maturational defects are quickly reversed, and normal development and maturation starts [137]. At $90^{th}$ day of the life of PTU treated rats, spermatogenesis and testicular architecture are histologically normal. The

diameter, length, and volume of seminiferous tubules increased by 11, 44, and 60%, respectively in response to the treatment. Moreover, the number of preleptotene spermatocytes and round spermatids was doubled at 90 days of life. The seminiferous epithelium was higher and denser, the lumen was smaller due to larger epithelium as germ cell occupied further towards lumen, Sertoli cells were located closer to each other and their number per unit area of the tubules was increased by 70% as compared to the control rats [136].

The number of Leydig cells in PTU treated rats increases by 69% at 180 days of age. However, their average volume and LH receptors decrease by 20% and 50%, respectively. Leydig cells have decreased steroidogenic capacity however their increased number compensates for this reduced capacity to maintain normal blood testosterone levels [138]. The absence of TNRs from Leydig cells suggests that their number increases in response to paracrine factors from an increased number of germ cells, peritubular myoid cells and particularly Sertoli cells [118]. ITH in mice by feeding 0.1% PTU from birth to day 25 led to 50% decrease in body weight during treatment though after cessation of the treatment the mice grew rapidly, however, adult body weight remained 15-20% lower than of the control. Serum testosterone was not affected, testes size increased by 30% and daily sperm production increased by 50% at 90 days of age. Sertoli cells proliferated for day 35 as compared to day 25 postnatal for control [139]. Moreover, PTU-ITH increased testes weight and sperm production in the hamster. Though the higher than rat PTU dose was required, testes weight increased by 30% and daily sperm production increased by 73% despite 40% decreases in adult blood levels of pituitary gonadotropins [140].

### 2.16 Possible mechanism of induced transient hypothyroidism

Two hypotheses have been proposed regarding the mechanism of action of TH on Sertoli cell. TH acts on Sertoli cells through cell cycle proteins cyclin-dependent kinase inhibitors (CDKIs) $p27^{Kip1}$ and $p21^{Cip1}$. TH upregulates $p27^{Kip1}$ (CDKN1B) and $p21^{Cip1}$ (CDKN1A) which then inhibit subsequent phosphorylation of activity of cyclins D and E. In proliferating Sertoli cells, cyclins D and E bind to cyclin-dependent kinases (Cdk) 4 or 6 and Cdk 2. Once the cyclin–Cdk complex is activated, it phosphorylates the pocket proteins, $p^{Rb}$, $p^{130}$, and $p^{107}$. This phosphorylation releases transcription factors of E2F family which activate downstream genes for progression of the cell through G1 restriction point [141]. This mechanism has been suggested by the findings that TH induces the expression of $p27^{Kip1}$ and $p21^{Cip1}$ in neonatal murine Sertoli cells, whereas ITH decreases $p27^{Kip1}$ [142,143]. Moreover, $p27^{Kip1}$ knockout (p27KO), $p21^{Cip1}$ KO (p21KO), and p27/p21 double-KO (DBKO) mice showed 42%, 27% and 86%, increase in testes weight. The Sertoli cell numbers were increased by 126%, 48% and 126%, respectively. Daily sperm production was also increased in all the knockouts. However, p27KO had 5% and DBKO had 1.5% seminiferous tubules with abnormal spermatogenesis as compared to only 0.5% for the wild-type control. Furthermore, in KO mice, developing germ cells, pyknotic spermatogenic cells and fewer spermatozoa were found within the epididymides [141].

Based upon the finding that PTU treatment of p27KO and DBKO mice results in increased testes weight and daily sperm production, [144] suggested that ITH actions on Sertoli cells involve some other mechanism also. Another possible mechanism involves Connexin43 (Cx43). Just before puberty, Sertoli cells form specific intercellular junctions with neighboring Sertoli cells and with adjacent germ cells [145]. Among these, connexin-based gap junctions form cell membrane channels thus allow intercellular communication are control cell proliferation and

differentiation [146]. Cx43 is the most abundant testicular gap junction protein [147]. Inhibitory effect of TH on Sertoli cell proliferation is associated with a time and dose-dependent increase in Cx43 and thus in increased gap junction communication. Oleamide and glycyrrhetinic acid, Specific blockers of gap junctions coupling, reverse the inhibitory effect of TH on Sertoli cell proliferation [148]. Sertoli cell-specific Cx43 knockout (SC-Cx43 KO) mice have continued Sertoli cell proliferation and delayed maturation in adulthood [149,150]. Moreover, SC-Cx43 KO mice are infertile and possess Sertoli cells only seminiferous tubules with actively proliferating early spermatogonia without initiation of spermatogenesis [151].

### 2.17 Application of induced transient hypothyroidism in livestock

The application of PTU-ITH in other species has potential difficulties and requires dose adjustment at a species-specific point in testicular development when Sertoli cells proliferate and express TNRs, to cope with innate species differences in developmental biology and pharmacological response to PTU. Application of this strategy to the bulls required consideration of differences between the bull and the rodents in Sertoli cell proliferation and maturation, and in overall testicular development [118].

## 2.18 References


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# CHAPTER THREE: ENHANCED SPERM PRODUCTION IN BULLS FOLLOWING TRANSIENT INDUCTION OF HYPOTHYROIDISM DURING PRE-PUBERTAL DEVELOPMENT

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## 3.1 Abstract

Male reproductive capacity is a critical component of cattle production and the majority of genetic gain is made via selective use of gametes from desirable sires. Thus, strategies that enhance sperm production increase the availability of elite genetics for use in improving production characteristics of populations on a worldwide scale. In all mammals, the amount of sperm produced is strongly correlated to the number of Sertoli cells in testes. Studies with rodents showed that the size of the Sertoli cell population is set during pre-pubertal development via signaling from thyroid hormones. Here, we devised a strategy to increase Sertoli cell number in bulls via induction of a transient hypothyroidic state just prior to and extending beyond the period of Sertoli cell proliferation that we found to normally cease between 4.5 and 5 months of age. In adulthood, these bulls produced a significantly greater number of sperm compared to age matched controls and their testes contained nearly two times more Sertoli cells. Importantly, sperm motility, morphology, fertilizing ability, and viability after cryopreservation were found to be no different for treated bulls compared to untreated control bulls. This strategy of transient induction of hypothyroidism during a defined period of pre-pubertal development in bulls could prove to be an efficacious approach for enhancing daily sperm production in genetically desirable sires that will, in turn, provide an avenue for improving the efficiency of commercial cattle production.

## 3.2 Introduction

In commercial cattle production, achieving genetic gain for improvement of production characteristics is made through selective breeding. Because millions of sperm are produced per day, the overriding mode for dissemination of genetics has been selective use of sperm from desirable bulls [1]. In addition, the advent of technologies for freezing and thawing bull sperm

followed by use in artificial insemination has provided a conduit for extended availability of elite genetics on a worldwide scale [2]. Furthermore, the application of semen sexing technologies has provided a key tool for precision breeding in cattle industries where a single gender is more desirable such as dairy cattle production [3]. However, an existing bottleneck has been that the demand for sexed semen from elite sires is greater than the number of sperm than can be collected. Thus, there is need for strategies to enhance the daily sperm production (DSP) level in bulls.

Spermatogenesis is the sum of germ cell maturation steps initiating with the transition of undifferentiated spermatogonia to differentiating spermatogonia [4,5]. A series of mitotic divisions amplifies the differentiating spermatogonial pool before meiotic prophase is initiated and the cells transition to spermatocytes that undergo two meiotic divisions to become haploid round spermatids that undergo spermiogenesis thereby yielding elongated spermatids and eventually spermatozoa [6]. The entire process occurs in seminiferous tubules and the sheer number of sperm produced in each round of spermatogenesis is strongly correlated to the size of a somatic support cell population termed Sertoli cells [7]. These “nurse” cells are the only somatic population that is in direct contact with germ cells and their density in adulthood is set by a defined period of proliferation in pre-pubertal development [8]. Therefore, strategies to increase Sertoli cell number can lead to enhanced DSP in males and would impact genetic gain in cattle populations via increasing the availability of sperm from desirable bulls for use in selective breeding.

In mice and rats, the length of the period of Sertoli cell proliferation during pre-pubertal development is set by the emergence of thyroid hormone signaling [9,10]. Transient induction of hypothyroidism from neonatal development through puberty leads to an extended period of

Sertoli cell proliferation resulting in increased population size and DSP compared to the normal physiological state [11–16]. In male rats, transient induction of hypothyroidism in the prepubertal development leads to testes with ~150% in Sertoli cell number and ~140% in DSP per gram of testis [12,16]. The number of Sertoli cells is correlated with the number of spermatogonial stem cell niches in mice which ultimately provides a larger foundation for continuity and robustness of spermatogenesis [17]. Intriguingly, a relationship between thyroid hormone levels in the systemic circulation and Sertoli cell proliferation has also been observed in the ram [18] and the boar [19]. Furthermore, in the bull the level of the thyroid hormone thyroxin ($T_4$) during pre-pubertal development is negatively correlated with testicular size at puberty [20]. In this study, we explored whether transient induction of hypothyroidism in pre-pubertal bull calves during the timeframe of when Sertoli cell proliferation normally occurs would impact spermatogenesis in adulthood.

## 3.3 Materials and Methods

### *3.3.1 Animals and treatments*

All animal procedures were approved by the Institutional Animal Care and Use Committee of Washington State University. Hybrid Angus X Wagyu bulls (n=6) from the same herd and of similar age were randomly assigned to a treatment or control group. The treatment group received the antithyroid drug Methimazole (1-methylimidazole-2thiol) in tablet form (2 mg/Kg body weight per os) twice daily from 4-6 mo of age. Control animals were fed the same diet and managed in identical conditions but did not receive Methimazole tablets. Body weight and scrotal circumference were measured every two from 4-28 mo of age.

### *3.3.2 Immunohistochemical staining of bovine testis cross-sections*

Testicular parenchyma (~100 mg in size) from testes of bull calves at 1-12 mo of age was fixed by immersion in Bouin's solution, serially dehydrated in ethanol and paraffin embedded. Cross-sections of ~5µm thickness were adhered to glass slides followed by deparaffinization and incubation in boiling Na citrate buffer (pH 6.0) for antigen retrieval. Nonspecific antibody binding was blocked by incubating the sections in 10% normal serum for 1 hr at room temperature. The sections were then incubated with primary antibody (anti-sox9, AB5535 Millipore, 1:100; or anti-Ki67, 550609 BD Biosciences, 1:500) diluted in PBS containing 0.5% BSA overnight at 4°C. On the next day, sections were washed in PBS and then incubated with either HRP or fluorophore conjugated secondary antibody at room temperature for 1 hr followed by washing in PBS. For the immunofluorescence stained sections, coverslips were mounted using aqueous solution containing DAPI (Life Technologies) for counter staining of DNA. For colorimetric staining, the sections were incubated with DAB solution (SK-4100 Vector Laboratories) followed by washing in distilled water, counter staining with hematoxylin, and then coverslips mounted with aqueous medium (H-5501 Vector Laboratories).

*3.3.3 Serum thyroid hormone analysis*

Blood was collected from all bulls every two weeks from day 0 of treatment until week 16 post treatment. Serum was isolated and stored at -80°C prior to assaying for the levels of Thyroxine ($T_4$) and Triidothyronine ($T_3$) by ELISA following the manufacturer instructions (BT0046 for $T_3$ and BT0047 for $T_4$, NeoBiolab).

*3.3.4 Semen collection and evaluation*

Semen samples were collected every two mo from all bulls by electroejaculation from 18 to 28 mo of age. Samples were collected into pre-warmed tubes and diluted 1:1 with Tris-citrate

based commercial extender, Triladyl® (Minitube). The volume of each semen sample was recorded and slides prepared for assessment of sperm morphology using eosin-nigrosin staining and light microscopy at 1000X magnification. Samples were also analyzed for sperm concentration and motility using a computer assisted semen analysis (CASA) system (Spermvision®, MOFA). In addition, semen was cryopreserved in liquid nitrogen at concentration of 1 X $10^8$ cells per ml in 0.5 ml straws and stored for at least 4 months. Straws were thawed at 37°C for 1 min and sperm viability (PI/SYBR staining, MOFA) and motility were assessed using CASA.

*3.3.5 In vitro fertilization of bovine oocytes*

To assess the fertilizing ability of fresh and cryopreserved sperm from all bulls, IVF was conducted as described previously [21], with minor modifications. Matured *Bos Taurus* oocytes obtained from a commercial service )(Applied Reproductive Technology Inc, address???) were washed in HEPES-TALP three times in an X-plate at 38°C followed by dispersion into IVF-TALP media in a 4-well plate (14444, ThermoFisher Scientific). Freshly diluted or frozen-thawed spermatozoa were prepared using percoll sedimentation (10min, 1000xg), washed in SP-TALP media, centrifuged at 200xg for 5 min and then re-suspended in IVF-TALP media. Spermatozoa and oocytes were then combined in a 4-well plate at a concentration of 1,000 spermatozoa/µl of the media followed by incubation at 38.5°C in an atmosphere of 5% $CO_2$ in air. Approximately, 18-20 hours post IVF, putative zygotes were denuded from cumulus cells using hyaluronidase (10, 000 IU/ml) and incubated in BSA and gentamicin supplemented KSOM+AA (Caisson Labs). Cleavage rate was calculated 24 hr post IFV and blastocyst rate was recorded 8 days post IVF.

*3.3.6 Postmortem analyses of testes*

At 29 mo of age, all bulls were euthanized and testes and epididymides were recovered and weighed. Samples of testicular parenchyma of approximately 100 mg in weight were collected and fixed in 4% PFA. Cross-sections of 5µm thickness were adhered to glass slides and processed by immunostaining for SOX9 expression followed by microscopic assessment. Images of cross-sections were captured and the number of SOX9+ Sertoli cell nuclei per seminiferous tubule cross section was quantified as well as measurement of tubule diameter. In total, 10 different cross-sections were analyzed per bull [22,23].

*3.3.7 Statistical analysis*

Results are presented as the mean±SEM. Differences between treated and control groups were determined statistically using the two-tailed unpaired t-test function of GraphPad Prism software (Graphpad, Inc.). A P-value of $<0.05$ was considered significant.

**3.4 Results**

*3.4.1 The postnatal period of Sertoli cell proliferation in Bos taurus bulls*

First, we aimed to determine the age at which Sertoli cell proliferation ceases in bull calves during pre-pubertal development. Previous studies that examined the testis cell population dynamics in Holstein bulls via cytological approaches suggested that Sertoli cell number plateaus between 6 and 7 mo of age [24]. To explore this in more depth and pinpoint when Sertoli cells exit the cell cycle, cross sections of testes from hybrid Angus bulls at 1 to 10 mo of age were analyzed by co-immunofluorescent staining for expression of the Sertoli cell marker SOX9 and the cell proliferation marker Ki67. Outcomes revealed Ki67 staining in Sertoli cells at 1-4.5 mo

age but absence of staining by 5 mo of age which persisted into adulthood (Fig. 1). These observations suggested that Sertoli cell proliferation ceases between 4.5 and 5 mo of age in *Bos taurus* breeds of cattle.

*3.4.2 Transient induction of hypothyroidism in bull calves during pre-pubertal development*

Having determined when Sertoli cell proliferation normally ceases in bull calves, we aimed to devise a strategy for induction of a transient hypothyroidic state just prior and extended beyond the age range (Figure 2A). To achieve this, we treated hybrid beef bulls (Angus X Wagyu, n=3) with the antithyroid compound Methimazole (2 mg/kg body weight) twice daily from 4 to 6 mo of age. Control bulls (n=3) were age matched from the same herd that did not receive Methimazole treatment but housed and managed in identical conditions. Based on serum levels of triiodothyronine ($T_3$) and thyroxine ($T_4$), induction of a hypothyroidic state occurred by 4 weeks of treatment, when the bulls had reached 5 mo of age (Figures 2B and 2C). Serum levels of $T_3$ and $T_4$ returned to normal by ~9 mo of age (i.e. 3 months after treatment was stopped). Thus, using the treatment strategy, we were able to effectively suppress the production of thyroid hormones just prior to and extending one month beyond the normal age range of when Sertoli cell proliferation ceases.

To assess the impacts of induced transient hypothyroidism on growth and development, we recorded body weight and testis size (assessed by scrotal circumference) every two mo for all bulls prior to the treatment period and up to two years of age. Neither overall weight gain nor testis size was different between the groups prior to or during the treatment period (Figures 2D and 2E). In contrast, during the age period when puberty is normally established (10-16 mo), mean body weight and scrotal circumference was significant ($P<0.05$) reduced for the treated bulls compared to the control group (Figures 2D and 2E). However, by 18 mo of age there was

no difference in either parameter between treated and control bulls. These findings indicate that transient induction of hypothyroidism during the period of Sertoli cell development delays the normal timeline for the onset of puberty in bulls.

### *3.4.3 Spermatogenic output in bulls subjected to transient induction of hypothyroidism during pre-pubertal development*

Next, we aimed to determine whether transient induction of hypothyroidism during and beyond the period of normal Sertoli cell development alters spermatogenic output in bulls in adulthood. To achieve this, we collected semen samples every two mo from 18 mo of age (when body weight was not different between the groups) to 28 mo of age. Overall, the number of total spermatozoa in ejaculates from treated bulls was found to be significantly ($p<0.05$) greater compared to control bulls (Figures 3A and 3B). Importantly, at 24-28 mo of age the samples from treated bulls contained ~30-180% (i.e. 1.3-2.8-fold) more spermatozoa compared to samples from the control bulls. Although the number of spermatozoa was different between the treated and control groups, the sperm motility and morphology of freshly collected samples were similar (Figures 3B and 3C). In addition, we tested the in vitro fertilization capacity of freshly collected sperm from one control and one treated bull at 27 mo of age and determined that there was no difference in cleavage or blastocyst rates (Supplemental Figure 1). Furthermore, the post-thaw quality (viability and motility) of semen collected at 24 mon of age and stored for at least 4 months was not different between control and treated bulls (Supplemental Figure 2). Moreover, the in vitro fertilizing capacity of the frozen-thawed sperm was found to be no different between control and treated bulls (Supplemental Figure 3). Collectively, these data indicate that transient induction of hypothyroidism during pre-pubertal development in bulls leads to enhancement of normal spermatogenesis in adulthood.

*3.3.4 Testicular dynamics of adult bulls subjected to transient induction of hypothyroidism during pre-pubertal development*

Lastly, at 29 mo of age, we euthanized all bulls and collected testes for examination of seminiferous tubule dynamics. We found that the mean paired testis weight was ~22% higher for treated bulls compared to controls but the difference was not statistically significant (Figure 4A). In addition, the paired epididymal weight was significantly ($P<0.05$) greater (~50% heavier) in treated bulls compared to controls (Figure 4B). Morphological assessment of cross-sections did not reveal any obvious differences between treated and control bulls and seminiferous tubule diameter was found to be not different (Figures 4C and 4D). To assess whether Sertoli cell number was altered in treated bulls, we immunostained cross-sections for the marker SOX9 and carried out a quantitative assessment (Figures 5A and 5B). Outcomes revealed that testes of treated bulls contained a significantly ($P<0.05$) greater number of Sertoli cells by ~2.3-fold compared to control bulls. These data demonstrate that similar to the observed effect in rodents, transient induction of a hypothyroidic state that spans and extends beyond the period of normal Sertoli cell proliferation leads to increased size of the population in adulthood.

**3.4 Discussion**

The primary approach for achieving genetic gain in cattle production has been through selective utilization of male gametes paired with artificial (1). In the United States, ~80% of dairy cattle are bred with artificial insemination and widespread adoption this technique is a major contributor to the greater than 4-fold increase in average milk production per animal since the 1940s, when artificial insemination was commercialized (1). Thus, strategies that increase

sperm production in bulls provides a means to enhance the availability of genetics from elite animals that will, in turn, improve the quality and production efficiency from cattle for the benefit of human consumption [25]. The human population is estimated to reach ~10 billion by 2050 and ensuring food security for this projected population growth will require enhanced production from livestock [26]. Improving the reproductive capacity of male livestock is a key avenue for making these advances.

An approach that has high potential for impacting cattle production is selection of X vs. Y bearing sperm (i.e. semen sexing) for use in an artificial insemination setting [27]. Sexed semen can improve the efficiency of herd expansion by shifting the gender ratio of offspring. For dairy cattle production, the efficiency and precision of producing replacement heifers is impacted by artificial insemination with X bearing sperm from desirable sires. In contrast, the generation of male calves at a higher ratio through artificial insemination with Y bearing sperm could impact beef cattle production where higher male ratios are of greater value in feedlot operations [27]. An important criterion for a bull to be selected as a sire for semen sexing is generation of an ejaculate containing six billion spermatozoa [28]. At present, many bulls with desirable genetics do not meet with minimum requirement and are therefore excluded from use as breeding stock in a sexed semen setting. In addition, many bulls do not reach peak sperm production until two years of age but attain puberty much earlier; thus, there is lost opportunity for use as a sexed semen sire. For these reasons, the potential impact of many elite bulls on advancing genetic gain in cattle production is not fully realized. Devising strategies that led to increased sperm production in bulls even at a younger adult age could overcome these bottlenecks.

Several previous attempts have been made to enhance sperm production in male livestock but none have proven to be of practical value [29]. Although unilateral castration leads to

increased size of the remaining contralateral testis, the total amount of sperm production does not exceed the pre-castration levels from both testes. Also, previous studies have suggested that immunization against the hormones inhibin and estradiol can lead to increased testicular size and daily sperm production in the bull; however, these approaches are transient and require multiple vaccinations thereby leading to limited commercial interest. As an alternative, approaches for increasing the number of Sertoli cells in testes during pre-pubertal development could produce a long-lasting impact on sperm production in adulthood [29]. The Sertoli cell population numbers in adult males is fixed, being established during a defined period of proliferation in postnatal development. In mice, Sertoli cells proliferate from birth until 12-14 days of age at which point mitotic arrest initiates in response to elevated thyroid hormone signaling [9,10]. In cattle, the number of Sertoli cell cells is highly correlated to daily sperm production [7] but the timing of population number establishment has been undefined. In the present study, we found that Sertoli cell proliferation ceases between 4.5 and 5 mo of age in *Bos taurus* breeds of cattle.

Due to a potent influence on inducing cell cycle arrest, suppression of thyroid hormone signaling in rats and mice via transient induction of hypothyroidism during the timing and extending beyond the period of Sertoli cell proliferation leads to enhanced population size in adulthood [11–14]. In the bull, higher thyroid hormone levels during neonatal life are negatively correlated with testicular size after puberty [20], but the impact of transiently inducing hypothyroidism during Sertoli cell development on sperm output as an adult has not been explored. Here, having defined when Sertoli cell proliferation ceases in bulls, we devised a strategy to test this idea. Outcomes of our studies demonstrate that the effects of transient induction of hypothyroidism in the bull during pre-pubertal development on spermatogenic output in adulthood phenocopies the effect in the rodents with higher than normal sperm

production and a doubling of the Sertoli cell population. These findings suggest that the mechanisms modulating development of the Sertoli cell population are conserved among mammalian species, at least at the level of hormone signaling.

A hurdle to overcome for effectively applying transient hypothyroidism in pre-pubertal bulls is the mode by which the condition is induced. In the present study, we attempted to feed the drug Methimazole, which is an antithyroid compound, in pill form to bull calves twice per day for two months by mixing in grain feed. Although effective at times, the animals often refused to ingest the pills thus necessitating the use of a balling gun. This approach is logistically challenging and unlikely to be implementable on a large scale. We propose that an alternative approach should be developed using a slow release implant to deliver the drug. Another potential challenge is the off-label use of PTU compounds in food animals. At present, Methimazole administration in livestock is approved in the US by the FDA for use in treating hypothyroidism only. Thus, administration for improving reproductive capacity leads to adulteration of the meat. However, previous studies that fed the drug to feedlot steers demonstrated clearance from tissues within 72 hours [30]. Considering that the withdrawal time between cessation of Methimazole treatment (6 mo of age) and when the breeding bull would enter the food chain is likely years, the off-label use is something that could be overcome. Moreover, the intent of treating bulls with the drug is to enhance sperm output rather than to generate an animal that would end up in the food chain. Thus, adulteration of the meat of a retired breeding sire should not be of concern, even if off-label use is not obtained by food animal oversight authorities.

Collectively, the methodology that we have devised in this study provides a novel and relatively simple avenue for enhancing sperm production in bulls. This approach has several possible applications to improve the reproductive capacity of pre-pubertal bulls that are

candidates for use as elite sires. First, an increased level of sperm production in bulls intended for use as sexed semen sires would correspond to less triage due to not meeting the minimum industry standard. Second, the sheer number of sperm available for bulls that are in high demand as artificial insemination sires would be less of a limiting factor for expanding utilization of desirable genetics. Both of these potential benefits would lead to enhanced genetic gain in commercial cattle populations and the ability to feed an expanding global population over the coming decades.

### 3.5 Acknowledgements

The authors thank members of the Oatley lab for helpful discussions. This research was supported by funds from Genus PLC.

### 3.6 Figure Legends

**Figure 1.** The period of Sertoli cell proliferation in *Bos Taurus* bull calves. Representative images of cross-sections from testicular parenchyma of bull calves at 4-5 mo and 12 mo of age immunostained for the Sertoli cell marker SOX9 (green staining) and proliferation marker Ki67 (red staining). Arrows denote actively dividing Sertoli cells and arrowheads denote actively dividing germ cells that do not express SOX9.

**Figure 2.** Transient induction of hypothyroidism in bull calves during pre-pubertal development. (A) Scheme for transient induction of hypothyroidism just prior to and extending one mo beyond normal cessation of Sertoli cell proliferation in bull calves by treatment with the drug Methimazole. (B and C) Serum levels of $T_3$ (B) and $T_4$ (C) in control and Methimazole treated bull calves during a 5-week treatment period. (D and E) Body weight (D) and scrotal

circumference (E) of control and Methimazole treated bulls from 4 to 28 mo of age. Data in B-E are mean±SEM for n=3 bulls per group and * denotes significantly different at P<0.05.

**Figure 3.** Effect of transiently induced hypothyroidism in bull calves during pre-pubertal development on spermatogenic output in adulthood. (A-C) Total number of sperm (A), percentage of motile sperm (B), and percentage of sperm with normal morphology (C) in ejaculates of control and Methimazole treated bulls at 18 to 28 mo of age. Data are mean±SEM for n=3 bulls per group and * denotes significantly different at P<0.05.

**Figure 4.** Impact of transiently induced hypothyroidism in bull calves on testicular dynamics in adulthood. (A and B) Paired weight of testes (A) and epididymides (B) from control and Methimazole treated bulls at 29 mo of age. Data are mean±SEM for n=3 bulls per group and * denotes significantly different at P<0.05. (C) Representative image of a seminiferous tubule cross-section from testicular parenchyma of a 29 mo old bull that was treated with Methimazole during pre-pubertal development. (D) Diameter of seminiferous tubules in cross-sections of testicular parenchyma from 29 mo bulls treated with Methimazole during pre-pubertal development. Data are mean±SEM for 30 different cross-sections from n=3 bulls per group.

**Figure 5.** Effect of transiently induced hypothyroidism in bull calves during pre-pubertal development on Sertoli cell number in adulthood. (A) Representative images of immunostaining for the Sertoli cell marker SOX9 in cross-sections of seminiferous tubules from testicular parenchyma of control and Methimazole treated bulls at 29 mo of age. (B) Quantification of SOX9+ Sertoli cell number in cross-sections of seminiferous tubules from testicular parenchyma of control and Methimazole treated bulls at 29 mo of age. Data are mean±SEM for 30 different cross-sections from n=3 bulls per group and * denotes significantly different at P<0.05.

**Figure 1**

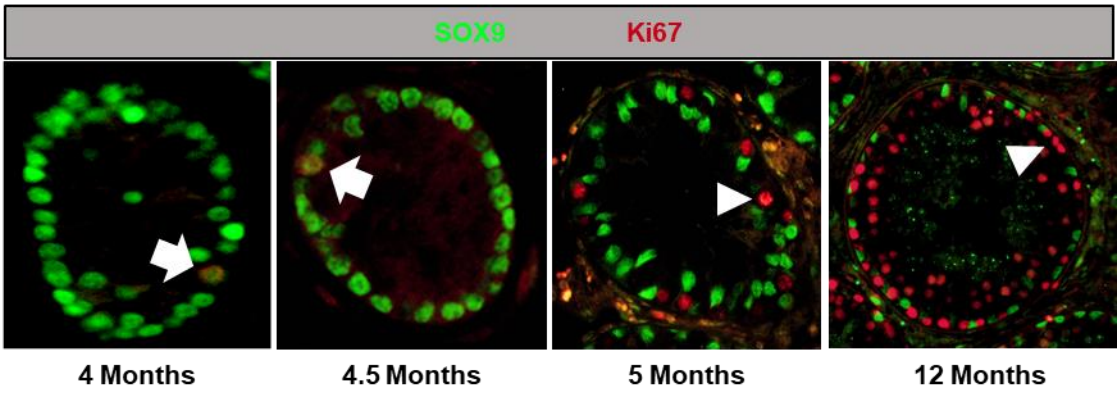


Figure 2

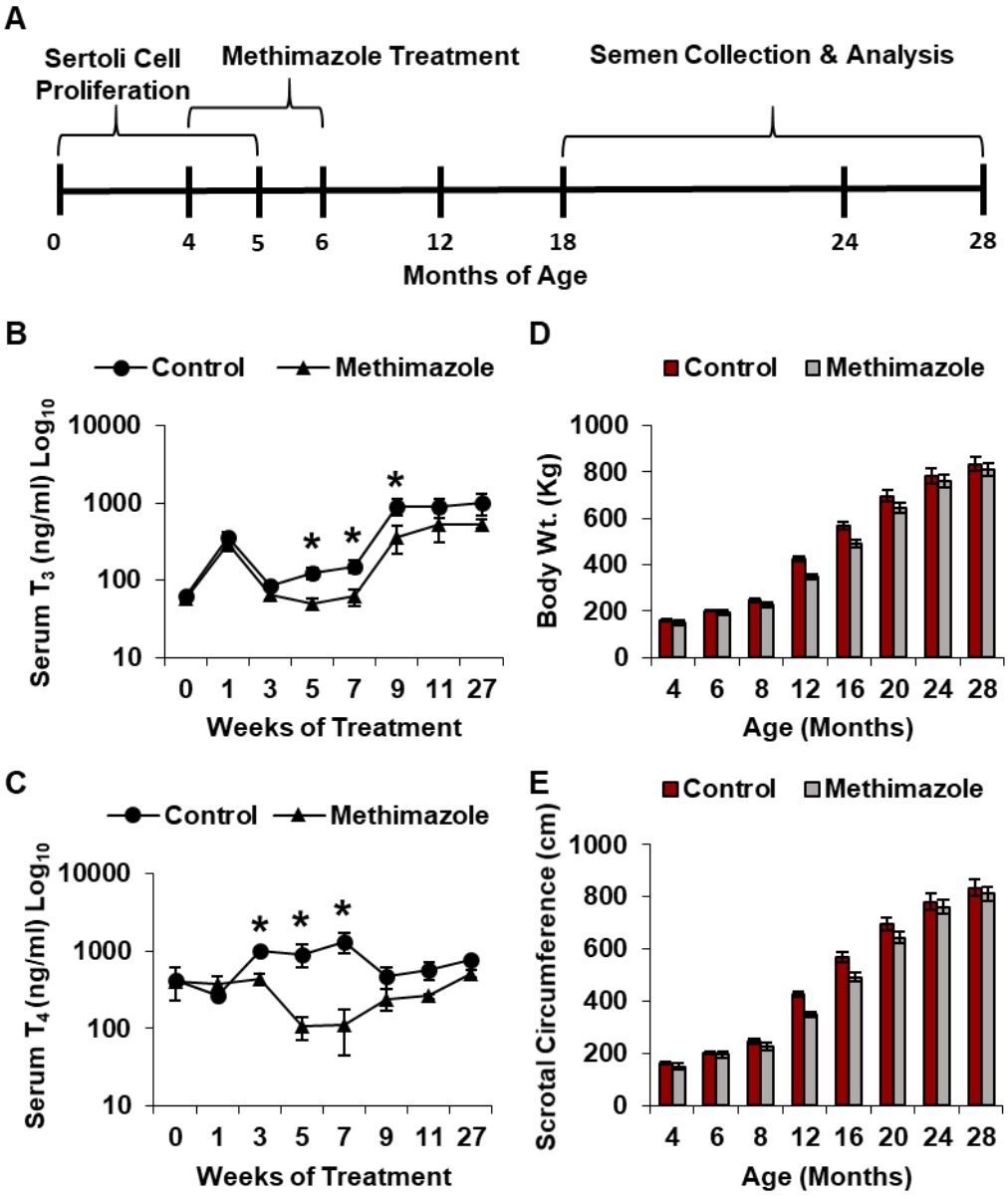

A
Sertoli Cell Proliferation
Methimazole Treatment
Semen Collection & Analysis
0
4
5
6
12
18
24
28
Months of Age
B
Control
Methimazole
Serum $T_3$ (ng/ml) $Log_{10}$
10000
1000
100
10
0 1 3 5 7 9 11 27
Weeks of Treatment
D
Control
Methimazole
Body Wt. (Kg)
1000
800
600
400
200
0
4 6 8 12 16 20 24 28
Age (Months)
C
Control
Methimazole
Serum $T_4$ (ng/ml) $Log_{10}$
10000
1000
100
10
0 1 3 5 7 9 11 27
Weeks of Treatment
E
Control
Methimazole
Scrotal Circumference (cm)
1000
800
600
400
200
0
4 6 8 12 16 20 24 28
Age (Months)

Figure 3

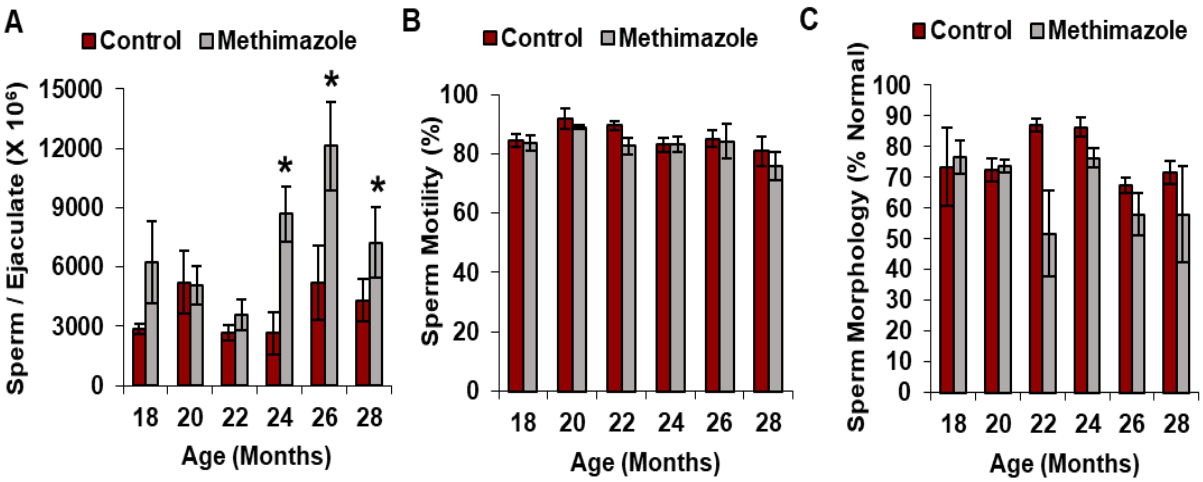

A
Control
Methimazole
Sperm / Ejaculate (X 10⁶)
15000
12000
9000
6000
3000
0
18 20 22 24 26 28
Age (Months)
*
*
*
B
Control
Methimazole
Sperm Motility (%)
100
80
60
40
20
0
18 20 22 24 26 28
Age (Months)
C
Control
Methimazole
Sperm Morphology (% Normal)
100
90
80
70
60
50
40
30
20
10
0
18 20 22 24 26 28
Age (Months)

**Figure 4**

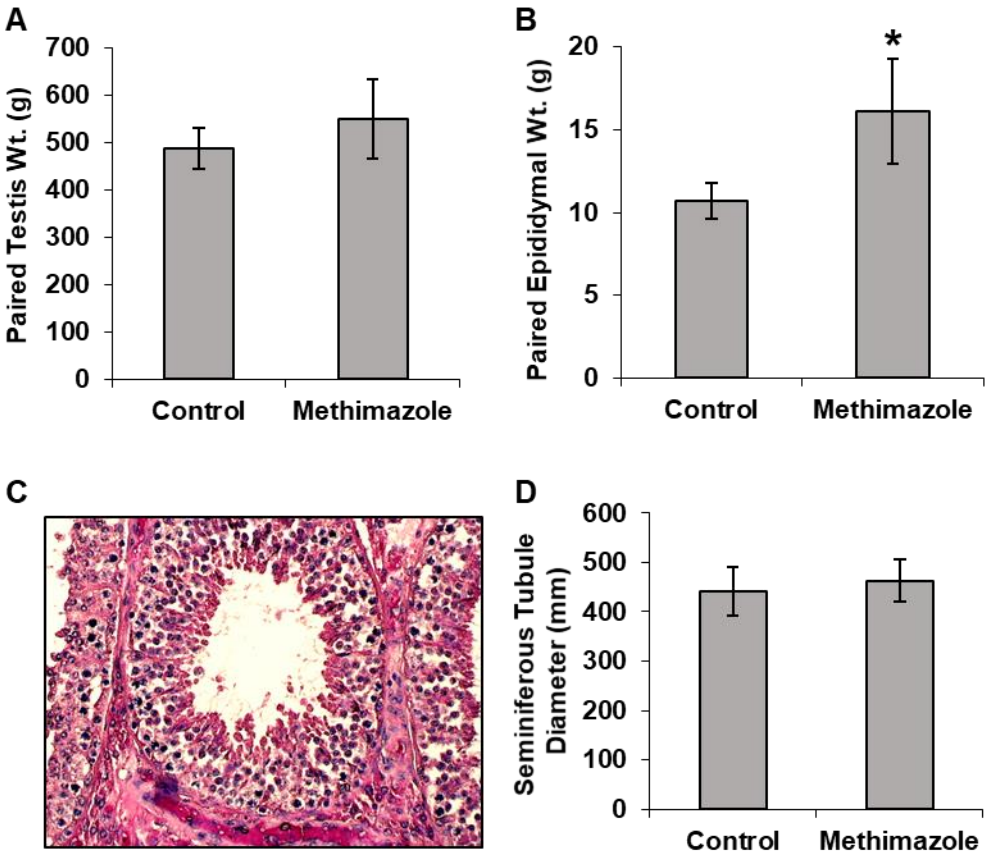
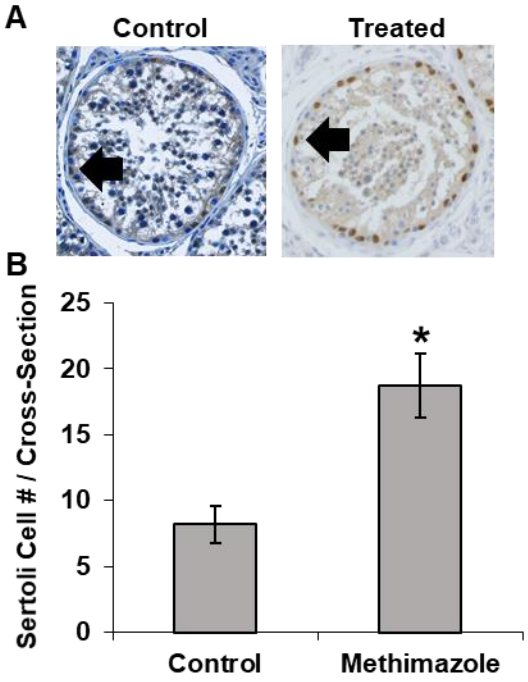

A
Paired Testis Wt. (g)
700
600
500
400
300
200
100
0
Control
Methimazole
B
Paired Epididymal Wt. (g)
20
15
10
5
0
*
Control
Methimazole
C
D
Seminiferous Tubule Diameter (mm)
600
500
400
300
200
100
0
Control
Methimazole

Figure 5


A
Control
Treated
B
Sertoli Cell # / Cross-Section
25
20
15
10
5
0
*
Control
Methimazole

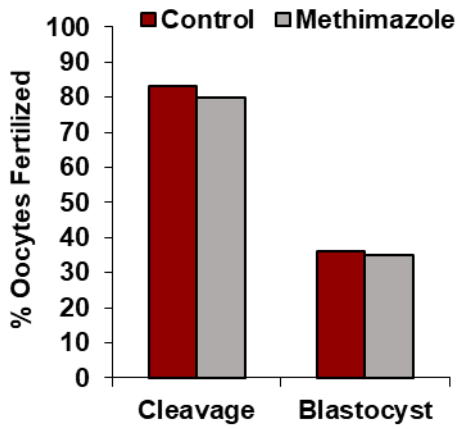
### 3.7 Supplemental Figure Legends

**Supplemental Figure 1.** Effect of transiently induced hypothyroidism in bull calves during pre-pubertal development on in vitro fertilization capacity of freshly collected spermatozoa from control and Methimazole treated bulls. A total of 130 oocytes were in vitro fertilized using spermatozoa from a control bull and 165 oocytes were in vitro fertilized using spermatozoa from a Methimazole treated bull at 27 mo of age. The data represent percent cleavage and blastocyst rate of total oocytes in vitro fertilized.

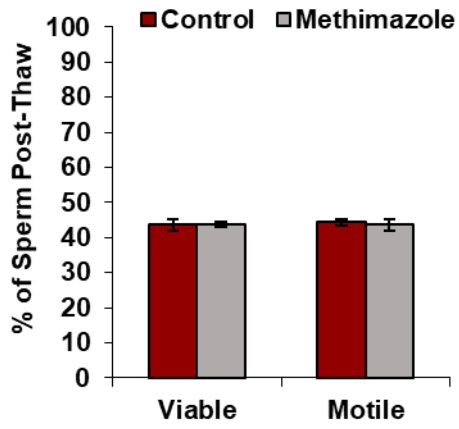
**Supplemental Figure 2.** Impact of transiently induced hypothyroidism in bull calves during pre-pubertal development on semen freezability at 24 mo of age. The data represent percentage of motile and viable spermatozoa in post-thaw semen of control and Methimazole treated bulls at 24 mo of age. Data are mean±SEM for 9 straws and n=3 bulls per group.

**Supplemental Figure 3.** Effect of transiently induced hypothyroidism in bull calves during pre-pubertal development on in vitro fertilization capacity of cryopreserved post-thaw spermatozoa from control and Methimazole treated bulls. A total of 462 oocytes were in vitro fertilized using post-thaw spermatozoa from a control bull and 422 oocytes were in vitro fertilized using post-thaw spermatozoa from a Methimazole treated bull at 24 mo of age. The data represent percent cleavage and blastocyst rate of total oocytes that were fertilized.

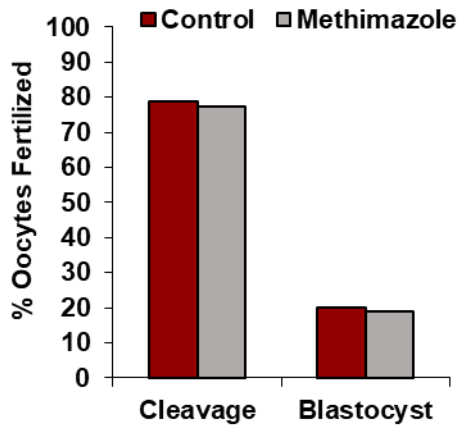
**Supplemental Figure 1**



**Supplemental Figure 2**



**Supplemental Figure 3**



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# CHAPTER FOUR: CONCLUSION AND FUTURE DIRECTIONS

## 4.1 Conclusion

Historically, the male of livestock species has been selected for breeding based upon scrotal size since long. Increased sperm production from the elite male will enhance the speed of genetic improvement in livestock through widespread use of desired genetics. Increased sperm production in response to induced temporary hypothyroidism (ITH) as we achieved would decrease the cost of semen production per bull at a fixed cost of maintenance. The bulls will produce more spermatozoa per ejaculate enhancing their utilization in semen sexing programs. The ultimate benefits will not be limited to cattle industry only, rather apply to swine and turkey production. Though the use of ITH to increase sperm production in horses, a seasonal breeder with the seasonal increase in Sertoli cell number, is untimely and needs further investigation, the success will enhance the number of semen doses available from an elite stallion during one breeding season.

Induced transient hypothyroidism (ITH) was used to increase sperm production in the rodents more than two decades ago. However, the technique has not been implemented to any livestock species although the first idea on it use in livestock was published about two decades ago. However as suggested by our findings, this increase will happen slowly increasing the age at maturity. Generally, yearling bulls are enrolled in sex sorting programs at or after two years of age because they cannot produce six billion spermatozoa required for each run of semen sexing process due to lower DSP at a younger age. Hence the current methodology to increase spermatozoa per ejaculate will allow bull enrollment to semen sexing program before two years of age. We found very first increased spermatozoal population per ejaculate at $18^{th}$ months of

age, however the increase was not consistent for subsequent months until from 21$^{st}$ months of age of the animals (Fig. 3C). Moreover, increased daily sperm production per bull will decrease the cost of semen doses and the cost of sexed semen. The technique is of commercial interest to semen sexing companies.

Increasing Sertoli cell number for the subsequent increase in daily sperm production is scientifically logical and preferable to some earlier tested but impractical methodologies including unilateral castration, immunization against inhibin and, estradiol. Application of CRISPR/Cas9 technology to livestock may be used to increase Sertoli cell number and daily sperm production. Knocking out $p27^{Kip1}$, $p21^{Cip1}$, or Cx43 may lead to an increase in Sertoli cell number and daily sperm production as seen in the mice. However, the knockout mice had abnormal spermatogenesis and were infertile. At present induction of transient hypothyroidism to increase Sertoli cell number is the most applicable method to increase daily sperm production in the bull.

### 4.2 Future directions

Dose optimization: Different doses of propylthiouracil in the rat gave the different magnitude of increase in sperm production and Sertoli cell number. Moreover, the magnitude of increase was different for rat and mice at the same dose rate. Furthermore, in the rat, the response to Methimazole was different than the response to propylthiouracil. We used methimazole® to induce hypothyroidism @2mg/kg boy weight of the animals in our study. Future studied with different doses of methimazole and propylthiouracil are recommended to discover optimum dose and drug for ITH in the bull. We fed methimazole® to the animal by mixing in grains twice a day. However, about half the times animal refused to swallow the tablets, spitted the tablets or

did not apprehend them at all. For such reasons, the tablets were fed to the animals using bolus gun. Handling animals twice daily for feeding through bolus gun would be cumbersome at commercial scale. An alternate approach to give methimazole to the animals would be ear implant of slow releasing methimazole. We used three animals as control and three as the treatment group. We observed about 200% increase in number of Sertoli cells per seminiferous tubule cross section. For an expected 200% difference in Sertoli cell number between the treatment and the control groups, at 80% power, 5% probability of type 1 error, statistically required sample size is seven animals per group. Future studies using slow releasing methimazole and with larger sample sizes are highly recommended before adopting this technique at commercial scale. In our study, in vitro fertilization capacity of fresh or post thaw spermatozoa from the treated bull was comparable to the control one. However, we did not attempt any natural insemination or artificial insemination to get viable calves. It is an area of future to confirm fertility of the spermatozoa from the treated bulls. Moreover, investigation of biochemical profile of the seminal plasma and spermatozoa from ITH treated bulls may be suggested.

Studies on induction of hypothyroidism in other livestock species will be of commercial interest and will add to the understanding of testicular biology in large animals. Moreover, ITH to increase Sertoli cell number in germ cell ablated NANOS2-knockout boars and bulls may increase the success rate of spermatogonial stem cell transplantation in the boar and the bull.