INTERRELATION BETWEEN THYROID HORMONES AND ONSET OF PUBERTY IN RAM LAMBS

A Thesis

Submitted to the Faculty

of

Graduate Studies of

the University of Manitoba

By

Amir Hooshang Fallah-Rad

In Partial Fulfillment of the

Requirements for the Degree

of

Doctor of Philosophy

Department of Animal Science

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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ABSTRACT

Fallah-Rad, Amir Hooshang. Ph.D. The University of Manitoba, May 1998. Interrelation between thyroid hormones and onset of puberty in ram lambs.

This project had two phases. The aim of the first phase of the study was monitoring concentrations and profiles of testosterone, thyroxine (T_4), tri-iodothyronine (T_3), insulin-like growth factor-I (IGF-I), glucose and testosterone pulse frequency during the peripubertal period and to investigate the relationship between these hormones and testicular function in the Suffolk (Sfk) and the more prolific Outaouais (Out) ram lambs. Average daily gain (ADG), relative growth (RG), scrotal circumference (SC), body weight (BW), gonadosomatic index (GSI) and blood glucose of the two breeds were similar throughout the experiment. Sperm cell concentration was higher in Out than in contemporary Sfk ram lambs. Mean testosterone concentration and pulse frequency during the 6 h period in each sampling day, T_4, T_3 and IGF-I were higher in Out than Sfk throughout the experiment. Analysis of the 20 min samples revealed no episodic release of IGF-I in both breeds. In conclusion, Out ram lambs had attained pubertal testicular function at an earlier age than Sfk ram lambs. Higher T_4, T_3, testosterone concentrations, testosterone pulse frequencies, and IGF-I in Out than Sfk may be associated with higher prolificacy and sperm concentration in the Out ram lambs.

The second phase was designed to investigate the influence of transient neonatal hyper- and hypothyroidism on the profiles of testosterone, follicle stimulating hormone (FSH), IGF-I, testicular development, function and onset of puberty in Sfk ram lambs.

Ram lambs were divided into four groups: control, PTU40, PTU55 and PTU+H. In the PTU+H group the lambs were induced hyperthyroid for 40 d. In the PTU40 and PTU55 the lambs were transiently hypothyroid for 40 and 55 d respectively. In all the treatment groups the level of FSH was lower during the treatment period. In the PTU55 group, FSH levels remained low after the treatment which is probably the result of direct effects of changes in the thyroid state on the higher brain centers. In both hypo- and hyperthyroid lambs, level of testosterone and pulse frequency did not change. Body weight and feed consumption in the treated animals were not different from those of controls. Hypothyroidism did not change testicular size, but decreased sperm production. Hyperthyroidism, on the other hand, increased sperm concentration and testicular size. Concentration of IGF-I was lower in both hypo- and hyperthyroid lambs, but only during the treatment period, indicating the negative impact of thyroid manipulation on IGF-I. In conclusion, the above results show the critical role of thyroid hormones on the function and development of the male reproductive system early in life and that hypo- and hyperthyroidism can change the profile of the reproductive processes in male sheep.

DEDICATION

This thesis is dedicated to the people of my beloved country Iran

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FOREWORD

This thesis has been written in the manuscript format. The first manuscript was submitted to the Canadian Journal of Animal Science, the second one to the Journal of Animal Science and the third one to the Biology of Reproduction.

Authors and the title of the manuscripts are:

1. Fallah-Rad, A. H. and M. L. Connor, 1997. Relationship of thyroid hormones and testosterone in ram lambs.
2. Fallah-Rad, A. H., M. L. Connor and R. P. Del Vecchio. Transient hyperthyroidism enhances onset of puberty in Suffolk ram lambs.
3. Fallah-Rad, A. H., M. L. Connor and R. P. Del Vecchio. Transient hypothyroidism suppressed testicular function in pubertal Suffolk ram lambs.

For the purpose of uniformity, all manuscripts presented here in are written to meet the format and style of the Journal of Reproduction and Fertility.

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LIST OF ABBREVIATIONS

Ab	Antibody
ABP	Androgen Binding Protein
ADF	Average Daily Feed Intake
ADG	Average Daily Gain
AEE	Acid Ethanol Extraction
ANOVA	Analysis of Variance
BW	Body Weight
°C	Degree Centigrade (Celsius)
CCAC	Canadian Council on Animal Care
cm	Centimeter(s)
COX	Cytochrome Oxidase
CPM	Counts Per Minute
CV	Coefficient of Variation
d	Day(s)
DDM	Daily Dry Matter Consumption
dl	Deciliter(s)
DM	Dry Matter
DPC	Diagnostic Products Corporation
DSL	Diagnostic Systems Laboratories
DSP	Daily Sperm Production
DHT	Di-Hydro Testosterone
E2	Estrogen
ELISA	Enzyme Linked Immuno Sorbent Assay
FSH	Follicle Stimulating Hormone
G	Gauge
g	Gram(s)
GGT	γ-glutamyl transpeptidase
GH	Growth Hormone
GLM	General Linear Model
GnRH	Gonadotropin Releasing Hormone
GSI	Gonado-Somatic Index
× g	g force
h	Hour(s)
HS	High quality Semen
I.D.	Inner Diameter
IGF-I	Insulin Like Growth Factor-I
IGF-II	Insulin Like Growth Factor-II
IGFBP	Insulin Like Growth Factor Binding Protein
Kg	Kilogram(s)
LDH	Lactate dehydrogenase
LH	Luteinizing Hormone
LS	Low quality Semen

LSD	Least Significant Difference
µg	Microgram(s)
mg	Milligram(s)
min	Minute(s)
MIS	Mullerian Inhibiting Substance
µl	Microliter(s)
ml	Milliliter(s)
mm	Millimeter(s)
MMI	Methimazole
m_{RNA}	Messenger RNA
N	Normal
ng	Nano gram(s)
nm	Nanometer(s)
NRC	National Research Council
NSB	Non Specific Binding
O.D.	Outer Diameter
Out	Outaouais
P_4	Progesterone
PBS	Phosphate Buffer Serum
PRL	Prolactin
PTU	6-Propyl 2-Thiouracil
r	Pearson Product-Moment (Correlation Coefficient)
RG	Relative Growth
RIA	RadioImmunoAssay
SAS	Statistical Analysis System
SC	Scrotal Circumference
SEM	Standard Error of the Mean
Sfk	Suffolk
ST	Seminiferous Tubules
std	Standard Deviation
T_3	Tri-iodothyronine
T_4	Tetra-iodothyronine; Thyroxine
TNH	Transient Neonatal Hypothyroidism
TNHI	Transient Neonatal Hyperthyroidism
TRH	Thyrotropin Releasing Hormone
TSH	Thyroid Stimulating Hormone
vol	Volume
vs	Versus
wt	Weight

1. INTRODUCTION

Puberty in the male mammal is manifested by developmental processes at the hypothalamic-pituitary-gonadal axis (Amann, 1983) with episodic release of testosterone (Renaville *et al.*, 1993). Testosterone may be considered as a primary regulator for other major physiological changes during this period. Since steroids have direct effects on the adenohypophysis, a rise in the blood testosterone level is accompanied by a discharge of growth hormone (GH; Wilson 1986, Copeland *et al.*, 1985). Other hormones such as IGF-I (Parker *et al.*, 1984) also contribute in this process. Moreover, gonadal steroids have the ability to increase IGF-I concentration irrespective of GH effects. In this respect, some researchers found that testosterone administration stimulated IGF-I production in prepubertal boys (Cuttler *et al.*, 1985).

Spermatogenesis is highly dependent on two major components of the seminiferous tubules; i.e. Sertoli cells and germ cells (Steinberger and Steinberger, 1971). Answers to many questions relevant to male physiology of reproduction, infertility and contraception are related to complex cellular and molecular interactions that take place in the testis in general and seminiferous epithelium in particular. There are two major fundamental concepts about seminiferous tubule morphology. First, the blood testis barrier which is formed by the tight junctions between the adjacent Sertoli cells (Francavilla *et al.*, 1991). Second, there is a specific relationship between Sertoli cells and germ cells in a developmental cycle called the *seminiferous epithelial cycle* (Mita *et al.*, 1982). This cycle is highly organized and requires delicate interaction of Sertoli and germ cells. In general, in higher vertebrates, spermatogenesis is dependent upon the function of Sertoli cells

(Berndtson *et al.*, 1987). In the male rat, germ cell maturation is regulated by the developmental state of the Sertoli cell, i.e. retardation of Sertoli cell maturation is followed by delayed maturation of the germ cells (Hess *et al.*, 1993).

The endocrine regulation of spermatogenesis is the result of hormone action on Sertoli cells and not germ cells (Griswold, 1995). Therefore, Sertoli cell number and integrity (i.e. ability to function normally) are major limiting factors for spermatogenesis. It was thought that the main gonadotropin affecting Sertoli cells is follicle stimulating hormone (FSH; Orth and Christensen, 1978). Although FSH is required for the onset of spermatogenesis, in the adult animal, much of the role of FSH is transferred to testosterone. Both of these hormones exert their effects on spermatogenesis by acting on their Sertoli cell receptors. There is no reported proof for the presence of androgen receptors in germ cells (for review see Griswold, 1995).

Recently, considerable research effort has been directed towards determining the effects of thyroid hormones on reproductive processes. The role of thyroid hormones in testicular physiology is far from clear (Cooke *et al.*, 1994 a). The effects of iodothyronines on the testis is highly dependent upon the age of the animal. Since thyroid hormones may affect testis function both directly and indirectly the scenario becomes more complicated. Based on studies of oxygen consumption; brain, spleen and testis were considered unresponsive to thyroid hormones (Oppenheimer *et al.*, 1974). Later this concept was rejected (Palmero 1988, 1989, Ulisse, 1992). A new concept involves the thyroid hormones in the development of the testes in early life. Transient neonatal hypothyroidism (TNH) in rat pups, induced by adding the reversible goitrogen 6-propyl-2-thiouracil

(PTU) to the mother's water from birth until Day 25, has been demonstrated to induce adult testicular hypertrophy and hyperplasia (Meisami *et al.*, 1992, Cooke *et al.*, 1992). This supports a critical role for thyroid hormones in the developing male gonads during the perinatal period. It is believed that thyroid hormones decelerate proliferation but accelerate differentiation of Sertoli cells (Cooke *et al.*, 1994 a). However, investigations thus far have been largely confined to some laboratory species.

The following review of literature will present general considerations about Sertoli cells and the role of relevant reproductive and metabolic hormones, and will discuss the new concept of transient neonatal manipulation of thyroid state in some species. Although most of the information has been derived from small rodents, some of the papers are pertain to the relationship of thyroid hormones with reproductive parameters in larger species which may be helpful in understanding the critical role of thyroid hormones in the perinatal period.

2. LITERATURE REVIEW

2.1. Anatomy of the testis

'Testis' is a Latin word meaning 'witness' or 'spectator'; from this meaning English words such as 'testify' and 'testament' were derived. It has been suggested that the use of 'testes' for the male gonads reflected an ancient belief that these organs were witnesses to an act in which they took no part. It seems more likely that the Romans considered the testes to be 'witness' or evidence of a man's virility (Setchell, 1978).

The testes of mammals fulfill two functions, the production of the male gametes (spermatozoa) and of the male sex hormones (androgens). Mammals are the only animals in which the testes descend from their point of origin into a scrotum. However, testicular descent occurs to a variable extent in the various orders of mammals. Primates, ruminants and marsupials are the species in which a pronounced scrotum develops. As the testis migrates it takes its blood and nerve supply with it, so that in the adult animal with scrotal testes, the blood vessels pass from abdominal cavity, through the inguinal canal and into the scrotum. The testis is encased in a tough fibrous capsule, usually referred to as the tunica albuginea. There are really three tunics: the tunica albuginea proper in the middle; a visceral tunica vaginalis on the outside; and a tunica vasculosa nearest to the parenchyma (Setchell, 1978).

The seminiferous tubules are basically two-ended loops with the two ends opening into the rete testis. The tubules contain various germinal cells and the somatic Sertoli cells.

They are surrounded by a well-defined boundary tissue composed of four layers (Steinberger and Steinberger, 1971). The Sertoli cells, named after the man who described them in 1865, apparently do not divide after puberty in man (Setchell 1978). Before puberty they lie immediately inside the boundary tissue of the tubules and surround the undeveloped germinal cells. In the adult, the cytoplasm of the Sertoli cells extends from the boundary tissue to the lumen of the tubule. The Sertoli cells share the surface of the boundary tissue with the spermatogonia. Sertoli cells have a complex nucleus with infolding or lobulation, and a prominent nucleolar complex. In the Sertoli cells, the mitochondria are numerous and there are other cell organelles including multiple separate Golgi elements and rough and smooth endoplasmic reticulum (Setchell, 1978).

The lumen of each tubule is filled with fluid, which carries the liberated spermatozoa off on their long journey to what de Graaf (1667) referred to as 'the place ordained by Almighty God for the reception of semen'. Spermatozoa are produced in the seminiferous tubules from germ cells and they develop through the processes of spermatocytogenesis to yield spermatids, and spermiogenesis which is the stage of metamorphosis of spermatids into spermatozoa. Spermatozoa leave the testis via the rete testis and efferent ducts and pass into the epididymis where they undergo maturation and are stored. From here the spermatozoa can move via the ductus deferens through the inguinal canal to the penis. For further details refer to Ashdown and Hafez (1993).

The seminiferous tubules are basically cylindrical, and, when a number of cylinders are stacked together, this creates a series of 3-sided spaces. These spaces, known as the interstitial tissue, contain the blood vessels, lymph vessels and nerves (none of which

penetrate the tubules), as well as the interstitial cells or Leydig cells. Leydig cells function to produce the majority of the androgens formed in the testis from cholesterol (Setchell, 1978). Therefore, testes are the reproductive organs in male mammals with the functional units of the testes being the Sertoli, Leydig, and germ cells which have a close inter-relationship necessary for normal spermatogenesis.

2.2. Hypothalamic-pituitary-gonadal axis and prolificacy

Changes in the responsiveness of ram testicles to luteinizing hormone (LH) are evident in the breeding season and this is associated with an increase in the number of Leydig cells and LH binding sites per Leydig cell (Barenton and Pelletier, 1983). Within a breed, the differences in the reproductive hormone profiles of individuals corresponds to differences in their prepubertal pattern of LH release. In the more prolific breeds of sheep, an earlier decrease in the sensitivity of the hypothalamic-pituitary axis to estradiol negative feedback may be the reason for more LH release (Land, 1978). As well, an age-dependent relationship between FSH concentration and breed fertility was demonstrated by Sanford *et al.* (1982). Maturational changes in FSH secretion during the prepubertal and pubertal periods of ram lambs may be completed relatively early in comparison with those for LH (Sanford *et al.*, 1982). Both LH and FSH serum concentration in 3-month-old ram lambs in the fall were positively related to breed prolificacy (Sanford *et al.*, 1982). In the more prolific breeds of sheep, the ram lambs, at certain ages had higher concentrations of LH and testosterone compared to those of low prolificacy (Land, 1978).

Therefore, reproductive hormonal profiles in more prolific breeds of sheep appear to be different than in less prolific ones. However, the etiology of these differences is not well defined. Because changes in the reproductive processes and cycles are dependent on pulse frequency, duration and amplitude of hormone release (Sanford *et al.*, 1982), it could be fruitful to investigate reproductive parameters and interrelationships from this angle.

2.3. Body weight, testicular size and function

Testicular weight is a good indicator of spermatogenesis. Scrotal circumference (SC) also provides a reliable indication of spermatogenic function. The SC was shown to be a highly repeatable, easily obtained indicator of testes size which was highly correlated with testes weight and sperm output in bulls of various ages (Almquist *et al.*, 1976). Likewise, a positive correlation exists between serum testosterone concentration, testicular size and daily sperm production (DSP; Yarney *et al.*, 1990). Berndtson *et al.* (1987) found that testes containing a larger total number of Sertoli cells are heavier than testes with fewer Sertoli cells while Orth *et al.* (1988) demonstrated that Sertoli cells are the major determinant of the magnitude of sperm production.

There is normally a close association between increase in testicular circumference and general body growth of ram lambs between 30 and 190 days of age (Yarney *et al.*, 1990). During the pubertal period, there is a positive correlation between body weight (BW), SC, DSP, testosterone and number of Sertoli cells. During this time, a major determinant of testicular function and size is the number of Sertoli cells. However, after

the onset of puberty, particularly in seasonal breeders, testicular growth and development is influenced primarily by age and daylength as well as other environmental factors (Schanbacher, 1979, Berndtson *et al.*, 1987).

2.4. Puberty and relevant hormone profiles

2.4.1. Puberty: definition and onset.

In the male, puberty can be defined as the time when spermatogenesis starts (Bearden and Fuquay, 1992). Renaville *et al.*, (1993) defined puberty as the physiological step, characterized by a pulsatile release of testosterone by the gonads, that precedes spermatogenesis. In practice, the first definition is more applicable because, some ram lambs may show libido and sexual desire accompanied by testosterone pulses without having any viable spermatozoa in their ejaculum. In rams, periods of elevated testosterone concentration are characterized by increases in LH and testosterone peak frequency and amplitude (Sanford *et al.*, 1978) as well as a transient increase in FSH secretion (Sanford *et al.*, 1977). Measurement of puberty in sheep is complicated by the seasonality of breeding which masks the influence of age. Animals which chronologically mature before the onset of the first breeding season of their lives will not normally show signs of this maturity until the start of the season; likewise, animals which are of a mature age soon after the end of the breeding season will not manifest sexual maturity until the onset of the next breeding season (Land, 1978).

2.4.2. Pubertal LH and testosterone concentrations and pulses

The prepubertal period starts with initiation of hypothalamic discharge of gonadotropin releasing hormone (GnRH) and gonadotropins by the anterior pituitary (Amann 1983). There is a close relationship between the concentrations of serum LH, LH pulses and testosterone concentration in young bulls and ram lambs (Sanford *et al.*, 1982, McCarthy *et al.*, 1979, Lunstra *et al.*, 1978, Yarney and Sanford, 1990). Increased testicular steroidogenic activity of rams in the breeding season is due in part to changes in both LH-peak frequency, LH peak height and testicular responsiveness to LH (Sanford *et al.*, 1977). Wilson and Lapwood (1979) found that mean LH levels in Romney ram lambs was generally high between 4 and 16 weeks of age and then decreased due to a reduction in the frequency of LH release. Sanford *et al.* (1977) noticed an increase in the frequency of pulses, but a decrease in peak height, in Suffolk ram lambs from 6 to 22 weeks of age. Decreases in circulating LH during the latter part of the pubertal period (after 16 weeks of age) to adult levels have been attributed to greater feedback inhibition by testicular steroids on the hypothalamic-pituitary axis (Wilson and Lapwood, 1979). In contrast, a steady increase in LH concentration has been reported in Finn, Merino and crossbred lambs during the prepubertal period (Land, 1978). Land (1978) also found that during the prepubertal period, the concentration of LH in peripheral plasma rose more rapidly in ram lambs from breeds of high prolificacy than from those of low prolificacy. It may be argued that the rapid rise in plasma LH and high rates of testicular growth may arise from a lower sensitivity of the highly prolific breeds to negative feed-back from gonadal steroids (Land, 1978).

In the literature there are some controversies about the age at which the concentration of testosterone reaches its maximum. According to Schanbacher *et al.*, (1974), maximum production of testosterone in the young Targhee crossbred rams occurred after 16 weeks of age. However, the first significant increase in testosterone concentration in Romney (Wilson and Lapwood, 1978) and Suffolk (Yarney and Sanford, 1990) ram lambs occurred by 24 weeks of age while progressive increases in all characteristics of LH release occurred between 4 and 16 weeks of age. Wilson and Lapwood (1979) found that the major increases in testosterone levels occurring after 24 weeks of age were preceded by a steady decrease in mean plasma LH concentrations from 16 to 22 weeks of age. Difference in time frame may be attributable to breed, and probably, seasonal effects. In conclusion, maximum concentration of LH have been observed before 16 weeks of age, after which the frequency of LH pulses increases while the pulse height and mean LH concentrations decrease. Concentrations of testosterone in all the experiments increased after 22 weeks of age, implying that T production is more dependent on the LH pulse than on serum LH concentration.

2.4.3. Pulse definition and importance of pulses

Santen and Bardin (1973) defined a pulse as an increment of hormone concentration that exceeds the preceding local nadir level by 20 %. Merriam and Wachter (1982) presented a new algorithm for the identification of episodic secretory pulses. Their method calculates a base line to represent the contribution of circadian rhythms and long-term trends free of ultradian fluctuations. It identifies peaks by criteria of both height and duration from the

base line. Veldhuis *et al.* (1984) observed that the criterion of "20 %" represented approximately 3 times the intra-assay coefficient of variation (CV). Instead of using a fixed 20 % threshold, they modified this algorithm to permit detection of pulses which were measurements of the hormone that exceeded the preceding local minimum by a value that was fourfold greater than the intra-assay CV of the RIA. Renaville *et al.* (1993) defined a testosterone pulse as an elevation in the hormone concentration which is at least equal to daily mean plus one standard deviation. The method used by Veldhuis *et al.* (1984) is more conservative because, it avoids the ultradian fluctuations which result from errors made during measurements. Moreover, this method ensures less false positive pulses.

2.4.4. FSH concentration, and testosterone level

In contrast to the pattern of progressive decreases in serum LH concentration with increasing age of rams, after the pubertal rise, FSH concentrations are remarkably similar for rams of different ages. However, FSH concentration is positively correlated with breed prolificacy (Land, 1978). Concentrations of FSH and the height of the testosterone peaks are consistently greater in highly prolific breeds compared to breeds of lower prolificacy. Pubertal increases in FSH are completed much earlier than those in LH (Sanford *et al.*, 1990) and are accompanied by increased blood testosterone levels (Lee *et al.*, 1976).

Testosterone concentration and ejaculate volume are highly correlated. Seasonal changes in the concentration of testosterone in seminal plasma tends to correspond to concurrent changes in the serum level of FSH rather than LH. This is because FSH

stimulates production of androgen binding protein (ABP) by Sertoli cells. ABP binds testosterone, thereby, increasing testosterone concentration in the seminal plasma. Increased serum FSH is associated with increased testicular growth, increased numbers of receptors for LH and FSH in both Sertoli and Leydig cells, and increased testosterone concentrations (Martin *et al.*, 1991). However, seasonal changes in sheep serum testosterone are related more to variations in serum LH rather than FSH (Sanford *et al.*, 1977). Spermatogonia have few FSH receptors. In Sertoli cells, which are the main target of FSH in seminiferous tubules, the basal compartment area is the major binding site for FSH (Orth and Christensen, 1978). The blood testis barrier seems to exclude FSH from the central part of the tubule, and from any receptors that may exist there. Therefore only those cells adjacent to the periphery of the seminiferous tubules have access to FSH.

2.5. Models to increase testicular size

Models for increasing testicular size and (or) DSP includes: immunization against inhibin (Martin *et al.*, 1991), prepubertal hemicastration (Setchell and Jacks 1974, Cunningham *et al.*, 1978), and immunization against estradiol (E_2) (Kosco *et al.*, 1987). All of these models resulted in transient or permanent increases in circulating FSH levels (Schanbacher *et al.*, 1987, Martin *et al.*, 1991).

2.5.1. Immunization against inhibin

Inhibin, a protein secreted by Sertoli cells of the testis, suppresses the level of FSH. FSH and inhibin concentrations are inversely related in plasma of rams (Schanbacher, 1988).

Inhibin regulates FSH secretion by a feedback mechanism, during both prepubertal and postpubertal periods, in males (Martin *et al.*, 1991). Bulls which were actively immunized against the α subunit of inhibin at 2 weeks of age tended to have increased peripheral FSH levels, as defined by higher sperm density at 390 days of age (Schanbacher, 1991). In general, immunization against inhibin in 3.2 month old bull calves, increased serum FSH and testosterone, decreased serum LH and increased DSP per gram of testis (Martin *et al.*, 1991). However, testicular weight (Schanbacher, 1991) and SC (Martin *et al.*, 1991) of bulls actively immunized against inhibin were not significantly increased over those of control bulls. Increased sperm production coincided with increased FSH and testosterone in bulls immunized against bovine inhibin, suggesting that both hormones may enhance testicular sperm production in bulls. FSH is the main gonadotropin affecting Sertoli cells (Orth and Christensen, 1978). Moreover, the increased secretion of FSH could have indirectly increased sperm production by increasing testicular sensitivity to circulating gonadotropins in general and LH in particular. Accordingly, high sensitivity of the testis to LH causes increase in the secretion of testosterone despite a decrease in the secretion of LH (Martin *et al.*, 1991). Some of the actions of inhibin on the secretion of steroids from gonads are attributed to local actions of inhibin in spermatogonial development (van Dissel-Emiliani *et al.*, 1989). Whether inhibin exerts its effects directly on the spermatogonia or through an action on Sertoli or interstitial cells is at this time unknown.

Berndtson *et al.*, (1987) stated that, because a positive relationship exists between DSP and the absolute number of Sertoli cells in the bull, success in increasing the sperm output will depend upon the ability to increase the absolute number of Sertoli cells.

Because Sertoli cell proliferation is confined to the prepubertal period (Steinberger and Steinberger, 1971), any research aimed at increasing DSP and testicular size should be focused at the prepubertal rather than the postpubertal period.

2.5.2. Hemicastration

Hemicastration of rats soon after birth, results in compensatory hypertrophy of the remaining testis (Hochereau *et al.*, 1976), but this is not seen if the operation is delayed until 10 days of age. However, compensatory hypertrophy occurs in the ram and other seasonally breeding animals even in adulthood provided that hemicastration occurs during the non-breeding season (Hochereau *et al.*, 1976). In ram lambs, removal of one testis at one week of age caused an increase in FSH which was maintained until the animals were 12 weeks old. However, in the hemicastrated lambs levels of testosterone and LH were similar to that of the intact animals (Walton *et al.*, 1978). It would seem, therefore, that the remaining testis of a hemicastrated lamb may be able to adapt by secreting extra quantities of testosterone or other steroid hormones and so maintain the concentration of LH and testosterone at a value similar to that observed in entire lambs.

The increase in the level of FSH in the plasma of hemicastrated lambs could be the manifestation of low levels of serum inhibin because, the remaining testis is the only source of inhibin secretion (Setchell and Jacks, 1974). The compensatory testicular growth observed is presumably the consequence of this increased level of FSH (Walton *et al.*, 1978). In intact animals administration of FSH is associated with increased testicular growth (Lee *et al.*, 1976). Therefore, in the hemicastrated lambs, changes in the serum

FSH and not testosterone or LH, are presumably responsible for the hypertrophy and hyperplasia of the Sertoli cells which ultimately cause enlargement of the remaining testis. Although hemicastration in the bull increased sperm production per gram of testis in the remaining testis, the overall sperm production was reduced compared to intact bulls (Schanbacher, 1988). In summary, hemicastration decreased inhibin and increased FSH while LH and testosterone did not change.

2.5.3. Immunization against estradiol

Immunization against E_2 in sheep increased testis size (Schanbacher *et al.*, 1987) and DSP by 25 % (Monet-Kuntz *et al.*, 1988). This effect was presumably through elevated FSH. In the ram, E_2 has a negative feedback on FSH secretion by the pituitary and immunization against E_2 increased circulating FSH levels (Schanbacher *et al.*, 1987).

In all of the above models, the FSH concentration was increased. FSH is known to be involved in the mitogenic processes of the testis therefore one may expect that any successful attempt to increase the size of the testis would be accompanied by an increase in the FSH concentration.

2.6. Sertoli cells

2.6.1. Role of Sertoli cells in spermatogenesis

Sertoli cells support the process of spermatogenesis through a variety of specialized functions. These include: the establishment of a blood-testis barrier (Francavilla *et al.*, 1991); the maintenance of high intratesticular testosterone concentrations via the

production and secretion of ABP (Palmero *et al.*, 1989); and the nourishment of the developing germ cells (Mita *et al.*, 1982).

Current views concerning the regulation of spermatogenesis propose that the Sertoli cell serves as an intermediary between plasma factors, including hormones and the germ cells. One way by which the Sertoli cell is thought to contribute to spermatogenesis is by regulating the milieu within which the germ cells develop. The blood testis barrier, formed by tight junctional complexes between adjacent Sertoli cells, is important for this function (Dym and Fawcett, 1970). The blood testis barrier excludes blood from the adluminal compartment of the tubules, therefore only the basal compartment of Sertoli cells have direct access to blood components (Orth and Christensen 1978). This would suggest that the Sertoli cell can respond to stimulation by circulating growth factors and mitogenic substances. In turn this stimulus is transduced to a form which results in stimulation of the germ cells through the intimate association between these two cell types (Borland *et al.*, 1984).

The activity of individual Sertoli cells as an ultimate contributing factor should not be totally neglected because Sertoli cells are typically associated with a limited number of developing germ cells (Hess *et al.*, 1993). Sertoli cells acquire their maximum supporting capacity in older animals. A marked progressive increase has been noted in sperm output from around puberty until at least 2 years of age in Angus and Herford bulls maintained on a frequent seminal collection schedule (Almquist *et al.*, 1976).

Because of the variety of supportive roles the Sertoli cells perform, it seems reasonable to speculate that the absolute numerical size of the Sertoli cell population

might establish the upper limit for spermatozoal production in any given testis. Indeed, DSP is generally related to the absolute number of Sertoli cells and this relationship is linear (Berndtson *et al.*, 1987). Age related increases in DSP coincide with proportional increases in the number of germ cells per Sertoli cell (Berndtson *et al.*, 1987). Moreover, highly significant increases in ejaculate volume, weekly total motile sperm output and a significant improvement in sperm concentration were found as bulls aged (Almquist and Cunningham, 1967). Sertoli cells are normally associated with a certain number of germ cells, which correspond to their highest capacity (Hess *et al.*, 1993). Therefore, it is possible that DSP increases up to the point at which individual Sertoli cells simply cannot support additional developing germ cells, at which time the absolute number of Sertoli cells would be more critical than at an earlier age. Increase in the surface area of the Sertoli cells may also contribute to the increases in DSP.

Both testicular size and DSP are positively correlated with the total number and size of Sertoli cells (Almquist *et al.*, 1976). Thus, it is not surprising that DSP and testicular weight are positively correlated with each other (Berndtson *et al.*, 1987). Therefore, one can speculate that by increasing the number of Sertoli cells in the testis, it would be possible to increase the size of the testes and sperm output. The newly developed method of "transient neonatal hypothyroidism" which will be discussed later, has achieved this in rodents.

2.6.2. Sertoli cell proliferation and differentiation

The issue of whether Sertoli cells lose mitotic potential at a young age is not completely resolved. Under normal conditions, in the rat, Sertoli cell proliferation peaks shortly before birth. This activity of Sertoli cells continues through the early postnatal period, then gradually diminishes and ceases entirely after spermatogenesis is established (Orth *et al.*, 1982). In the stallion, the number of Sertoli cells increases up to 4 or 5 years of age with dramatic fluctuations in their population between the breeding and non-breeding season (Johnson and Thompson, 1983). However, in stallions it is required that, Sertoli cells increase during the reproductive season and that they retain mitotic potential throughout adulthood.

The number of Sertoli cells increases 15-fold during days 1-15 in neonatal rats (Zhengwei *et al.*, 1990, Orth 1982). This increase is limited thereafter so that mitogenesis declines at 15-18 days of age and complete cessation occurs at 50 days of age. Studies with the ram, goat, bull, boar, horse, and human also support the general conclusion that Sertoli cell proliferation is maximal at an early age (Gondos and Berndston, 1993). The fact that Leydig cells are the source of β-endorphin which directly suppresses the proliferative response of perinatal Sertoli cells to FSH, adds to the complexity of the process (Orth, 1986). Considerable evidence points to the existence of multiple two way lines of communication among the various cells of the testis, including germ cells, Sertoli cells, myoid cells, and Leydig cells (Orth and Boehm, 1990, Weber *et al.*, 1983).

The maximal proliferation of rat Sertoli cells, which occurs *in utero* during late gestation and in the perinatal period, coincides with the onset of fetal thyroid function

(Jannini *et al.*, 1990) which indicates the probable relationship of thyroid hormones and Sertoli cells. Therefore, in most of the species studied, Sertoli cell proliferation is maximal during a short period after birth but, the rate of mitotic divisions varies in different species.

In many species, the Sertoli cells are not differentiated at birth. Later they undergo extensive changes leading to structural and functional maturation. These changes are expressed by appearance of hormone receptors and reactivity to different hormones, production of many proteins (ABP, inhibin, IGF-I, etc.) and metabolism of hormones (e.g., aromatization of testosterone). In the lamb, there is a four-fold increase in Sertoli cell area between 25 and 100 days of age (Monet-Kuntz *et al.*, 1984). This increase in volume is mainly due to increase in the cytoplasm of the cells. Major contributions to the increase in cytoplasmic volume come from mitochondria, endoplasmic reticulum, lipid droplets and lysosomes and extension of apical cytoplasm toward the developing tubular lumen. Although not expanding as rapidly as cytoplasmic volume, nuclear dimensions also undergo an increase during postnatal development (Monet-Kuntz *et al.*, 1984). In the lamb, the increase in nuclear size during pubertal development is somewhat more gradual. The above findings suggests that both hypertrophy and hyperplasia of the Sertoli cells occur during the peripubertal period and it may be possible to change the adult testicular size and function by manipulation of this process during the appropriate time period.

Although in rats and mice Sertoli cell proliferation and differentiation occurs during the perinatal period, there are major differences between these species and farm species like sheep and cattle. During the fast proliferation period, the labeling index (percentage of the proliferating cells from the total) of rat Sertoli cells was 20% and that

of bull calf was 2 to 5% (Cooke *et al.*, 1994a). This means that manipulation of Sertoli cell proliferation during a certain period affects a greater number of Sertoli cells in rats than in calves. Therefore, methods to influence this proliferative phase may require a longer duration in ruminants than in rats.

2.6.3. Proteins secreted by Sertoli cells

During early postnatal life, Sertoli cells begin to produce increasing amounts of secretory proteins characteristic of the juvenile and adult cell (Bunick *et al.*, 1994, Kirby *et al.*, 1992, Palmero *et al.*, 1989). Production of receptors for FSH, testosterone, insulin, β-adrenergic hormones and L-T_3 (or T_3) are among other functions of the Sertoli cells (Gondos and Berndston, 1993). The gene encoding thyroid hormone receptor, C-erbAα, is produced by the juvenile Sertoli cells (Hirobe *et al.*, 1992). The production of certain proteins such as IGF-I (Smith *et al.*, 1987), ABP (Palmero *et al.*, 1989), clusterin and inhibin-β_b (Cooke *et al.*, 1994 b) has been shown in Sertoli cell cultures. Apart from the production of the proteins mentioned above, Sertoli cells are involved in regression of the Mullerian ducts. Production of Mullerian inhibiting substance (MIS) is associated with dividing Sertoli cells (Hirobe *et al.*, 1992).

The function of the seminiferous tubular epithelium in the postnatal testis is to produce spermatozoa through continuous proliferation and maturation of the germ cell under the regulation of pituitary FSH. In the fetal period the Sertoli cells, in contrast, work actively to prevent spermatogenesis. At the same time they have to maintain the germ cells in good shape for their future chores (Pelliniemi *et al.*, 1993). In recent years, a

number of techniques have been developed that allow for the investigation of molecular events taking place in Sertoli cells and germ cells during the seminiferous cycle. All of these techniques have shown that the secretions by Sertoli cells and thus the immediate environment in the seminiferous epithelium varies as a function of the stage of the cycle. Sertoli cells associated with germ cells at one stage of the cycle express genes different from those in other parts of the cycle. One mode includes the stages of meiotic division, and the other mode is associated with the spermiation stage. In each mode, production of specific proteins is maximal. These two modes of biochemical activity correlate well with the two morphological types of Sertoli cells described by Russell (1983). It can be concluded that Sertoli cells react to the same stimulus in different ways depending on the age of the animal and stage of seminiferous cycle. Therefore, to manipulate the function of these cells, knowledge of timing of the events is required.

2.7. Thyroid gland

2.7.1. Anatomy and hormones.

Thyroid (Gr. Thyreoeides, Fr. Thyreos, an oblong shield) means "shield" or something resembling a shield (Stedman's medical dictionary 1972). In man the thyroid gland is located on the thyroid cartilage in the anterior part of the larynx like a shield. The thyroid gland is derived from cranial endoderm of the embryo and it has two lobes which are connected together by an isthmus. The functional components of the thyroid gland are the individual thyroid follicles, which consist of a cuboidal epithelium arranged as a single layer surrounding a lumen which contains a colloid material (Hadley, 1992).

Thyroid hormones are involved in brain and body development (McEwen *et al.*, 1991). It is well recognized that in newborn infants, lambs, calves or piglets, the hypothalamic-pituitary-thyroid axes is already well developed and of considerably high functional capacity (Brzezinska-Slebodzinska and Krysin, 1990). However, newborn rabbits and rats are delivered hypothyroid (Dubois and Dussault, 1977). In neonatal rats, total T_3 and thyroxine (T_4) concentration is lower than in adult rats. In sheep (a precocial animal), but not rats and rabbits (altricial animals), the link between hypothalamus, pituitary, thyroid and gonads is developed at the time of birth (Ferreiro *et al.*, 1988). Therefore, the time window for induction of transient neonatal hyper- or hypothyroidism may be different in different species.

There is a huge pool of thyroid hormones bound to blood proteins. Therefore, there is no pulsatile release similar to what is seen in LH. Nevertheless, in golden hamsters, a long day breeder, a circadian rhythm in thyroid hormone concentration was reported which was dependent upon seasonal changes in day length (Ottenweller *et al.*, 1987). Moreover, adrenal, thyroidal, and testicular rhythms were suppressed on the short photoperiod in golden hamsters (Ottenweller *et al.*, 1987). One of the major factors stabilizing the thyroid hormone concentration in the blood is thyroid hormone binding proteins. Sex steroids are known to affect the concentrations of a number of plasma proteins, including thyroxine-binding globulin and sex steroid-binding protein (Meyer *et al.*, 1982). Therefore, in any experiment which involves thyroid hormones, age of the animal and altricial or precocial state of the species should be considered.

2.7.2. Thyroid hormone metabolism

The ontogenesis of T_3 metabolism in peripheral tissues has already been studied extensively in sheep (Wu *et al.*, 1986) and rat (Cheron *et al.*, 1980). In the rat a large proportion of circulating T_3 is generated by at least two tissue-specific pathways of T_4 monodeiodination (Silva *et al.*, 1982). While T_4-5′-monodeiodination has been demonstrated in most tissues, the liver and kidneys are thought to be the main sites of the enzyme activity in mature rats and rabbits (Brzezinska-Slebodzinska and Krysin, 1990). Moreover, brown adipose tissue in immature rats is one of the active sites in this respect. Approximately half of the T_3 bound to receptors in the pituitary gland is produced within the anterior pituitary gland by 5′-monodeiodination of T_4 in normal adult rats (Silva *et al.*, 1978). Intrapituitary 5′-monodeiodination is required for the inhibition of TSH secretion by the hypothalamus (Larsen *et al.*, 1979).

Based on *in vitro* assay systems, iodothyronine 5′-monodeiodinase activities have been tentatively classified as type I or type II. Type I activity catalyzes the 5′-deiodination of 3,3′,5′-tri-iodothyronine, (reverse T_3 or r-T_3) more efficiently than that of T_4. Type II 5′-deiodinase, which is present in the brain, showed preference for T_4 over r-T_3 as a substrate and was insensitive to 6-propyl 2-thiouracil (PTU) inhibition both *in vivo* and *in vitro* (Silva *et al.*, 1987). In the liver and lung, type I 5′-deiodinase mostly predominates (Ferreiro *et al.*, 1988). PTU caused almost complete suppression of hepatic and pulmonary 5′-deiodinase activity (type I), but had no effect on the fractional conversion of

T_4 to T_3 in the brain, indicating that T_3 production in the central nervous system occurs through a PTU-insensitive mechanism (Silva *et al.*, 1987).

In the newborn lamb, total T_4 utilization rate on days 3 and 4 was greater than days 10 and 11. Because of the 'hyperthyroid' state which existed in the developing lamb, rectal temperature was above the adult values (i.e. above 39.3) (Symonds *et al.*, 1989). After about 3 weeks of age, due to a gradual improvement in thermal efficiency, the plasma concentration of T_3 reduces (Symonds *et al.*, 1989, Glade and Reimers, 1985). There is a gradual fall in the metabolic rate over the first 8 weeks of life in lambs maintained at a thermoneutral ambient temperature (Nathanielsz, 1970).

In summary, T_4 is produced exclusively in the thyroid gland. However, T_3 can be produced in the thyroid gland, in the central nervous system and in peripheral tissues.

2.7.3. Thyroid hormone receptors and their mode of regulation

Thyroid hormone receptor is a protein present in the nucleus, cytosol (Weinberger *et al.*, 1986) and cell membrane (Segal, 1989). There is considerable evidence demonstrating the presence of specific binding sites for thyroid hormone at plasma membranes from rat liver, kidney, and thymocytes, rabbit adipocytes, cultured fibroblasts, and other cells (Segal, 1989). These receptors facilitate transport of a vast range of nutrients including water, ions, amino acids and glucose. The effects of thyroid hormones which occur at the plasma membrane are prompt and independent of new protein synthesis.

The nuclear receptor is a protein product of the proto-oncogene C-erbAα (Weinberger *et al.*, 1986). The appearance of receptors in the fetus occurs from day 70 to

110 of gestation in human (Bernal and Pekonen, 1984), day 50 to 80 in sheep (Ferreiro *et al.*, 1987) and day 13 of gestation in rats (Ferreiro *et al.*, 1988). T_3 enters a target cell either by passive diffusion or by an undefined carrier-mediated process. Within the cytoplasm T_3 interacts with a proteinacious cytosol receptor. The actions of T_3 may not be restricted to a genomic locus; rather, several pathways contribute to an integrated cellular response of this hormone (Sterling, 1989). The actions of thyroid hormones on the plasmalemma and mitochondria are rapidly initiated events which control increased heart rate (in a synergistic action with catecholamines), oxygen consumption and ATP production. On the other hand, interaction of T_3 with chromatin results in stimulation of protein synthesis which is not as rapid as the plasmalemmal effects.

The interdependence of hormones and their receptors, resulting in the alteration of receptor numbers after exposure to the homologous hormone, has been well documented. The regulatory effect of thyroid hormone on its own receptor is multifaceted (up-regulation, down-regulation, no change), controversial, and appears to be tissue-specific and age-dependent (Palmero *et al.*, 1992). Hamada & Yoshimasa (1983) reported that the number of T_3 receptor sites was increased in brains of T_3 treated rats (up-regulation), while Palmero *et al.*, (1991) found that increase in the serum thyroid hormones which occur naturally in neonatal rats caused a decline in the number of newly formed thyroid receptors (down-regulation). Both up and down regulation may occur in the same tissue (i.e. pituitary) but, at different ages (Coulombe *et al.*, 1983, Franklyn *et al.*, 1985).

In contrast to a lack of effect in the adult testes, thyroid hormones appear to exert more influence on the developing rat testes. Thyroid deficiency during development affects

testis growth and maturation deleteriously (Meisami *et al.*, 1994). There is also evidence for the presence of T_3 binding sites in human granulosa cells which are the Sertoli cell counterparts in the ovarian follicles (Wakim *et al.*, 1993). Palmero *et al.*, (1988, 1991) demonstrated the existence of a nuclear receptor for T_3 in rat Sertoli cells which is expressed maximally during late fetal life and the first 2 weeks of post-natal life. These receptors decrease progressively until puberty and virtually disappear in the adult (Jannini *et al.*, 1990). In immature rats, Sertoli cells are the only cell type in the seminiferous tubules that contain nuclear receptors for T_3 (van Haaster *et al.*, 1992). Moreover, these receptors have been determined both by binding assays and analysis of mRNA levels for the C-erbAα gene encoding the thyroid hormone receptor in the rats (Bunick *et al.*, 1994) and pigs (Palmero *et al.*, 1993). The number of nuclear T_3 receptors in Sertoli cells from immature porcine testes is modulated by thyroid hormone (Palmero *et al.*, 1991). Postnatal hypothyroidism was associated with a significant increase in the number of T_3 receptors in Sertoli cells. Moreover, replacement therapy with T_3 re-established the number of receptors, which was consistent with *in vitro* down regulation of nuclear T_3 receptors by thyroid hormone in cultured porcine Sertoli cells.

The ratio between the number of Sertoli cells and the total testicular tissue is higher in immature than in the mature rat, owing to the relative increase in germ cells which correlates with the postnatal decline of nuclear T_3 receptors in the testis (Palmero *et al.*, 1994). The absence of nuclear T_3-specific binding in the interstitial cell fraction and its presence in cultured porcine Sertoli cells demonstrate that the Sertoli cell is the T_3 target in the testis . Conclusively, thyroid receptors in the testis are mostly located in the Sertoli

cells and their presence is higher during neonatal life and declines thereafter (Cooke *et al.*, 1994 b). Therefore, changes in the thyroid state of the animal during the perinatal period influence testicular parameters which are linked to the functional Sertoli cells.

2.7.4. Role of thyroid hormones in Sertoli cells

Thyroid hormones play a key role in Sertoli cell proliferation during early postnatal development (Bunick *et al.*, 1994). At this stage, thyroid hormones normally act directly on Sertoli cells to inhibit mitogenesis but stimulate differentiation (Cooke *et al.*, 1994 a). However, in neonatal hypothyroid rats Sertoli cell proliferation continues until approximately day 35 (van Haaster *et al.*, 1992, Cooke *et al.*, 1992) while proliferation is minimal by 20 days of age in euthyroid rats. Nevertheless, alterations in neonatal thyroid hormone status produce numerous metabolic and endocrine changes that could also possibly affect Sertoli cell development (Kirby *et al.*, 1992, van Haaster *et al.*, 1993). Cooke *et al.* (1994 b) indicated that T_3 directly inhibited mitogenesis of cultured neonatal rat Sertoli cells and directly stimulated clusterin and inhibin-β_b mRNA production even in the absence of FSH. Moreover, T_3 potentiated the actions of FSH on inhibin-β_b mRNA production. The above results indicate that the increased Sertoli cell mitogenesis and retarded differentiation that occur following neonatal hypothyroidism, and the converse effects produced by neonatal hyperthyroidism, are the outcome of direct effects of thyroid hormones on the Sertoli cells.

The effect of thyroid hormone on the availability of glucose in the rat Sertoli cells gives further evidence for a direct effect of iodothyronines in the modulation of Sertoli cell

functions (Ulisse *et al.*, 1992). In the seminiferous micro-environment the mobilization of energetic resources must be strictly controlled since the activity and survival of germ cells depend on the supply of lactate produced from glucose oxidation by the Sertoli cells.

One of the major functions of Sertoli cells is conversion of testosterone to its metabolites. In the rat, by 3 and 4 weeks of age, 5α-reductase of the Sertoli cells converts testosterone into its metabolites such as dihydro-testosterone (DHT), 3α-diol and androsterone. However, in 3-week-old hypothyroid animals there was a reduction in DHT and 3α-diol and an increased formation of the two other 5α-reduced metabolites, androstenedione and androsterone. Both of these steroids have poor androgenic activity (Panno *et al.*, 1994). This means that TNH may postpone puberty by decreasing net hormonal efficacy of androgens. In addition to other physiological effects, FSH simulates aromatization of androgens by neonatal Sertoli cells, but Sertoli cells from older rats do not respond to FSH in this manner, reflecting a developmental change in responsiveness (Cooke *et al.*, 1994 a). Since the period in which Sertoli cells multiply, corresponds with the ability of these cells to produce E_2 (Panno *et al.*, 1994), it is possible to speculate that the enhanced and prolonged aromatase activity observed in hypothyroid rats may be associated with increased and sustained mitogenic activity of Sertoli cells.

There are many functions of the neonatal Sertoli cells which are attributed to thyroid hormones. Major functions are Sertoli cell proliferation, Sertoli cell differentiation and the related events. Making any changes in the thyroid status at this period could be a potentially powerful tool to change gonadal function thereafter.

2.7.5. Thyroid hormones and testicular metabolism

Due to the low level of nuclear thyroid hormone receptors in the adult testes and to the lack of increase in oxygen consumption and low level of activity in mitochondrial α-glycerophosphate dehydrogenase following thyroid hormone treatment, the testis was considered for many years a tissue unresponsive to thyroid hormone (Oppenheimer *et al.*, 1974). Later, other researchers found that Sertoli cells are reactive to thyroid hormones (Palmero, 1988, 1989; Ulisse, 1992). The thyroid hormone-induced increase in the oxygen consumption of the neonatal rat testis takes place mainly at the Sertoli cell level (Palmero *et al.*, 1994). Similarly, cytochrome oxidase (COX) activity and mitochondrial protein content were significantly lower in hypothyroid rats than in normal controls (Lanni *et al.*, 1992). *In vivo* studies showed that the intracellular ATP content of Sertoli cells from hypothyroid rats were significantly decreased with respect to the control (Palmero *et al.*, 1994). On the other hand, replacement therapy with T_3 resulted in a significant increase in the ATP level, even if control values were not achieved. These findings highlight the direct actions of thyroid hormones on the Sertoli cells. Conclusively, in the rats, the effects of thyroid hormones on the male gonads are mainly restricted to the neonatal period.

Thyroid hormones influence the function of most organs either through a nuclear effect by regulating gene expression and (or) through an extranuclear action at the level of the plasma membrane, by regulating the transport of nutrients into the cells (Oppenheimer 1985) or through cytosolic organelles like mitochondria (Ferreiro *et al.*, 1988). Studies with cultured rat Sertoli cells show that stimulation of glucose transport by T_3 requires *de-novo* protein synthesis. On the other hand there are many reports that thyroid hormones

act at the level of the plasma membrane to increase cellular sugar uptake (Segal 1989). These effects are extranuclear in nature, are produced by physiological doses, are thyroid hormone specific, and therefore are of physiological importance. The resulting, thyroid hormone enhanced, increase in intracellular glucose would provide additional substrate for glycolysis (Hall and Mita, 1984). However, FSH represents the main physiological regulator of glucose transport and lactate production in Sertoli cells, acting through a fast mechanism (cAMP production) that does not require protein synthesis (Hall and Mita 1984). In many mammalian cells, active transport of glucose is a major factor in the regulation of glycolysis so that the response explained above is likely to account, at least in part, for the increased synthesis of lactate stimulated by FSH. It may be concluded that FSH regulates glucose transport and metabolism by Sertoli cells through a fast mechanism without protein production, while thyroid hormone involvement in that process requires nuclear response with *de novo* protein production as well as a plasmalemmal response without new protein synthesis. Practically, thyroid hormone is acting as an amplifier for FSH in glucose transport and glycolysis in Sertoli cells.

2.7.6. Thyroid and GH-IGF-I axis

Thyroid hormone has been shown to rapidly stimulate the rate of rat growth hormone gene transcription in the pituitary gland which parallels the kinetics of binding of L-T_3. This suggests that the L-T_3-receptor complex interacts with DNA sequences in the environs of the growth hormone gene to elicit direct control of transcription (Casanova *et al.*, 1985). In the rats, hypothyroidism induced by feeding PTU, caused a reduction in GH

mRNA level in the pituitary gland (Samuels *et al.*, 1989) and hyperthyroidism induced by a single dose of T_3 or T_4 at physiological levels (i.e. 100 $\mu g\ kg^{-1}$ BW) has maximum stimulatory effects on GH gene transcription. *In vitro* studies using various lines of GH producing cells from rat pituitary gland and different levels of thyroid hormones supports the *in vivo* results (Martinoli and Pelletier, 1989).

Growth hormone stimulates IGF-I production in the liver and other tissues (McGuire *et al.*, 1992). While Sertoli cells were able to synthesize and secrete IGF-I, production of the somatomedin by these cells was significantly affected by the thyroid status of the rat (Smith *et al.*, 1987). The IGF-I may act at the testicular level as an autocrine and (or) paracrine differentiating factor (Borland *et al.*, 1984). As well Palmero *et al.* (1990, 1991) reported that TNH resulted in a severe impairment of IGF-I production by cultured Sertoli cells. Additionally, T_3 induced *de novo* synthesis of the IGF-I by Sertoli cells.

It may be concluded that thyroid hormones affect GH and IGF-I secretion directly and (or) indirectly. While GH stimulates production of IGF-I by the hepatic cells and other somatic cells, thyroid hormones *per se* may affect local testicular production of IGF-I. Therefore, any attempt to manipulate thyroid hormones may change the GH and IGF-I concentrations locally or generally in the body.

2.8. Transient neonatal hypothyroidism (TNH)

2.8.1. TNH; a model to increase testicular size and function

Hypothyroidism can be induced by adding different kinds of goitrogens to the diet or drinking water. The reversible goitrogen PTU is an inhibitor of type I, 5′-monodeiodinase enzyme (Ferreiro *et al.*, 1988) and is a more potent thyroid blocker than methimazole (MMI), another reversible goitrogen. PTU prevents both thyroid hormone synthesis in the thyroid gland and the peripheral deiodination of T_4 to T_3 by reducing 5′-deiodinase enzyme activity, while MMI only blocks thyroid hormone synthesis in the thyroid gland (Kirby *et al.*, 1992). PTU can pass freely through the placenta (Marchant *et al.*, 1977) of pregnant animals and mammary glands (Cooke *et al.*, 1991) of suckling mothers to affect the neonates. In PTU treated rats, serum concentration of T_4 decreased throughout the treatment period T_3 concentration was suppressed to a lesser degree (Kirby *et al.*, 1992). Transient neonatal hypothyroidism in rats (Cooke and Meissami, 1991, Cooke *et al.*, 1991) and mice (Joyce *et al.*, 1993) pups, induced by adding PTU at the rate of 0.1 % to the mother's water from birth until Day 25, resulted in increased adult testis size and sperm production in the rats by 80 % and 140 %, respectively, and in the mice by 30 % and 50 %, respectively. Kirby *et al.*, (1993) induced hypothyroidism in hamster pups by adding 0.1 % PTU in the drinking water supplemented with 0.4 % PTU in the feed of their mother from the day of birth until day 35 (time of weaning). At 150 days of age, the animals showed a 30% increase in testis size and a 73% increase in DSP. However serum FSH and LH levels were reduced significantly.

TNH during the suckling period significantly increased subsequent sperm production in adult rats by 140 % at 160 days of age. Therefore, it appears that not only can the deleterious effects caused by early hypothyroidism be reversed, but if neonatal thyroid deprivation is followed by recovery, it may enhance sperm production in the adult (Cooke *et al.*, 1991). Cooke *et al.*, (1992) hypothesized that an increase in the number of Sertoli cells could be the critical aspect of the PTU effect for the enlargement of the testis in the adult rat. Sertoli cells are the major determinant of the magnitude of sperm production and in rats, these cells are mitogenically active during early postnatal life (Orth *et al.*, 1988). It has been demonstrated that neonatal Sertoli cell proliferation was extended, up to 15 days, in PTU-treated rats (van Haaster *et al.*, 1992) and mice (Joyce *et al.*, 1993), leading to large increases in their adult numbers (Hess *et al.*, 1993). The influence of neonatal hypothyroidism in poultry or large farm animals is unknown (Meisami *et al.*, 1992).

The critical developmental period during which PTU treatment can increase adult testis size and sperm production in rats (Meisami *et al.*, 1992, Cooke *et al.*, 1992), and mice (Joyce *et al.*, 1993), has been determined to be from 4-24 days of age, but, this time window is not known in larger mammals. Since hypothyroidism in the rat must be induced during early postnatal life to increase Sertoli cell proliferation (Meisami *et al.*, 1992, Cooke *et al.*, 1992), it means that thyroid hormones inhibit neonatal Sertoli cell proliferation. The stimulatory effects of TNH on the proliferation of Sertoli, germ, Leydig and other testicular cells was not evident immediately after recovery from PTU treatment (after 24 days of age). However, these effects do manifest later in adulthood (Cooke *et*

al., 1994 b). In practice, immediately after cessation of PTU treatment, the testicles looked immature and the seminiferous tubules were retarded, i.e., lumen formation was delayed or absent, and Sertoli cells were unable to support advanced stages of spermatogenesis (Cooke *et al.*, 1994 a). However, the increased Sertoli cell population was then believed to be responsible for secondary changes such as increased germ cell number, testis weight, and sperm production that characterized adult rats treated neonatally with goitrogens (Cooke *et al.*, 1994 a, Hess *et al.*, 1993).

Formation of the tubular lumen is thought to reflect the secretion of the fluid by the differentiated Sertoli cells (Russell *et al.*, 1989). Moreover, a relationship exists between the temporal appearance of testicular fluid flow and the establishment of the blood-testis barrier between Sertoli cells; both of these phenomena are in fact well developed in the euthyroid rat by the end of the third week of life. Therefore, the lack of a patent lumen in the seminiferous tubules of 21-day-old hypothyroid rats was evidence of delayed maturation of Sertoli cells (Francavilla *et al.*, 1991).

Hypothyroidism decreased binding of T_3 in 21 day rat testis (Bunick *et al.*, 1994). Therefore, expression of several Sertoli cell mRNA genes like ABP, clusterin, inhibin-β_b and hemiferrin was delayed in hypothyroidism. The other gene showing prolonged expression in early hypothyroid rats is C-erbAα, which encodes a member of the thyroid hormone receptor gene family (Bunick *et al.*, 1994).

TNH has been shown to be a reliable method to improve our understanding of the relationship between thyroid function and testicular development and change, as well as

testicular size and function in some rodent species. The thyroid-gonadal development relationship and the reliability of this method has not been reported for other species.

2.8.2. TNH; testicular development and histology

Sertoli cells are believed to mediate the developmental effects of thyroid hormones on the testes (Palmero *et al.*, 1994). It was shown that increases in the testicle size in PTU treated rats was accompanied by increases in the DNA content of the tissues (Meisami *et al.*, 1994, Cooke and Meisami, 1991), meaning that the increase in weight was mainly the result of increase in the number of the Sertoli cells. However, testicular changes could, perhaps, be due to retarded differentiation of the Sertoli cells, inducing a marked delay in spermatogenesis. Indeed, the Sertoli cells of 3-week-old hypothyroid rats were less differentiated than those from untreated animals; their cytoplasm failing to show lipid deposits which are markers of pubertal maturation (Russell *et al.*, 1989). Hess *et al.* (1993) demonstrated that increased testicular size in adult rats recovering from transient early hypothyroidism resulted from proportional increases in interstitial and tubular volumes.

Withdrawal of PTU, after transient early hypothyroidism in the rat, caused a dramatic stimulation of spermatogenesis and primary spermatocyte formation in such a way that, by 90 days of age, the total number of seminiferous tubules were 54 % higher than in normal controls (Meisami *et al.*, 1994). In the hypothyroid rats, at 25 days, the testes were highly undifferentiated, but, by 90 days of age the germinal epithelium appeared thickened, showing numerous germ cells at various stages of differentiation.

Many tubules contained sperm in their lumen, even more frequently than the age-matched control testes (Meisami *et al.*, 1994). Hess *et al.*, (1993) showed that in adult rats, Sertoli cell numbers were increased 157 % in PTU treated rats compared to those in controls. Despite great enlargement, no pathological alterations were observed in the testes of the treated rats. However, clear morphological changes in the seminiferous tubules were demonstrated, which account for the increased testicular size and sperm production induced by the neonatal goitrogen treatment.

In the transient neonatal hypothyroid rats, the histological profile of the testis shows hyperplasia of the cellular components of seminiferous tubules without pathological changes. However, increases in the number of the Sertoli cells is the major criteria which changes adult testicular size and function.

Neonatal hypothyroidism leads to an increase in the number of Leydig cells in the adult rat, but reduced the ability of these cells to produce testosterone. Because, the increase in the number of Leydig cells in adulthood, was accompanied by a profound continuous suppression of FSH and LH during both the treatment period and subsequent adult life (Kirby *et al.*, 1992), the increased Leydig cell number did not result from an elevation in serum gonadotropins (Hardy *et al.*, 1993). The increase in the number of the Leydig cells was presumably a direct (Hardy *et al.*, 1996) and (or) an indirect (Palmero *et al.*, 1992) effect of thyroid hormones.

Other factors that promote differentiation or proliferation of Leydig cells such as; interleukin-1β, steroid-inducing proteins, transforming growth factor-α, and IGF-I should be considered (Hardy *et al.*, 1993). It is also possible that the observed effects on Leydig

cells were mediated locally by the Sertoli cells. Thyroid hormones stimulated IGF-I production by Sertoli cells (Palmero *et al.*, 1990, 1991), which increases DNA synthesis in Leydig cells (Bernier *et al.*, 1986, Hardy *et al.*, 1993). Since Sertoli cells are affecting other cells in the testis, it seems that Sertoli cells are the primary target of PTU treatment and that Leydig cells are affected secondarily. Despite the increase in Leydig cell number after PTU treatment, the capacity of these cells to produce testosterone was markedly reduced; this decrease might be due in part to the decreased number of LH receptors per Leydig cell and a reduction in the cell volume (Hardy *et al.*, 1993). Low testosterone production in hypothyroid rats might be due to lower LH and FSH secretion and the decrease in the steroidogenic potential of the Leydig cells was probably resulting from decreased activity of one or more steroidogenic enzymes involved in testosterone production (Cooke *et al.*, 1994 a). It seems that although TNH had no pathological effects on seminiferous tubules, it resulted in lowered testosterone production by Leydig cells in spite of a significant increase in their number. No deleterious effects of TNH on the accessory sex organs have been reported.

In addition to the increase in the growth and ultimate size of the testis, TNH also caused enlargement of the epididymis, seminal vesicles, and ventral prostate. The occurrence of organomegaly seen in animals recovering from early hypothyroidism appeared to be confined to the reproductive system as it was not seen in non-reproductive organs such as the brain, liver and kidney (Cooke and Meisami, 1991).

2.8.3. TNH and Sertoli cell secretions

The production of certain proteins such as IGF-I has been shown to be reduced in Sertoli cells from hypothyroid rats (Palmero *et al.*, 1990). IGF-I is involved in the thyroid hormone dependent differentiation of testicular functions (Palmero *et al.*, 1990). Therefore, Sertoli cells from hypothyroid rats have low levels of enzymes and protein content and low ABP production (Palmero *et al.*, 1989). Evidently, the receptors for thyroid hormones are generated in the dividing Sertoli cells, then, thyroid hormones act on these receptors to stimulate production of other proteins.

The production of the mRNA for clusterin and inhibin-β_b and the circulating levels of immunoreactive inhibin-α, γ-glutamyl transpeptidase (GGT), ABP and lactate were decreased in neonatal hypothyroid rats (Bunick *et al.*, 1994, Kirby *et al.*, 1992). As a result, these proteins may be used as *in vivo* markers of differentiation in hypo- and hyperthyroidism because they are affected by the thyroid status of the animal (Cooke *et al.*, 1994 b). However, daily administration of T_3 for 1 week to 21 day old euthyroid rats did not affect body and testis growth nor the level of Sertoli cell specific biochemical markers, such as GGT and lactate dehydrogenase (LDH) activities or lactate secretion (Palmero *et al.*, 1989).

Stimulatory (Palmero *et al.*, 1989, Panno *et al.*, 1994) and (or) inhibitory (Fugassa *et al.*, 1987) effects of T_3 on the production of ABP and its mRNA in cultured Sertoli cells from young rats have been reported. This apparent contradiction may be the result of the dual mode of Sertoli cell function at different ages and stages of the seminiferous cycle (Griswold, 1995).

2.8.4. TNH and gonadotropins

While previously described models for increasing testicular size and sperm production were associated with increasing FSH levels, neonatal goitrogen treatment was shown to decrease circulating gonadotropin levels in rodents (Kirby *et al.*, 1992). FSH stimulates differentiation of Sertoli cells therefore, the reduced FSH levels after use of PTU could result in retarded Sertoli cell differentiation. At the beginning, some researchers (Valle *et al.*, 1985), were convinced that alterations of the hypothalamic-pituitary-testicular axis at an immature age was the main effect of hypothyroidism resulting in changes in FSH levels. Francavilla *et al.*(1991) showed that rats which were hypothyroid from early post-natal life until 25 days of age, or later (i.e. 35-50 days after birth), had delayed maturation of seminiferous tubules and lower serum levels of gonadotropins during puberty compared to control rats. Therefore, it was concluded that a low level of FSH was the reason for delayed pubertal maturation of seminiferous tubules. Moreover, this was supported by the finding that thyroid hormones synergized *in vitro* with FSH to exert direct stimulatory effects on the functional differentiation of porcine granulosa cells which are the female counterparts of the Sertoli cells (Maruo *et al.*, 1987). Rats which remained hypothyroid for a longer time (i.e. 35-50 days after birth), showed lower serum levels of gonadotropins during puberty compared with control rats (Francavilla *et al.*, 1991).

High levels of T_3 early in the life decreased serum FSH levels as well (van Haaster *et al.*, 1993). Since FSH levels are reduced in both hypo- and hyperthyroid states, it seems unlikely that the opposite effects of high and low T_3 levels on the terminal differentiation of Sertoli cells are mediated by FSH. Furthermore, Kirby *et al.* (1992) observed that both

FSH and LH were suppressed permanently in neonatal hypothyroid rats. Therefore, they concluded that enlargement of rat testes is not mediated by either transient or permanent increases in serum FSH levels. Apparently, FSH is capable of modulating the rate of Sertoli cell proliferation without interfering in the terminal differentiation process (Haisenleder *et al.*, 1989).

The timing of goitrogen treatment that lead to increased adult testicular size coincides with the period of prepubertal changes in gonadotropin secretion (Dohler, 1975, Ackland, 1991). The prepubertal period starts with initiation of hypothalamic discharge of GnRH and anterior pituitary gonadotropins (Amann, 1983). Evidently, TNH affects gonadotropin secretion from the anterior pituitary in the male animal which, in turn is followed by alterations in the production and release of testosterone, inhibin and activin. Since these are the main hormones controlling the function of the gonads, it is useful to follow the changes in the levels of these hormones after use of PTU (Cooke *et al.*, 1994 b).

Apart from changes in the concentration of the gonadotropins, neonatal hypo- or hyperthyroidism also caused other metabolic and hormonal effects, including changes in the concentrations of TSH, IGF-I, inhibin, GH, and prolactin (PRL; van Haaster *et al.*, 1992, 1993, Kirby *et al.*, 1992).

The reason for the reduction in FSH levels was not understood and is still unclear. Moreover, it was not known whether the low level of FSH was the result of changes in the level of inhibin or direct effect of T_3 on the higher brain centers. Van Haaster *et al.* (1992) found that serum inhibin levels were also reduced up to day 30 in hypothyroid rats and

concluded that FSH levels were reduced as a result of a direct effect of hypothyroidism on pituitary development and not because of serum inhibin concentration. Likewise, Kirby *et al.* (1992) demonstrated that a decrease in the serum level of inhibin was temporary and happened only during PTU treatment inhibin concentration returned back to normal after termination of treatment. Therefore, reduction in the inhibin production by Sertoli cells was due to low level of T_3 and delayed maturation of these cells, accordingly, one may expect high levels of inhibin production in Sertoli cells of hyperthyroid animals. This hypothesis is supported by the work of van Haaster (1993).

As expected, hypothyroidism caused a sharp increase in the level of TSH (Kirby *et al.*, 1992). TSH, a heterodimeric peptide hormone made up of a common α-subunit, shared with FSH and LH, and a unique β-subunit, has previously been shown to mimic some of the effects of FSH on rat Sertoli cells *in vitro* (Hutson, 1981). Thus it was hypothesized that TSH may stimulate the proliferation of Sertoli cells during the period of neonatal hypothyroidism (Kirby *et al.*, 1992). However, in neonatally T_3-treated rats, TSH levels were decreased, and Sertoli cell differentiation was accelerated while the proliferation period was shortened (van Haaster *et al.*, 1992). These findings made it unlikely that TSH was an important regulator of Sertoli cell differentiation or stimulator of proliferation.

The mechanism of suppression of FSH secretion is not well understood but, thyroid hormone control over pituitary GH producing cells are well known. The probable mechanism involves the L-T_3-receptor complex interaction with DNA sequences in the vicinity of the gonadotropin hormone gene in the pituitary to bring direct control of

transcription. This mechanism has been defined for the control of thyroid hormone over pituitary GH producing cells both *in vitro* (Casanova *et al.*, 1985) and *in vivo* (Martinoli and Plletier, 1989). Cooke *et al.* (1995) stated that reduction in the circulating gonadotropins appears to be due to either a reduced responsiveness to GnRH stimulation or an overall decrease in the synthetic capacity of gonadotropes in the pituitaries of treated males.

2.8.5. TNH and testosterone concentration

While immunization against E_2 resulted in increased testis size and a 10- fold increase in serum testosterone (Monet-Kuntz *et al.*, 1988), Cooke and Meisami (1991), could not find any significant changes in the serum testosterone concentration between neonatally hypothyroid and control rats at all ages examined. However, the testis and accessory sex organs were larger in the adult TNH rats. Therefore, it was concluded that increased testis growth can occur in the absence of supranormal testosterone concentrations.

Hepatic cytochrome P-450 (P-450) enzymes are sex specific (Waxman *et al.*, 1989), are involved in steroid metabolism and their production in the hepatic cells is reduced at puberty. In males, these enzymes convert testosterone into various metabolites to inactivate them. For example, $P\text{-}450_{6\beta\text{-}1\text{-}4}$ catalyses microsomal testosterone 6β-hydroxylation and $P\text{-}450_{7\alpha}$ is a microsomal testosterone 7α-hydroxylase. Age dependent production of these enzymes is regulated by GH, steroids and thyroid hormones. GH increases production of P-450 enzymes by hepatic cells while thyroid hormones suppress their production (Yamazoe *et al.*, 1990).

In conclusion, TNH reduces pituitary GH production which causes a decrease in the production of P-450 enzymes, while a decrease in the level of thyroid hormones has a stimulatory effect on the production of P-450 enzymes in the liver. Hence, the net effect is no change in serum testosterone concentration in PTU treated rats.

2.9. Transient neonatal hyperthyroidism

Transient neonatal hyperthyroidism (TNHI) decreased proliferation rate, but, enhanced differentiation of Sertoli cells. Palmero *et al.* (1992) found that excess T_3 inhibited DNA synthesis and presumably proliferation in cultured Sertoli cells obtained from prepubertal rats. Moreover, van Haaster *et al.* (1993) demonstrated that high T_3 levels in immature rats reduced the postnatal period of Sertoli cell proliferation, resulting in a 50% reduction in the number of Sertoli cells formed and a 48% reduction in adult testis weight. Furthermore, T_3 treatment accelerated the formation of a tubular lumen. Stimulatory effects of thyroid hormone on the maturation of porcine granulosa cells which are the female counterparts of the Sertoli cells provides another reason for this claim (Maruo *et al.*, 1987). Hence, neonatal T_3 levels are important for determination of the final number of Sertoli cells and, consequently, adult testis size.

Cooke *et al.* (1994 b) stated that the early suppression of Sertoli cell proliferation, the reduced adult number of these cells, and the enhanced onset of Sertoli cell secretory activity in hyperthyroid young rats were all due to direct actions of increased thyroid hormones on the Sertoli cells.

So far from the literature it can be concluded that at least in the rats, mice and hamsters; during the perinatal period, thyroid hormones are one of the major hormones determining the pubertal testicular function and size. This effect is manifested by changing the proliferation and maturation time period for Sertoli cells. Moreover, these changes are not accompanied by an increase in the concentration of gonadotropins and (or) testosterone, but are mainly due to changes in the thyroid state of the animals.

2.10. GH-IGFs axis, body growth and development

2.10.1. GH and somatomedins

GH secretion in the ruminant is pulsatile in nature and nutritional factors have a major impact on GH secretion (Breier *et al.*, 1986). The original somatomedin hypothesis ascribed an endocrine action of insulin like growth factors (IGFs). In this scenario, GH has the dominant endocrine influence on plasma concentrations of the IGFs which are produced in the liver. IGF-I and -II, low-molecular-weight peptides of 70 and 67 amino acids, respectively, are structurally similar to pro-insulin. Both peptides are produced by, and have mitogenic action on, a large variety of tissues and cell types. Concentrations of somatomedins in the peripheral circulation of humans are relatively stable with no obvious diurnal rhythm due to the long biological half-life (Hall and Sara, 1984). However, in ruminants, circulating concentrations of somatomedins do vary according to physiological state and are responsive to factors such as endocrine and nutritional status (McGuire *et al.*, 1992).

Sertoli cells possess receptors showing high affinity for IGF-I and lower affinity for insulin and IGF-II (Borland *et al.*, 1984, Mita *et al.*, 1985). Genetic as well as nongenetic factors may affect somatomedin concentrations. Genetic factors include, breed and (or) line and sex e.g., male lambs have IGF-I concentrations 1.5 to 2 times higher than females (Medrano and Bradford, 1991). A number of non-genetic factors such as age and gonadal steroid production also contribute to variation in plasma IGF-I concentrations. Clark *et al.* (1994) demonstrated that IGF-I is a plausible regulator of Leydig cell function and steroidogenesis in the testis. Furthermore, there is evidence that the rise in plasma IGF-I concentrations at puberty may be related to a rise in circulating gonadal steroids, as plasma IGF-I levels were increased by treatment with physiological doses of steroids (Breier *et al.*, 1988, Jasper, 1985).

2.10.2. IGF binding proteins

The insulin-like growth factors (IGF-I and IGF-II) circulate in plasma in association with IGF-binding proteins (IGFBPs)(Butler and Gluckman, 1986). Functions of IGFBPs are postulated to be several: circulatory transport vehicles; retardation of somatomedin degradation; transvascular IGF movement and direct modulation of the actions of somatomedins at the target cell either by enhancing or blocking the activity of somatomedins (McGuire *et al.*, 1992).

Many different size binding proteins are present in ovine serum or plasma. In the adult sheep, the circulating form of IGFBP is not identical to that of the fetus and is of smaller size (Butler and Gluckman, 1986). The liver is the main site of binding protein

production. Human and rat liver are capable of synthesizing IGFBPs with the same molecular weight but with different affinities for IGF-I and IGF-II. It is interesting that the BP preparation from fetal human liver also had a preferential affinity for IGF-I, whereas that produced from adult human liver had a stronger affinity for IGF-II (Binoux *et al.*, 1982).

2.10.3. IGFs in fetal and perinatal life

According to Young Lee *et al.* (1991), in rats, IGF-I and IGF-II are partially GH-dependent and nonhepatic production of these factors is high prenatally. Hepatic production increases postnatally suggesting that the endocrine mode of IGF-I action has increased significantly after birth. Serum IGF-I concentrations were highly correlated with hepatic GH receptor binding in rats and domestic animals (Young Lee *et al.*, 1991). Thus it is likely that GH affects IGF-I production beginning in late fetal life and perhaps coincident with GH receptor ontogeny (Young Lee *et al.*, 1991). However, subsequent work showed that in addition to the liver, many tissues contain the message for IGF-I, although levels were much lower than those found in the liver (Murphy *et al.*, 1987, Young Lee *et al.*, 1991). Therefore, it was postulated that in addition to an endocrine action, IGF-I must have the ability to act in an autocrine and (or) paracrine fashion (McGuire *et al.*, 1992). In the dairy cow, exogenous GH also increased IGF-I concentrations, but only after several hours following its administration (Gluckman and Breier, 1987). This suggests that the effects of GH on IGF-I production in the cow was through stimulation of *de novo* synthesis rather than through release from a storage pool.

In sheep, IGF-I concentrations had a positive association with birth weight and length of the neonate (Gluckman *et al.*, 1983a). Similarly, a positive correlation between serum somatomedin-like activity and relative weight gain in lambs has been reported (Olsen *et al.*, 1981). Furthermore, variation in plasma concentration of IGF-I is commonly associated with variation in growth rate or mature body size (Roberts *et al.*, 1990). Nevertheless, measurement of plasma IGF-I is not likely to be a very effective aid to selection for growth rate in these species (Medrano and Bradford, 1991). The reasons may be the great variation due to effects of sex, age, state of nutrition and other physiological conditions in the level of the somatomedin.

IGF-I and -II concentrations are different during prenatal and neonatal life. During fetal development, IGF-II is a major growth factor, whereas IGF-I is considered more important during postnatal growth (Clark *et al.*, 1994). It is of interest that somatomedin concentrations change most prominently during the perinatal period. Generally, in most species, IGF-I levels are lower during fetal life compared to post partum levels, suggesting that the endocrine mode of IGF-I action increases significantly after birth (Gluckman and Butler, 1983, Parker *et al.*, 1984). Mesiano *et al.*, (1989) found that, IGF-II levels were higher during fetal life and decreased immediately after birth in rats and sheep but, its concentration was lower than IGF-I at any time.

Gluckman and Butler (1983) showed that the concentration of IGF-I was lower in the youngest ovine fetuses, with a progressive rise in IGF-I concentration with advancing gestation. This prenatal level of somatomedin was still lower than the adult levels. In neonatal lambs, on the first 2 days after birth, concentration was similar to those in late

gestation. However, by 3-7 days after birth, IGF-I concentration increased and was maintained at a higher level until 4 weeks of age. By 40 days postpartum, the values of IGF-I fell to adult concentrations. This decrease may represent the effect of nutritional changes associated with weaning and the reliance on full rumen function (Gluckman and Breier, 1987).

There is ample evidence that GH does not play a significant role in influencing fetal growth (Gluckman *et al.*, 1983 b). This suggests that GH is not the dominant influence on fetal IGF secretion. Concentration of IGF-I in serum of newborn piglets continued to increase, and at 3 weeks of age exceeded those in maternal serum (Young Lee *et al.*, 1991). The transient nature of the postnatal rise in IGF-I concentration suggests, however, that other factors are also involved in the changing secretion of IGF-I after birth.

It can be summarized that GH and other hormones, (i.e. steroids and thyroid hormones), stimulate IGF-I production in the liver and other tissues. During fetal life IGF-II predominates while IGF-I is the major somatomedin in postnatal life.

2.10.4. GH, IGFs and steroids

There is no doubt that in man, IGF-I and gonadal steroid levels both rise dramatically at puberty (Parker *et al.*, 1984). In primates, physiological concentrations of gonadal steroids are thought to regulate GH and IGF-I secretion during development (Wilson, 1986, Copeland *et al.*, 1985). This is in agreement with the findings of Cuttler *et al.* (1985) that there was no significant elevation of IGF-I in adolescence patients with gonadal dysgenesis. Estrogen is the primary steroid involved in the regulation of IGF-I in females

(Copeland *et al.*, 1985, Simard *et al.*, 1986). Likewise, Wilson (1986) found that during puberty in female primates, GH may play a permissive role in IGF-I secretion but, absolute levels of IGF-I are regulated by E_2 and not by further elevations in GH. However, the mechanism by which E_2 increases serum IGF-I levels probably involves a peripheral stimulation of IGF-I secretion and not a mediation through increases in basal GH secretion (Wilson, 1986). Inhibitory or stimulatory effects of E_2 on plasma IGF-I was reported (Gluckman *et al.*, 1987) which was dose and species dependent. Therefore, E_2 therapy has a bi-phasic effect on immunoreactive IGF-I levels; at low doses it stimulates IGF-I generation and at high doses inhibits its production (Harris *et al.*, 1985). According to Wilson (1986) pubertal increases of IGF are a consequence of and not a prerequisite to sexual maturation and increased growth rates are not a determinant of the final stages of puberty, but are, rather, the result of sexual maturation.

During puberty, in bull calves (Renaville *et al.*, 1993), humans (Parker *et al.*, 1984, Harris *et al.*, 1985) and chimpanzees (Copeland *et al.*, 1985), there is a marked increase in the concentration of IGF-I along with a rise in the concentrations of gonadal and adrenal androgens. According to these findings, IGF-I rather than testosterone concentration in the serum can be used as an index for onset of puberty with the advantage of its relatively constant concentration during a 24 hr period (Renaville *et al.*, 1993).

If testosterone does play a role in the pubertal generation of IGF-I, it could exert its effect in one or more of four different ways: 1) It may have a direct effect on IGF-I production. 2) It may act by augmenting or potentiating the effects of GH on IGF-I

generation. 3) It may act synergistically with hormones other than GH. 4) It may increase IGF-I levels by causing increased pituitary GH secretion (Parker *et al.*, 1984).

There is evidence supporting the role of increased pituitary GH secretion as the cause of the phenomenon (Jasper, 1985). Testosterone stimulated IGF-I secretion in boys who's GH levels were normal and not in those who were GH deficient (Parker *et al.*, 1984). Likewise, in young human subjects, GH deficiency resulted in delayed puberty and GH was necessary for normal pubertal development while the onset of puberty was significantly enhanced with GH treatment (Darendeliler *et al.*, 1990). Moreover, no increase in IGF-I levels was evident in normal human adults given pharmacological doses of testosterone (Meyer *et al.*, 1982), This indicates that the response of somatotropes to testosterone and their mode of secretion changes after puberty.

Evidently GH is required for IGF-I production in the liver but, peripheral production of IGF-I at a pubertal age is mainly stimulated by gonadal steroids. However stimulation of IGF-I production by E_2 is dose dependent and permissive action of GH is required.

2.10.5. Testicular immunoreactive IGF

Gonads can be considered as GH target organs which can respond to GH via an intra-gonadal mediator peptide, IGF-I, (Davoren *et al.*, 1986). Production of an IGF-I like protein by cultured Sertoli and Leydig cells has been reported (Smith *et al.*, 1987). Furthermore, cultured rat Leydig (Lin *et al.*, 1986) and Sertoli (Smith *et al.*, 1987) cells possess IGF-I receptors, and those on Leydig cells are regulated by LH analogs (Borland

et al., 1984). The presence of both IGF-I and -II receptors in the testis of the fetal and immature pig provides more evidence for the specific role of IGF in the testis (Clark *et al.*, 1994).

Sertoli cells possess receptors showing high affinity for IGF-I and lower affinity for insulin and IGF-II (Mita *et al.*, 1985). Insulin stimulated the production of lactate by cultured Sertoli cells by an action exerted via high affinity receptors for IGF-I (Borland *et al.*, 1984). Also, in Sertoli cells, the native IGF-I receptor showed striking structural similarities to the insulin receptor but not for the IGF-II receptors (Massague and Czech, 1982). Moreover, responses to insulin observed with Sertoli cells was the result of binding of insulin to the receptors for IGF-I (Borland *et al.*, 1984). In other words, in Sertoli cells, it seems that insulin acts via IGF-I receptors. Since the Sertoli cells in Borland's experiment were obtained from immature rat testes, it is possible that they share with growing tissue the ability to respond to IGF-I. Also, this demonstrates that in the testis, similar to other tissues, during neonatal life IGF-I is the major somatomedin rather than IGF-II.

2.10.6. Diurnal variations or rhythms in IGF-I

Although largely determined by episodes of GH secretion, the concentration of IGF-I in blood is generally constant under circumstances of normal nutrition and hormonal status. Studies in many mammalian species have shown, despite distinctly episodic secretion of GH, IGF-I concentrations change little over 24 hr (Danaghue *et al.*, 1990). Breier *et al.*, (1986,1988) found that in the steers plasma IGF-I concentrations from the serial sampling

over 24 h had no diurnal variation but, serum IGF-I level was very sensitive to the level of nutrition.

2.11. CONCLUSION

The testes are the reproductive organs in male mammals with the functional units of the testes being the Sertoli, Leydig, and germ cells which have a close inter-relationship necessary for normal spermatogenesis. In the male, puberty can be defined as the time when spermatogenesis starts which is manifested by developmental processes at the hypothalamic-pituitary-gonadal axis with the discharge of gonadotropins and episodic release of testosterone. Changes in the male reproductive processes and cycles are dependent on pulse frequency, duration and amplitude of the above hormones. The endocrine regulation of spermatogenesis is the result of actions of sex hormones on Sertoli cells. Therefore one can speculate that by increasing the number of Sertoli cells in the testis, it would be possible to increase the size of the testes and sperm output. It has been demonstrated that transient neonatal hyper- and hypothyroidism is a reliable method to improve our understanding of the relationship between thyroid function and testicular development. So far from the literature it can be concluded that at least in the rats, mice and hamsters; during the perinatal period, thyroid hormones are one of the major hormones determining the pubertal testicular function and size. Thyroid hormones decelerate proliferation but accelerate differentiation of Sertoli cells. However, investigations thus far have been largely confined to some laboratory species. Moreover, these changes are not the results of an increase in the concentration of gonadotropins and (or) testosterone, but are mainly due to changes in the thyroid state of the animals.

3. Manuscript I

Relationship of Thyroid Hormones IGF-I and Testosterone in Ram Lambs With Low and High Prolificacies.

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ABSTRACT

Twelve Suffolk (Sfk) and twelve Outaouais (Out) ram lambs were selected to study the relationship between testosterone, thyroid hormones, IGF-I, T_3, T_4, glucose, semen production and testicular histology at the onset of puberty in low prolific (Sfk) and high prolific (Out) breeds. Scrotal circumference (SC), body weight (BW), average daily gain (ADG), relative growth (RG) and dry matter (DM) consumption were recorded weekly. Semen was collected by electro-ejaculation from 32 to 35 weeks of age and evaluated. Blood was collected weekly by indwelling jugular catheters, every 20 minutes from 0900 to 1520 h to measure the concentration of glucose, T_4, T_3, IGF-I and testosterone. At week 35, histological sections of testes were evaluated. Number of spermatozoa in the semen of Out was higher than Sfk ($P<0.01$). Average BW, ADG, RG, SC, DM consumption and blood glucose of the two breeds were not different throughout the experiment ($P>0.1$). However, there were negative correlations between levels of glucose, T_4, T_3 and age of the lambs. Testosterone concentrations and pulse frequencies were higher in Out than Sfk ($P<0.05$). There were positive correlations between testosterone concentration, testosterone pulse frequencies and age of the lambs in the two breeds. Moreover, level of T_4, T_3 and IGF-I were higher in Out than Sfk throughout the sampling period ($P<0.01$). Although there were fluctuations in the level of IGF-I in the blood, no pulses were detected. Higher levels of testosterone, T_4, T_3, IGF-I and testosterone pulses in Out may be associated with greater prolificacy in this breed.

3.1. INTRODUCTION

Puberty in the male mammal is manifested by developmental processes at the hypothalamic-pituitary-gonadal axis (Amann, 1983) with episodic release of testosterone (Renaville *et al.*, 1993). Testosterone may be considered as a primary regulator for other major physiological changes during this period. Steroids have direct effects on the adenohypophysis and a rise in the blood testosterone level is accompanied by a discharge of growth hormone (GH) (Wilson, 1986, Copeland *et al.*, 1985). In humans, peripubertal GH administration accelerates onset of puberty. Insulin-like growth factor-I (IGF-I) is the major mediator of the somatotropin action (McGuire *et al.*, 1992) and its blood concentration increases at the onset of puberty in a fashion similar to the increase in blood testosterone (Parker *et al.*, 1984). Moreover, gonadal steroids have the ability to increase IGF-I concentration irrespective of the effects of GH. In this respect, Cuttler *et al.* (1985) found that administration of testosterone stimulated IGF-I production in prepubertal boys. Therefore, IGF-I is affected by steroids directly and (or) indirectly through the effect on GH.

Spermatogenesis is highly dependent on the complex cellular and molecular interactions that take place in the testis between Sertoli and germ cells (Steinberger and Steinberger, 1971). However, the endocrine regulation of spermatogenesis is the result of hormone action on Sertoli cells and not germ cells (Griswold, 1995). In general, in higher vertebrates, spermatogenesis and germ cell maturation is dependent upon the function (Berndtson *et al.*, 1987) and developmental state of the Sertoli cells (Hess *et al.*, 1993). Therefore, Sertoli cell number and their state of maturation are the major limiting factors

for spermatogenesis. The main gonadotropin affecting Sertoli cells is FSH (Orth and Christensen, 1978). Although FSH is required for the onset of spermatogenesis, in the adult animal much of the role of FSH is transferred to testosterone. There is no evidence reported for the presence of androgen receptors in germ cells (Griswold, 1995). Apart from primary effects on the reproductive processes, sex steroids are known to be, and are used as, growth promoters (Breier *et al.*, 1988).

While all the regular changes in the reproductive parameters were attributed to primary sex hormones (e.g. FSH, LH, testosterone, estrogen etc.) and metabolic hormones (e.g. GH, T_3, ACTH etc.), a critical role of thyroid hormones in the testicular development of neonatal rats has been demonstrated (Cooke and Meisami, 1991; van Haaster *et al.*, 1992). At the testicular level, thyroid hormones are influential during the perinatal period which coincides with the development of the rat gonads (Bunick *et al.*, 1994). Moreover, the effects of iodothyronines on the testis is highly dependent upon the age of the animal and testis functions can be affected both directly and indirectly. Transient neonatal hypo- (Cooke and Meisami, 1991) (TNH) and hyper-thyroidism (TNHI) (van Haaster *et al.*, 1993) in rat pups has been demonstrated to induce changes in the adult male reproductive parameters. These findings suggest a critical role for thyroid hormones in the developing male gonads during the perinatal period. It is believed that thyroid hormones decelerate proliferation but accelerate differentiation of Sertoli cells (Cooke and Meisami, 1991). The aim of the present study was to monitor concentrations of T_4, T_3, IGF-I and testosterone profiles and to investigate the relationship between these hormones and testicular function during the peripubertal period in two breeds of sheep with different

prolificacies: Suffolk (Sfk) and a more prolific breed, Outaouais (Out)(Shafto *et al.*, 1996 a, b).

3.2. MATERIALS AND METHODS

3.2.1. Animals, housing and environment

Twelve Sfk and twelve Out ram lambs, born between February 15 and March 15, were used in this study at 90 ± 2.3 (mean ± SEM) days of age and BW of 22.4 ± 1.6 kg for Sfk and 21.0 ± 0.5 kg for Out. Lambs were paired by breed and housed in 180 × 150 cm pens on plastic coated expanded metal flooring in the Animal Science Research Unit of the University of Manitoba. Water, chopped hay and a barley based concentrate, meeting NRC (1985) requirements for fast growing lambs, were available *ad libitum*. Ram lambs were maintained in accordance with the guidelines of the CCAC (1993). Photoperiod in the experimental room was adjusted weekly to correspond to the natural photo-period.

3.2.2. Blood sampling

To monitor changes in the glucose, T_3, T_4, IGF-I and T, blood was collected once a week beginning at week 14 until week 36 of age from 20 lambs, 10 from each breed, every 20 min from 0900 to 1520 h by indwelling clear vinyl jugular catheters (Medical Grade, I.D. 1.00 mm, O.D. 1.50 mm, Cat. No. SV.70, Dural Plastics, Dural, Australia). The catheters were placed according to the procedure of Sakurai *et al.* (1993). Hematocrit were determined every 2 h from 0900 to 1520 h on the sampling days. At each sampling, two 5-ml blood samples were collected for serum and plasma. Serum was collected after

centrifugation for 20 min at 2000 g (Joan CR 3000 centrifuge, Vinchester, VA USA) within 16 h from samples kept at 4°C. Plasma was collected from heparinized samples within 30 min of collection. Plasma and serum were stored at –20°C.

3.2.3. Growth parameters

On a weekly basis, wool was clipped from the scrotum and scrotal circumference (SC) was measured in the lambs by a cloth measuring tape. The lambs were restrained in a sitting position and the testes were pulled fully into the scrotum before measurement. Hay and concentrate intake were measured weekly until 35 week of age and dry matter (DM) consumption d^{-1} $animal^{-1}$ was calculated. Weekly body weight (BW), average daily gain (ADG) relative growth (RG) (increase in body weight as a percentage of the total body weight) and gonado-somatic index (GSI = SC/BW) were calculated. The fleece was removed once in July and adjustments were made for the reduction in body weight.

3.2.4. Semen collection and evaluation

Semen collection, using an electro-ejaculator (Bailey Ejaculator, Western Instrument Company Denver, CO) started from 32 to 35 week of age. A semen sample was examined immediately with a light microscope and ranked. The microscope slides were pre-warmed (+ 30°C) to reduce the likelihood of cold shock to sperm. The volume of the ejaculate was recorded. To determine the sperm concentration a 20-µL sample of the ejaculate was fixed in acetic acid and the number of the sperm was counted by using a hemocytometer (Reichert Scientific Instruments, Buffalo, NY).

3.2.5. Testicular histology

At 36 weeks of age 3 rams from each breed were castrated and histologic sections of the testes were prepared by hematoxilin and eosin staining method. The state of maturity, number of cell layers in the seminiferous tubules (ST), number and diameter of ST were determined microscopically using a calibrated slide (Carl Zeiss, Germany). Due to the large diameter of the testicles, it was not possible to count the total number of ST in the cross section of the testis. Therefore, the number of the ST per view field of the microscope were counted at ×100 magnification. At least 10 view fields were observed to obtain an average number and diameter of ST in the view field. The longitudinal sections were avoided as much as possible.

3.2.6. Hormone values and pulse calculations

Mean hormone values for each sheep were calculated from 19 samples (every 20 min samples, for testosterone) and (or) from 4 hourly samples (for T_4, T_3, and IGF-I). The ratio of T_4/T_3 throughout the experiment was calculated. To calculate IGF-I pulses, the 20 min samples of week 14, 22 and 28 of age were analyzed from 4 lambs (two from each breed with either high or low semen quality). Week 14 was the start of frequent sampling. Week 22 was near the beginning of the pubertal period as manifested by a prominent increase in the testosterone concentration in most of the lambs and week 28 was the time during which IGF-I reached a plateau in the two groups. Pulses of testosterone and IGF-I were calculated according to the method described by Veldhuis *et al.* (1984). Veldhuis *et al.* (1984) modified the algorithm used by Merriam and Wachter (1982) and based their

detection of pulses on the measurements of the hormone that exceeded the preceding local nadir by a value that was fourfold greater than the intra-assay coefficient of variation (CV) of the radioimmunoassay (RIA). This method eliminates noise produced by the error of the experiment and produces less false positive pulses compared to other methods.

3.2.7. Testosterone radioimmunoassay

Sera samples were assayed in duplicate for testosterone using RIA method described by Sanford (1974). In brief the procedure was as follows: for extraction 200 µl of serum was added to 4 ml of di-ethyl ether (anesthesia grade USP; Mallinckrodt Chemical Inc. Paris, KY), vortexed for 1 min and left to stand for 15 min. To maximize recovery, vortexing was repeated once in order to separate the 2 phases in the mixture before freezing over a dry ice and ethanol bath (– 40°C). The ether was then decanted and evaporated in a water bath (30°C). The residue was re-suspended in 1.1 to 1.25 ml PBS with gel (depending on the expected concentration of the hormone). Efficiency of recovery of testosterone, validated within each assay by using a known aliquot of ^{3}H-testosterone [1,2,6,7,- ^{3}H(N)] (New England Nuclear, Boston, MA) was 92.74 ± 0.82% (mean ± SEM). For the assay the standard solution of testosterone was freshly prepared by adding 1000 pg of testosterone stock (4-androsten-17 β-ol-3-one) (Steraloids Inc., Wilton, NH) into 500 µl of PBS, from which serial dilutions were made ranging from 12.5 to 1000 pg testosterone 500 µl $^{-1}$. The testosterone antibody (Ab) (Sanford *et al.*, 1974) was raised in sheep immunized against testosterone-3-carboxy-methyloxime conjugated to bovine serum

albumin and 0.5 ml of the frozen Ab was diluted with 200 ml of PBS giving a 1:4000 dilution. The ^{3}H testosterone was diluted in PBS to 11000 cpm 100 μl^{-1}. BetaMax™ ES (ICN Biomedicals Inc., Aurora, Ohio) was used as the scintillation cocktail. Maximum binding of the Ab was between 40 and 50% while non-specific binding was below 3%. The sensitivity of the assay at 90% maximum binding was 12.5 pg testosterone 500 μl^{-1}. The mean intra-assay CV for reference pools were calculated according to Wilson and Lapwood (1979). These values for high, medium, and low plasma pools were 8.2, 9.5, and 8.7% respectively. Mean inter-assay CV was 60%. The values of testosterone are expressed as ng ml^{-1}.

3.2.8. T_3 and T_4 RIA

Total T_3 and T_4 were assayed by RIA using Coat-A-Count® kits [total $T_3$1 501 for T_3 and total $T_4$1 1081 for T_4 Diagnostic Products Corp. Los Angeles, CA]. The procedure for the assay was very similar for both hormones and has been used by others (Wrutniak *et al.*, 1985; Milter and Albyl, 1985) for sheep. Mean T_4 and T_3 intra-assay CV were 4.8% and 4.4% respectively while inter-assay CV were 8.5% and 9.0% for T_4 and T_3. The values of T_4 and T_3 are expressed as μg and ng dl^{-1} respectively. The sensitivity of the assay was 0.25 μg dl^{-1} for T_4 and 7 ng dl^{-1} for T_3.

3.2.9. IGF-I ELISA

Serum samples were assayed for IGF-I by using active IGF-I ELISA kits (Diagnostic Systems Laboratories, Inc. Webster, Texas). This method involves acid ethanol extraction (AEE) which has been confirmed to be the most reliable method of extraction (Breier *et al.*, 1991, Daughaday *et al.*, 1989). The amino acid sequence of ovine IGF-I is identical to the human IGF-I except for amino acid number 66 which in sheep is alanine instead of proline (Francis *et al.*, 1989). Similarity of purified ovine IGF-I to human IGF-I in structure and biological activity has been demonstrated (Breier *et al.*, 1991, Francis *et al.*, 1989, McNeil, 1989, Wallace *et al.*, 1989).

In the present assay, IGF-I displacement comparison graphs were plotted at wavelengths ranging from 200 to 400 nm and at different concentrations. Also parallelism was tested using a mixture of half standard and half sheep serum and compared to human standards. The displacement was parallel and the absorbency in the two lines were highly correlated ($r = + 0.993$). Therefore, the kits were assumed to give reliable results. The mean intra-assay CV for IGF-I was 9.0% and inter-assay CV was 5.6%. The sensitivity of the assay was 0.03 ng ml^{-1}. The values of IGF-I are expressed as ng ml^{-1}.

3.2.10. Glucose measurement

Quantitative colorimetric determination of glucose in plasma at 620 to 650 nm was performed using standard kits (Procedure No. 635, Sigma Diagnostics, Sigma Chemical Co, St. Louis, MO). Mean intra-assay CV for high and low plasma pools were 5.0 and

7.5% respectively while inter-assay CV was 7.2%. The values of glucose are expressed as mg dl^{-1}.

3.2.11. Statistical analysis

All the data are shown as mean ± SEM unless stated otherwise. Hormone levels between the two breeds were compared by analysis of variance (ANOVA) and Students t-test. To test the effects of age on the level of hormones, mean hormone levels of each sampling day of the group were analyzed by ANOVA in a split plot design and, if the difference was significant, the means were compared by least significant difference (LSD) test (Steel and Torrie, 1960). Hematocrit measurements from 0900 and 1500 h were analyzed using paired t-test assuming equal variances. Correlation coefficients (r values) were calculated by using a computer software (Quatro-pro, version 5, Borland international, USA) and if the absolute value was more than 0.5, it was considered as a significant correlation.

3.3. RESULTS

3.3.1. Growth performance and feed consumption

Frequent blood collection did not change mean hematocrit measurements of all the lambs between 0900 and 1500 h ($P>0.1$). The overall weekly BW, ADG and RG of the two breeds were similar throughout the experiment ($P>0.1$). Mean final BW were 66.3 ± 1.2 and 70.1 ± 2.47 kg for Sfk and Out respectively. ADGs were within the expected range (NRC 1985). There were negative correlations between age and RG of the lambs (r = –

0.840 and – 0.843 for Sfk and Out respectively) showing the effects of age on growth (Figure 1). During the experimental period, hay and concentrate consumption was similar in the two breeds (P>0.1). Consumption of DM kg^{-1} BW had a negative correlation with age (r = – 0.6425 Vs – 0.677 for Sfk and Out) indicating that feed consumption was higher at younger ages (Figure 2). Dry matter consumption was within the expected ranges (NRC 1985).

3.3.2. Testicular growth and function

Comparison of weekly SC measurements and GSI of the two breeds revealed no significant differences throughout the experiment (P>0.1). The initial SC of the lambs were 16.7 ± 0.5 and 18.1 ± 0.4 cm for Sfk and Out, respectively while the final SC was 36.1 ± 1.5 and 36.1 ± 1.6 cm for Sfk and Out (Figure 3). In the last semen collection, one Out and four Sfk lambs had poor quality semen (sperm count $<5 \times 10^5$ ml^{-1} and motility <50%; LS lambs) the remaining lambs had good quality semen (sperm count $>1 \times 10^9$; motility >85%; HS lambs). Histology of the testis showed that the LS lambs had immature and less developed seminiferous tubules than HS lambs. Average number of ST in the view field of the microscope at × 100 magnification were 22 ± 1.1 and 30 ± 0.9 for HS and LS (respectively). The diameter of the seminiferous tubules in LS lambs were smaller than HS lambs (250-350 μm for HS and 100-200 μm for LS) and the number of cell layers in the seminiferous tubules was also lower in LS lambs (6 ± 1.2 vs 2 ± 1 for HS and LS). The lumen of the ST in the LS lambs were filled primarily with fluid while the lumen of the HS lambs were full with sperm which indicated the higher state of maturation.

3.3.3. Testosterone concentration and pulses

Although coefficients of correlation for age and testosterone concentration were similar in the two breeds (r = + 0.95 vs + 0.94 for Sfk and Out), mean concentration of testosterone was higher in Out than Sfk (P<0.01; Figure 4). At 15 weeks of age, the mean testosterone concentration was slightly higher in Out (2.1 ± 0.2 ng ml^{-1}) than Sfk (1.7 ± 0.1 ng ml^{-1}) but at 29 week of age, testosterone concentration was much higher in Out (5.1 ± 0.7 ng ml^{-1}) than Sfk (2.2 ± 0.3 ng ml^{-1}). Testosterone pulse frequencies in the 6 h sampling period d^{-1} were higher in Out than Sfk (P<0.05) and LSD test showed the significant differences were at week 19, 23, 25, 27 and 29 (Figure 5). There was a positive correlation between age of the lambs and testosterone pulse frequencies (r = + 0.62 for Out and + 0.23 for Sfk).

3.3.4. T_4 and T_3

There was a negative correlation between T_4 (Figure 6), T_3 (Figure 7) and age in the two breeds (r = − 0.88 vs − 0.35 for T_4 and r = − 0.88 vs − 0.89 for T_3 in Sfk and Out respectively) indicating the effects of age on thyroid hormones. Level of T_4 was higher in Out than Sfk throughout the sampling period (P<0.01). The mean concentration of T_4 at weeks 15 of age was 8.0 ± 0.6 μg dl^{-1} for Sfk and 8.4 ± 0.8 μg dl^{-1} for Out while T_4 concentrations at 29 weeks of age were 5.8 ± 0.6 and 8.6 ± 0.7 μg dl^{-1} for Sfk and Out, respectively. Concentrations of T_3 were higher in Out than Sfk throughout the sampling period (P<0.05). At week 15, the average level of T_3 was 157.2 ± 9 vs 133.8 ± 8 ng dl^{-1}

for Out and Sfk and, at week 29 it was 110 ± 5 and 85 ± 6 ng dl^{-1} for Out and Sfk, respectively.

3.3.5. IGF-I concentration and pulses

IGF-I concentrations were higher in Out than Sfk throughout the sampling period ($P<0.05$) (Figure 8). Moreover, there was an effect of age on the level of IGF-I in the two breeds. Mean IGF-I level in the Sfk was lowest at week 15 of age (525 ± 60 ng ml^{-1}), highest at week 23 (771 ± 62 ng ml^{-1}) then declined to 479 ± 30 ng ml^{-1} at week 31 of age. IGF-I levels in Out were 769 ± 48 ng ml^{-1} at week 15, reached a maximum at week 19 (911 ± 29 ng ml^{-1}) and declined to 700 ± 60 ng ml^{-1} at 31 week of age. Correlation coefficients between IGF-I and thyroid hormones were positive throughout the sampling period ($r = +0.04$ vs $+0.12$ for T_4 and T_3 in Sfk and $r = +0.35$ vs $+0.64$ for T_4 and T_3 in Out) showing simultaneous reduction among IGF-I, T_4 and T_3 levels in the two breeds. Assay of the hourly samples at week 15, 23 and 29 for IGF-I revealed fluctuations suggesting presence of pulses. Frequent serum samples (every 20 min for 6 h) from Out ($n = 2$) and Sfk ($n = 2$) lambs with either high and (or) low quality semen were assayed and no episodic release of IGF-I in the selected samples were found. The intra-assay CV for week 15, 23 and 29 of age were 10.8%, 7.6% and 8.8% respectively and were used to calculate IGF-I pulses.

3.3.6. Plasma glucose concentration

Glucose concentrations were similar in the two breeds throughout the experiment ($P>0.1$) (Figure 9). There was a negative correlation between age and glucose concentration ($r = -0.85$ vs -0.86 in Sfk and Out). Correlation coefficients for glucose and IGF-I were positive in both breeds ($r = +0.30$ vs $+0.65$ in Sfk and Out). There were positive correlations between glucose and T_4 ($r = +0.88$ and $+0.38$ for Sfk and Out, respectively), and between glucose and T_3 ($r = +0.92$ vs $+0.87$ in Sfk and Out) indicating simultaneous reduction in the levels of glucose, IGF-I and thyroid hormones.

3.4. DISCUSSION

As expected both relative growth and dry matter consumption kg^{-1} BW decreased as the lambs became older. Although the size of the testes in the two breeds were similar, semen in Out lambs had higher quality than Sfk at similar ages. By last semen collection at 35 weeks of age, 89% of Out and 56% of Sfk ram lambs had viable sperm. Histological sections of the testicles showed that, regardless of the breed, ram lambs with low quality semen had seminiferous tubules which were less developed including smaller diameter, larger lumen filled with liquid and fewer cell layers than lambs with high quality semen.

Higher testosterone concentrations and pulse frequencies may be reasons for the higher degree of testicular activity in Out. On the average, the Out attained pubertal testicular function at an earlier age than Sfk ram lambs. Land (1978) found that at corresponding ages, ram lambs in the more prolific breeds of sheep had higher testosterone concentrations than those of low prolificacy. Moreover, in rams, periods of

elevated testosterone concentrations are accompanied by increases in testosterone pulse frequency (Sanford *et al.*, 1978). Therefore, higher concentrations of testosterone may be due to higher frequencies of testosterone pulses in Out than Sfk. Moreover, lambs of more prolific breeds have higher peak height and numbers than the lambs of less prolific breeds at the same age (Land, 1978; Sanford *et al.*, 1982) which is consistent with our data.

Thyroid hormones play a key role in Sertoli cell proliferation and differentiation (Cooke *et al.*, 1994 a). During early postnatal development, Sertoli cells are the major sites of thyroid hormone receptors in the male gonads. At this stage, thyroid hormones normally act directly on Sertoli cells to inhibit proliferation but stimulate maturation and production of secretory proteins such as ABP, IGF-I and inhibin, characteristic of adult cells (Bunick *et al.*, 1994, Kirby *et al.*, 1992, Palmero *et al.*, 1989). Moreover, it has been demonstrated that high levels of thyroid hormones, early in the life, enhances Sertoli cell differentiation (van Haaster *et al.*, 1993). Panno *et al.* (1994) found that TNH delayed puberty by decreasing net hormonal efficacy of androgens. In other words, high levels of thyroid hormones enhance the efficacy of androgens which may enhance puberty. One way of increasing androgen efficacy is through secretion of ABP which binds to testosterone (Martin *et al.*, 1991), thereby, increasing testosterone concentrations in the ST. High local testosterone concentrations in the testes, provides a suitable environment for spermatogenesis (Palmero *et al.*, 1989) and is necessary for the nourishment of the developing germ cells (Mita *et al.*, 1982). Therefore, it is conceivable that in Out lambs with an earlier rise and higher testosterone and thyroid hormones concentrations and

testosterone pulse frequency, Sertoli cells may mature at a younger age than in Sfk thereby leading to earlier sexual maturity.

There is a close relationship between anabolic sex steroids and hypophyseal GH production. A rise in the blood testosterone level is accompanied by a discharge of GH (Wilson 1986, Copeland *et al.*, 1985). Likewise, it has been demonstrated that hypothyroidism, in the rats, reduced pituitary GH production and that a single dose of T_3 or T_4 at physiological levels stimulated maximum production of GH (Samuels *et al.*, 1989). Furthermore, GH stimulates IGF-I production in the liver and other tissues, as well (McGuire *et al.*, 1992). Thus, it may be assumed that higher levels of thyroid hormones (and testosterone) caused more GH secretion in Out than in Sfk leading to increase in the IGF-I production by the hepatic cells or other somatic cells. Although it has been reported that injection of high levels of T_4 and T_3 into steers decreased IGF-I level (Elsasser *et al.*, 1991) the employed levels were far beyond the normal physiological levels. In the Out lambs with intrinsic high, but normal, levels of thyroid hormones, IGF-I concentrations were naturally high. It is possible that, the physiological reactions of the pituitary and liver in animals with natural high levels of thyroid hormones is not similar to artificially elevated situations in experimental animals.

Postnatal reduction in the level of blood glucose normally happens in ruminants (van Soest 1994) which is consistent with our data. As lambs become older, other sources of energy partially replace glucose (e.g. volatile fatty acids produced in the rumen) (Blaxter, 1969). Positive correlations between glucose, IGF-I, T_3 and T_4 are indications of

both reduction in the energy expenditure and less dependence of the body to glucose as the major source of energy in the peripubertal sheep (van Soest, 1994).

Lack of presence of pulses in the IGF-I concentrations in ruminants is attributed to high level of binding proteins for these hormones (McGuire *et al.*, 1992). IGF-I and IGF-II circulate in plasma in association with IGF-binding proteins (IGFBP; Butler and Gluckman, 1986). The somatomedin concentrations in the peripheral circulation of humans are relatively stable with no obvious diurnal rhythm due to the long biological half-life (Hall and Sara 1984). On the other hand, in ruminants, circulating concentrations of somatomedins do vary according to physiological state and are responsive to factors such as endocrine and nutritional status (McGuire *et al.*, 1992). However, no episodic release of the somatomedin was found in cannulated young steers sampled every 15 min for 24 h (Breier *et al.*, 1986 and 1988). Similar results were reported in pigs (Sillence and Etherton, 1986) and rats (Donaghue *et al.*, 1990) sampled every 15 min for 6 h. Sillence and Etherton (1986) noticed significant quantity of "noise" in their IGF-I measurements over the same period. In the routine IGF-I assay using commercial kits, a final dilution of 1:100 is required therefore, any error in the pipetting could generate dramatic fluctuations in the final results which may look like a pulse. To eliminate the error of the assay, and the so called fluctuations, the method of Veldhuis *et al.* (1984) was used in our pulse analysis. Merriam and Wachter (1982) suggested using the moving average procedure in the analysis of the data to reduce noises but, this method was not used in our data analysis because it reduces the sensitivity of the analysis. Thus, our results confirm that IGF-I pulsatile release is not evident in ram lambs.

3.5. CONCLUSIONS

During perinatal development of male gonads, there is a close relationship between thyroid hormones and Sertoli cells. Perinatal Sertoli cells are the major targets of thyroid hormones in the gonads. Therefore, high levels of thyroid hormones can have potential impacts on the functional Sertoli cells. These effects can be either direct and (or) indirect, such as local production of IGF and ABP. Direct influence of Sertoli cells on the production of androgens by Leydig cells, and synthesis of ABP by Sertoli cells, maintains testosterone concentration in the seminiferous tubules at a high level. There is an intimate association between thyroid hormones, testosterone and GH-IGF-I axis. Therefore, higher serum concentrations of IGF-I in Out than Sfk may partly be the result of higher testosterone and thyroid hormone levels in this breed. The higher prolificacies in the Out may be also the result of higher concentrations of thyroid hormones, IGF-I, testosterone and testosterone pulse frequencies as compared to Sfk.

Figure 1. Relative growth in Out □ and Sfk ■ throughout the pre- and peripubertal period. Each data point is the average of the group. There were no significant differences in the relative growth between the two breeds throughout the experiment ($P>0.1$).

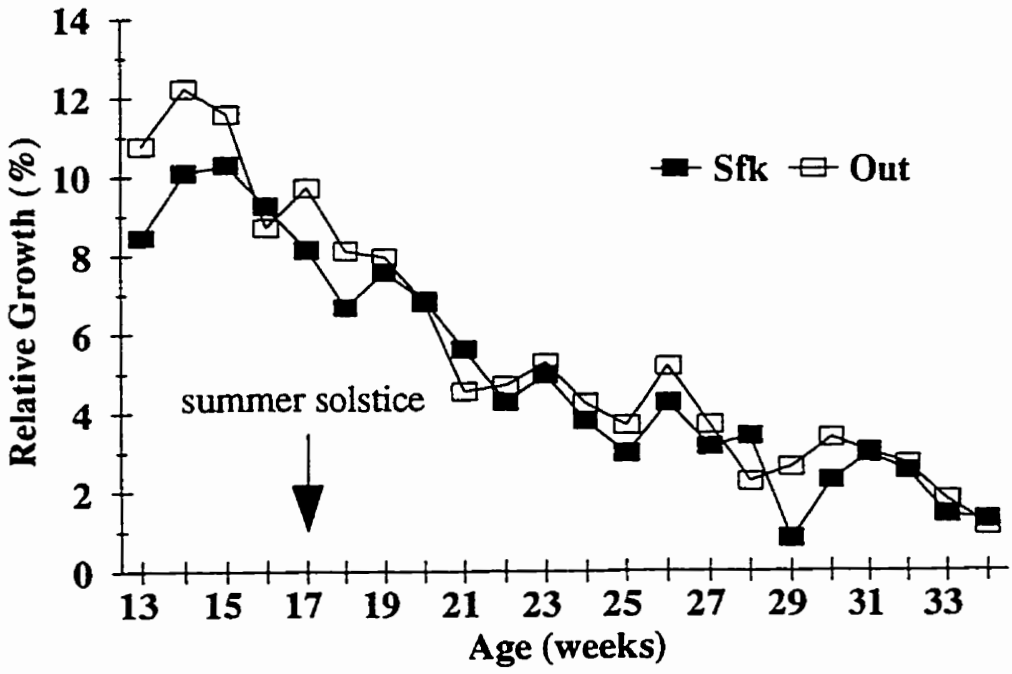

14
12
10
8
6
4
2
0
Relative Growth (%)
Sfk
Out
summer solstice
13
15
17
19
21
23
25
27
29
31
33
Age (weeks)

Figure 2. Daily dry matter consumption of Out □ and Sfk ■ lambs. Data points represent averages of the group. There were no significant differences in daily dry matter consumption between the two breeds throughout the experiment ($P>0.1$).

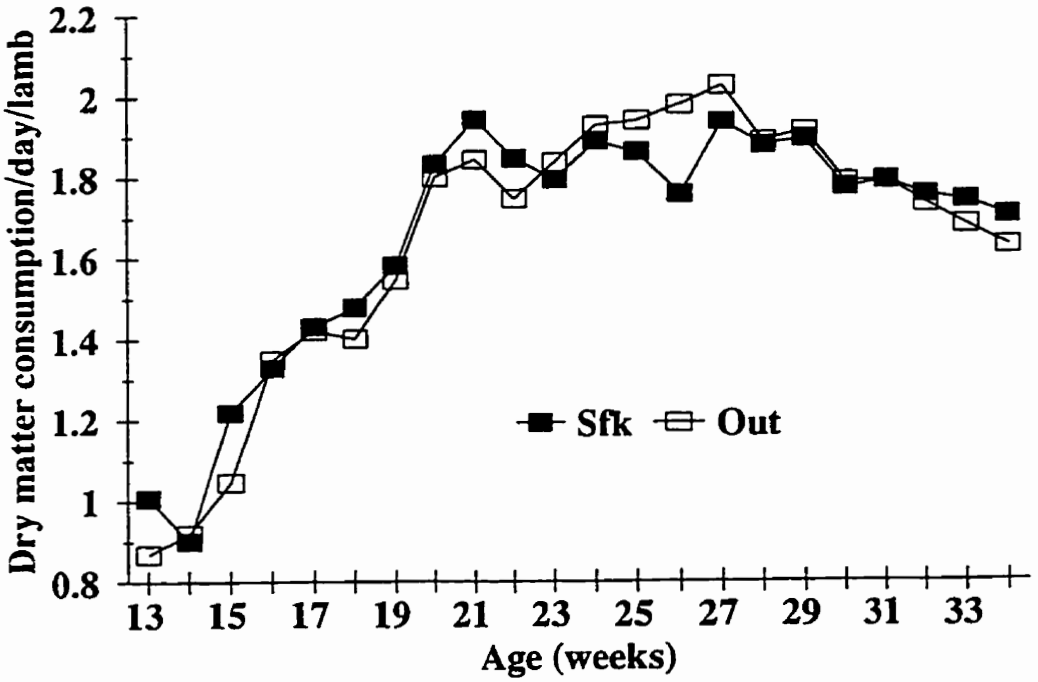

Dry matter consumption/day/lamb
2.2
2
1.8
1.6
1.4
1.2
1
0.8
13
15
17
19
21
23
25
27
29
31
33
Age (weeks)
Sfk
Out

Figure 3. Scrotal circumference in Out □ and Sfk ■ lambs. There were no significant differences in scrotal circumference between the two breeds throughout the experiment ($P>0.1$).

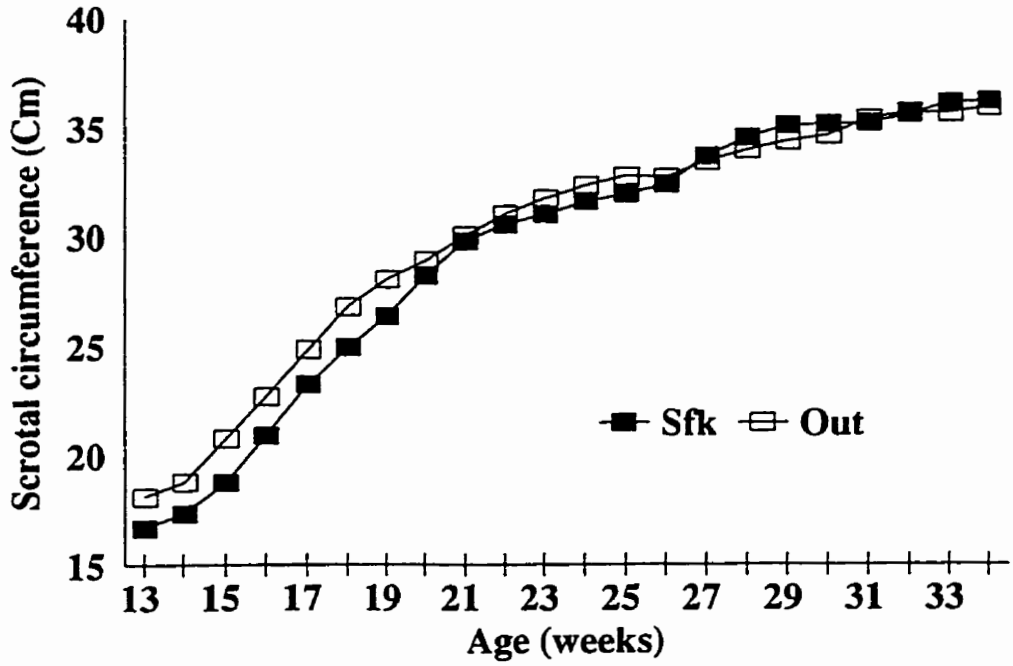

Scrotal circumference (Cm)
40
35
30
25
20
15
13
15
17
19
21
23
25
27
29
31
33
Age (weeks)
Sfk
Out

Figure 4. Serum concentrations of testosterone during the sampling period in Out □ and Sfk ■ lambs. Levels of the hormone was significantly higher in Out than Sfk at week 25, 27 and 29. ✳ indicates difference between the treatment groups at $P<0.05$.

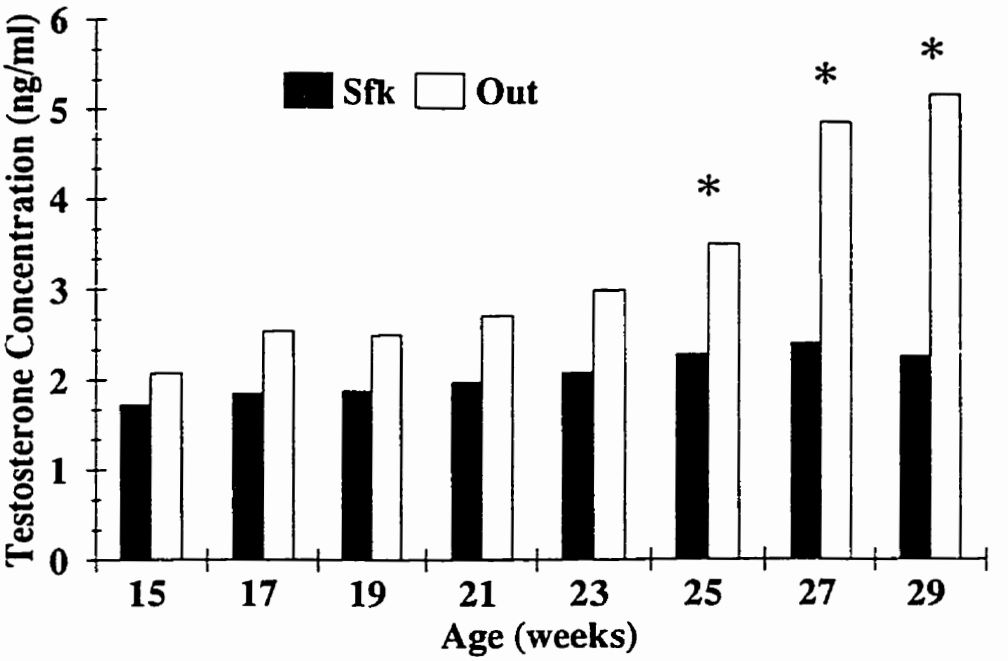

Testosterone Concentration (ng/ml)
0
1
2
3
4
5
6
Sfk
Out
*
*
*
15
17
19
21
23
25
27
29
Age (weeks)

Figure 5. Testosterone pulse frequencies in Out □ and Sfk ■ lambs. The data points are averages of pulse frequencies in the group during 6 h sampling period in each sampling day. Pulse frequencies tended to be higher in Out than Sfk at any point and significantly higher at weeks 19, 23, 25, 27 and 29 of age. ✳ indicates difference between the treatment groups at $P<0.05$.

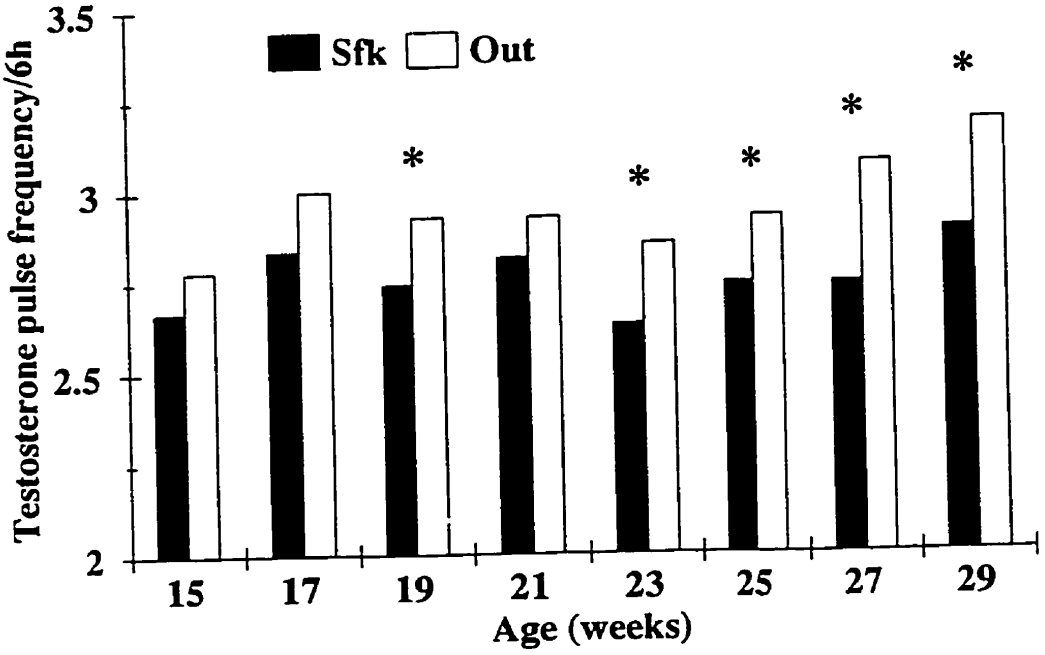

Testosterone pulse frequency/6h
3.5
3
2.5
2
Sfk
Out
*
*
*
*
*
15
17
19
21
23
25
27
29
Age (weeks)

Figure 6. Serum concentrations of T_4 in Out □ and Sfk ■ lambs throughout the sampling period. Levels of T_4 tended to be higher in Out than Sfk at any point but significantly higher from week 23 to the end of experiment. ✳ indicates difference between the treatment groups at $P<0.05$.

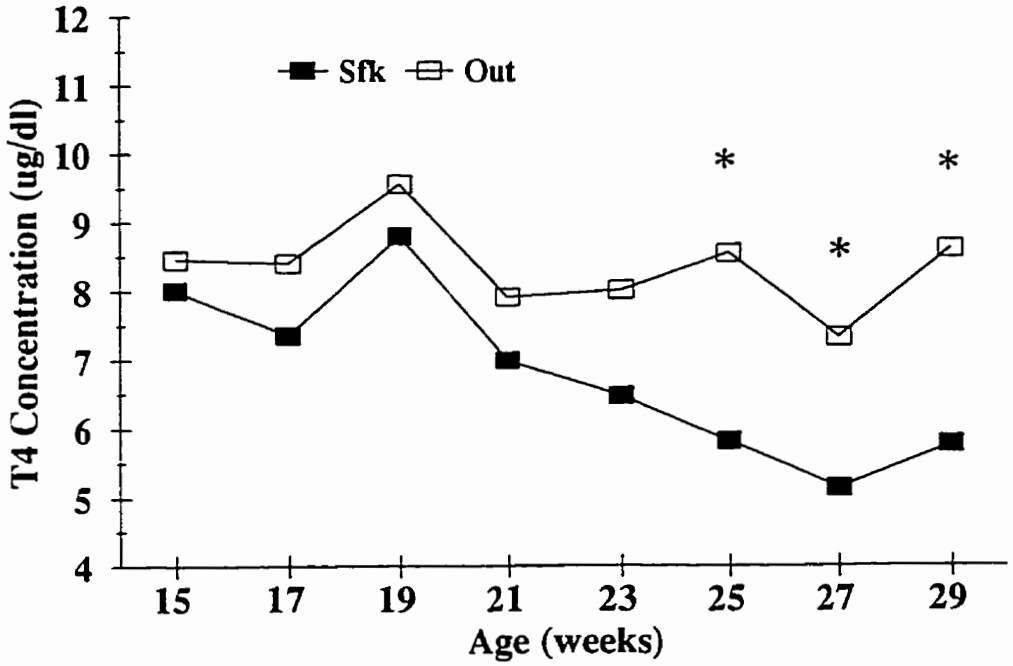

12
11
10
9
8
7
6
5
4
T4 Concentration (ug/dl)
Sfk
Out
*
*
*
15
17
19
21
23
25
27
29
Age (weeks)

Figure 7. Serum concentrations of T_3 in Out □ and Sfk ■ lambs. Levels of T_3 tended to be higher in Out than Sfk at any point but significantly higher at weeks 15, 19 and 29. * indicates difference between the treatment groups at $P<0.05$.

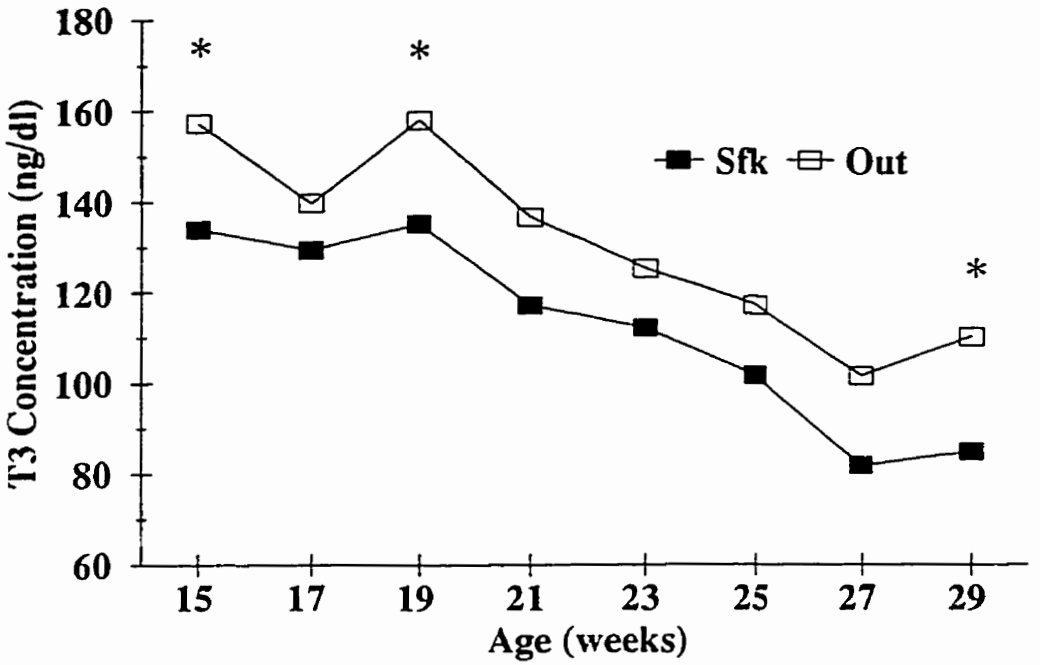

180
160
140
120
100
80
60
T3 Concentration (ng/dl)
*
*
*
Sfk
Out
15
17
19
21
23
25
27
29
Age (weeks)

Figure 8. Serum IGF-I concentrations in Out □ and Sfk ■ lambs. Levels of IGF-I tended to be higher in Out than Sfk at any point but significantly higher at weeks 15, 17, 19, 21 and 31. ✳ indicates difference between the treatment groups at $P<0.05$.

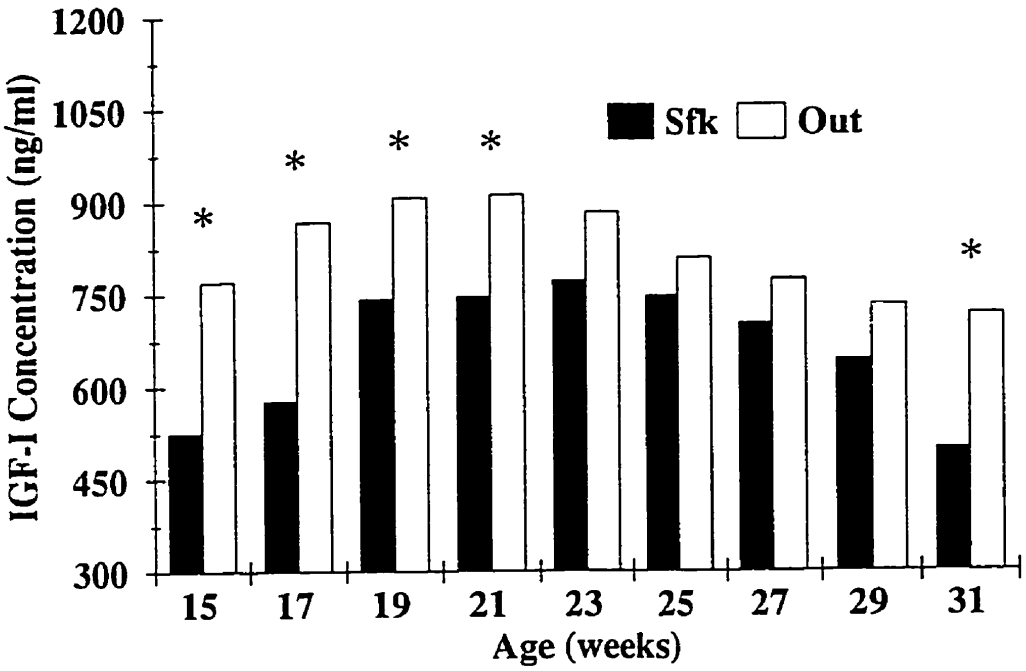
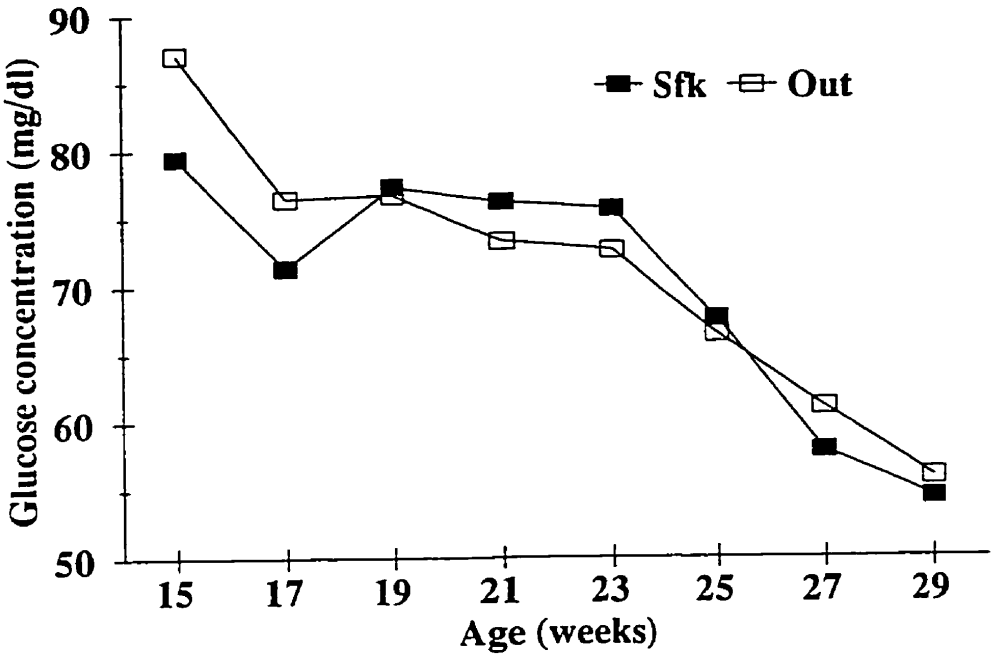

1200
1050
900
750
600
450
300
IGF-I Concentration (ng/ml)
Sfk
Out
*
*
*
*
*
15
17
19
21
23
25
27
29
31
Age (weeks)

Figure 9. Plasma glucose concentrations in Out □ and Sfk ■ lambs throughout the sampling period. Glucose concentrations were similar throughout the experiment in the two breeds ($P>0.1$).


90
80
70
60
50
Glucose concentration (mg/dl)
Sfk
Out
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Age (weeks)

4. Manuscript II

Transient Early Hyperthyroidism Enhances Onset of Puberty in Suffolk Ram Lambs.

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ABSTRACT

To study thyroid function and sexual development, eight 6-week-old Suffolk ram lambs were divided into either control or PTU+H groups and housed indoors. From 42 to 82d of age, four lambs in the PTU+H received 15 mg kg^{-1} BW d^{-1} of PTU orally to suppress normal thyroid function. To achieve a hyperthyroid state, during the same period, subcutaneous injections of T_4 and T_3 were given at the rate of 8 and 16 μg kg^{-1} BW d^{-1}, respectively. Four control lambs received normal saline subcutaneously and excipient orally. Feed and water were *ad lib*. Room and rectal temperatures were recorded three times a week. In order to determine levels of T_4, T_3, FSH, testosterone and IGF-I, blood was collected by indwelling jugular catheters once a week, every 20 minutes from 0900 to 1520 h. Scrotal circumference, relative growth, gonadosomatic index and feed intake were recorded weekly. From week 16 to 36, semen was collected by electro-ejaculation. Lambs were castrated at week 36 and testicular histology was examined. During the treatment period only, level of thyroid hormones were higher in PTU+H than control ($P<0.05$). From week 6 to 9 only, PTU+H lambs had lower FSH ($P<0.05$). IGF-I was lower in PTU+H than control from week 10 to 13 ($P<0.05$). Frequency of testosterone pulses were higher ($P<0.01$) in the PTU+H rams but concentrations were similar ($P>0.1$) in the control and PTU+H throughout the experiment. Average daily gain and feed intake were not different between the two groups ($P>0.1$). Relative growth was lower in PTU+H than control in week 6 through 11 ($P<0.05$) but not thereafter. Gonadosomatic index was higher in week 6 and 7 in control but it was higher in PTU+H from 27 to 36 week of age ($P<0.05$). Scrotal circumference was larger in PTU+H rams from week 26 to 36 ($P<0.05$).

PTU+H lambs produced viable sperm earlier than control lambs. At week 36, sperm concentration in PTU+H was higher than control ($P<0.01$) but semen volumes were similar ($P>0.1$). Diameter of the seminiferous tubules in PTU+H were larger than in control ($P<0.05$). In conclusion, transient neonatal hyperthyroidism, decreased FSH and IGF-I temporarily, increased testosterone pulses and sperm production and enhanced puberty in Suffolk ram lambs.

4.1. INTRODUCTION

Thyroid hormones may affect testis functions both directly and indirectly (Palmero *et al.*, 1988), but these effects are highly dependent upon the age of the animal (Meisami *et al.*, 1994). Testis of adult animals were considered unresponsive to thyroid hormones (Oppenheimer *et al.*, 1974). However, recent evidence supports the concept of a critical role for thyroid hormones in testicular development during the perinatal period. High L-tri-iodothyronine (T_3) levels in immature rats reduced the postnatal period of Sertoli cell proliferation and the final testicular size (van Haaster *et al.*, 1993). Conversely, a 6-propyl-2-thiouracil (PTU; a potent reversible goitrogen) induced transient neonatal hypothyroidism (TNH) resulted in increased adult testis size and sperm production in rats (Cooke and Meisami, 1991), hamster (Kirby *et al.*, 1993) and mice (Joyce *et al.*, 1993). PTU treatment in the rats decreased T_4 concentration throughout the treatment period but suppressed T_3 to a lesser degree (Kirby *et al.*, 1992). Serum FSH and LH levels were reduced significantly in hypothyroid rats without affecting testosterone level.

Sertoli cells are the major determinant of the magnitude of sperm production (Orth *et al.*, 1988). Neonatal Sertoli cell proliferation was extended up to 15 d in PTU-treated rats (van Haaster *et al.*, 1992) and mice (Joyce *et al.*, 1993), leading to large increases in their numbers in the adult (Hess *et al.*, 1993). On the other hand, transient neonatal hyperthyroidism (TNHI) decreased proliferation rate, but enhanced differentiation, of Sertoli cells (van Haaster *et al.*, 1993). Similarly, Palmero *et al.* (1992) found that excess T_3 inhibited DNA synthesis and presumably proliferation in the rat prepubertal Sertoli cell culture. Immediately after cessation of PTU treatment in hypothyroid rats, the testicles

looked immature and the lumen formation was delayed or absent. Conversely, T_3 treatment accelerated the formation of a tubular lumen (van Haaster *et al.*, 1993) which reflected the secretion of the fluid by the differentiated Sertoli cells (Russell *et al.*, 1989). Cooke *et al.* (1994 b) stated that the early suppression of Sertoli cell proliferation, the reduced adult number of these cells, and the enhanced onset of Sertoli cell secretory activity in hyperthyroid young rats were all due to direct actions of thyroid hormones on the Sertoli cells.

The aim of the present study was to apply TNHI in sheep to further our understanding of the role of thyroid hormones and testicular development in peripubertal rams. Due to major differences in the rate of Sertoli cell proliferation of neonatal rats and sheep (Orth *et al.*, 1982; Gondos and Berndston, 1993) it is of interest to document the reaction of ovine testis to the effects of TNHI. In addition, sheep hypothalamic-pituitary-thyroid axis is well developed at the time of birth (Brzezinska-Slebodzinska and Krysin, 1990) but not in rats (Dubois and Dussault, 1977) therefore, there are potential differences between rodents and sheep in the application of TNHI which should be investigated.

4.2. MATERIALS AND METHODS

4.2.1. Animals, housing and environment

Eight Suffolk ram lambs, selected from a large flock after spring weaning, were brought to the Animal Science Research Unit of the University of Manitoba at 40 ± 2 d of age and weighing 14.4 ± 0.6 kg (mean ± SEM). Lambs were randomly assigned to either control (n = 4) or PTU+H (n = 4) groups. Each group was housed in a mechanically ventilated

room in a 180 × 300 cm pen on a concrete floor covered by wood shavings. Room light duration was adjusted weekly to correspond to the natural photoperiod. Lambs had free access to fresh water and were fed chopped hay and a barley based concentrate *ad lib.* The ration was formulated to meet 1985 NRC requirements for fast growing lambs and they were maintained in accordance with the Canadian Council on Animal Care guidelines (1993). Room and rectal temperatures were taken 3 times a week during the experiment.

4.2.2. Treatments

To suppress normal thyroid function, PTU was administered to the PTU+H lambs at the rate of 15 mg kg^{-1} BW d^{-1} from 42 to 82 d of age (week 6 to week 12). The PTU solution was prepared as a 0.1% (weight/volume) solution with 1% sugar added to make it more palatable. The daily dose was divided into two equal parts administered by drench at 0800 and 1600. During the same period, to achieve a hyperthyroid state, PTU+H lambs were also given daily subcutaneous injections of T_3 and T_4 (Sigma Chemical Co. St. Louis, Mo) at the rate of 8 and 16 μg kg^{-1} BW, for T_3 and T_4 respectively. PTU suppresses hepatic and peripheral T_3 production. Therefore, T_3 was also injected into the lambs to attain a hyperthyroid state. T_3 and T_4 solutions were prepared by dissolving both hormones in alkalinized saline as described by Lynch and Moore (1983) but with some modifications as follows: 2500 μg of T_4 and 1250 μg of T_3 were dissolved in a mixture of 5 μl of 1 N NaOH and 495 μl of 95% ethanol and vortexed. This was mixed with a solution composed of 2.5 ml of propylene glycol and 7 ml of normal saline. The final solution had approximately 250 μg T_4 and 125 μg of T_3 ml^{-1}. The control lambs were given placebo and injected subcutaneously with normal saline.

4.2.3. Blood sampling

To measure the concentration of T_4, T_3, FSH, insulin-like growth factor-I (IGF-I) and testosterone, blood was collected by indwelling jugular catheters once a week, at 20 minute intervals from 0900 to 1520 h. The catheters were put in place according to the procedure of Sakurai *et al.* (1993).

Hematocrits were determined every 2-h during the sampling d. Blood samples were stored up to 16-h at 4°C. Serum was collected after centrifugation for 20 min at 2000 g (Joan CR 3000 centrifuge, Vinchester, VA USA) and stored at –20°C.

4.2.4. Growth measurements

Scrotal circumference (SC) of the lambs was measured from 6 to 36 weeks of age with a cloth measuring tape while the lambs were restrained in a sitting position. Scrotal wool was clipped off and the testes were pulled fully into the scrotum before measurement. Weekly body weight (BW), relative growth (RG), average daily gain (ADG), gonadosomatic index (GSI = SC/BW), and average daily feed intake (ADF) were measured and calculated until 36 week of age. Total body fleece was removed once in July and adjustments made to calculations for the reduction in body weight.

4.2.5. Semen collection and evaluation

Attempts to collect semen were made twice a week from week 16 to 36 by electro-ejaculation (Bailey Ejaculator, Western Instrument Company Denver, CO) and semen samples were evaluated immediately. To determine the sperm concentration, a 20 µl

sample of the ejaculate was fixed in acetic acid and the number of the sperm cells were counted by using a hemocytometer (Reichert Scientific Instruments, Buffalo, NY). Semen was considered low quality if the sperm count was $< 5 \times 10^5$ ml^{-1} and good quality if sperm count was $> 1 \times 10^9$. Volume of the semen was recorded and percentage of non-motile sperms in the total counts was calculated.

4.2.6. Testicular histology

At 36 weeks of age, rams were castrated surgically, the testicles weighed and histologic sections prepared by hematoxilin & eosin staining method to examine the number and diameter of seminiferous tubules (ST), and number of cell layers in the ST. Diameter of ST was determined with the aid of a calibrated microscopic slide (Carl Ziess, Germany). Number of cell layers was determined by counting the average number of cells standing in a column from the periphery to the lumen of ST in four different directions. Due to the large diameter of the testicles, it was not possible to count the total number of ST in the cross section of the testis, therefore the number of the ST per view field of the microscope were counted at 100 magnification and at least 10 view fields were observed to obtain the average number and diameter of ST The longitudinal sections were avoided as much as possible.

4.2.7. Hormone concentrations and pulse calculations

Mean hormone values for each sheep were calculated from the 19 samples per sampling d (for testosterone) and (or) from 4 hourly samples (for T_4, T_3, FSH, and IGF-I). Pulses of

testosterone were calculated according to the method described by Veldhuis *et al.* (1984). Veldhuis *et al.* (1984) modified the algorithm used by Merriam and Wachter (1982) and based their detection of pulses on the measurements of the hormone that exceeded the preceding local minimum by a value that was fourfold greater than the intra-assay coefficient of variation (CV) of the radioimmunoassay (RIA). This method allows elimination of the noises produced by the error of the assay and leaves less false positive pulses compared to other methods. The ratio of T_4/T_3 during and after treatment period was calculated.

4.2.8. Testosterone RIA

Concentrations of testosterone were determined in duplicate by RIA following the procedure of Sanford (1974). In brief the procedure was as follows: 200 µl of serum was added to 4 ml of di-ethyl ether (anesthesia grade USP; Mallinckrodt Chemical Inc. Paris, KY), vortexed for 1 min and left to stand for 15 min. To maximize recovery, this was repeated once in order to separate the 2 phases in the mixture before freezing over a dry ice and ethanol bath (– 40°C). The ether was then decanted and evaporated in a water bath (30°C). The residue was re-suspended in 1.1 to 1.25 ml PBS with gel (depending on the expected concentration of the hormone). Efficiency of recovery of testosterone as validated within each assay by using a known aliquot of ^{3}H-testosterone [1,2,6,7,-^{3}H(N)] (New England Nuclear, Boston, MA) was 92.7 ± 0.8 % (mean ± SEM). For the assay, the standard solution of testosterone was freshly prepared by adding 1000 pg of testosterone stock (4-androsten-17 β-ol-3-one; Steraloids Inc., Wilton, NH) into each 500 µl of PBS

from which, serial dilutions were prepared ranging from 12.5 to 1000 pg testosterone 500 μl^{-1}. The testosterone antibody (Ab; Sanford *et al.*, 1974) was raised in sheep immunized against testosterone-3-carboxy-methyloxime conjugated to bovine serum albumin and 0.5 ml of the frozen Ab was diluted with 200 ml of PBS giving a 1/4000 dilution. ^{3}H testosterone was diluted in PBS to 11000 cpm 100 μl^{-1}. BetaMax™ ES (ICN Biomedicals Inc., Aurora, Ohio) was used as the scintillation cocktail. Maximum binding of the antibody was between 40 and 50% while non-specific binding was below 3%. The sensitivity of the assay at 90% maximum binding was 12.5 pg testosterone 500 μl^{-1}. The mean intra-assay CV for high, medium, and low reference pools of serum were calculated according to the Wilson and Lapwood (1979) procedure and were 9.9%, 9.2%, and 12.7% respectively while, mean inter-assay CV were 14.2%. The values of testosterone was expressed as ng ml^{-1}.

4.2.9. T_3 and T_4 RIA

Total T_4 and T_3 assay were performed by RIA using Coat-A-Count® kits [total $T_4$1 1081 and total $T_3$1 501 for T_4 and T_3, respectively, Diagnostic Products Corp. (DPC) Los Angeles, CA]. Similar procedures have been used by others (Wrutniak *et al.*, 1985; Milter and Albyl, 1985) in sheep. Mean T_4 and T_3 intra-assay CV were 4.8% and 4.4% respectively while inter-assay CV were 8.5% for T_4 and 9.0% for T_3. The values of T_4 and T_3 are expressed as µg and ng dl^{-1} respectively. Sensitivity of the assay were 0.25 µg dl^{-1} for T_4 and 7 ng dl^{-1} for T_3.

4.2.10. IGF-I ELISA

IGF-I concentrations were determined by using IGF-I ELISA kits [Diagnostic Systems Laboratories, Inc. (DSL) Texas]. This method involves acid ethanol extraction (AEE) which has been confirmed to be the most reliable method of extraction (Breier *et al.*, 1991, Daughaday *et al.*, 1989). The amino acid sequence of ovine IGF-I is identical to the human IGF-I except for amino acid number 66 (Francis *et al.*, 1989). Similarity of purified ovine IGF-I to human IGF-I in structure and biological activity has been demonstrated (Breier *et al.*, 1991, Francis *et al.*, 1989, McNeil, 1989, Wallace *et al.*, 1989).

In the present assay, displacement comparisons have been plotted at wavelengths ranging from 200 to 400 nm and at different concentrations. To test the parallelism a mixture of half standard and half sheep serum was used. The absorbency of the two lines were highly correlated (r = 0.993). Therefore, the above mentioned kits were assumed to give reliable results. The mean intra-assay CV for IGF-I was 9% and inter-assay CV was 5.6%. The values of IGF-I are expressed as ng ml^{-1}. The sensitivity of the assay was 0.03 ng ml^{-1}.

4.2.11. FSH RIA

Serum samples were assayed for FSH according to the method described by Evans *et al.* (1992). The average intra- and inter-assay CV were 3.1% and 7.8% respectively. Sensitivity of the assay was 0.1 ng ml^{-1} .

4.2.12. Statistical analysis

The design of the experiment was a completely randomized design (CRD). All the data are shown as mean ± SEM unless stated otherwise. Statistical analysis included analysis of variance (ANOVA) and Student t-test. To compare the hormone values at different ages within a group, (e.g. different sampling week), mean hormone levels of each sampling d of the lambs in the same group were analyzed by ANOVA in a CRD and, if the difference was significant, the means were compared by least significant difference (LSD) test (Steel and Torrie, 1960) corrected to hold experimental error at 0.05. Hematocrit measurements from 0900 and 1500-h were analyzed using a paired t-test assuming equal variances. Correlation coefficients (Pearson product-moment) were calculated by using a computer software (Quatro-pro, version 5, Borland International, USA). Due to changes that occurred in the level of thyroid hormones during treatment period, correlation coefficients have been calculated from the end of treatment to the end of experiment.

4.3. RESULTS

4.3.1. Growth performance

Mean BW was initially 16.9 ± 1.1 vs 12.2 ± 0.4 kg for PTU+H and control and 83.4 ± 2.5 and 85.4 ± 3.0 kg at the end for PTU+H and control, respectively. The RG (daily weight gain as a percentage of the total BW) was lower ($P<0.05$) in the PTU+H than control during the treatment period at 6 through 11 week of age (Figure 10). However, the ADF kg^{-1} BW and ADG were similar ($P>0.1$) between groups and within the acceptable range for rapidly growing ram lambs (NRC, 1985). As would be expected, RG was higher

(P<0.05) at younger ages and declined as the lambs became older. Average rectal temperature of the lambs in both groups were similar throughout the experiment (P>0.1).

4.3.2. Testicular growth

The initial mean SC of the control and PTU+H groups were identical (12.5 ± 0.3 cm) and remained similar until 21 weeks of age (P>0.1). From week 21 to week 26, testes of PTU+H lambs tended to grow faster than control lambs but the difference was not significant until week 26 through to the end of experiment which was higher in PTU+H than control (P<0.05; Figure 11). At the end of the experiment, average SC was 37.5 ± 2.7 and 42.4 ± 1.9 cm for control and PTU+H respectively. At 31 week of age SC plateaued in both groups. The GSI was higher for control at 6 and 7 week of age (P<0.05) however, it was higher for PTU+H from 27 to 36 week of age (P<0.05; Figure 12). The average final testicular weight of the two groups were different at 513.3 ± 126.3 gm and 674.5 ± 99.4 gm for control and PTU+H respectively (P<0.05). There was no within group difference between right and left testicular weight (P>0.1).

4.3.3. Semen quality

Semen was collected between 17 and 33 weeks of age. In the final ejaculation, semen volume was similar in the two groups (1.05 ± 0.09 vs 1.2 ± 0.09 ml^{-1} for control and PTU+H; P>0.1) while sperm cell concentration was different ($2.07 \times 10^9 \pm 0.06$ vs $2.54 \times 10^9 \pm 0.09$ ml^{-1} for control and PTU+H respectively; P<0.05). In the control group, viable sperm cell was first found at 20 weeks of age in first lamb and at 24 weeks of age in the

second lamb. The third control lamb showed motile sperm at 25 and the forth one at 30 weeks of age. In the PTU+H group, the first and second lambs had viable sperm at 18 week of age and third and fourth lamb had viable sperm at 19 week of age.

4.3.4. Histology of the testis

At 36 week of age, testicular development was greater in the PTU+H lambs than control lambs so that there were more sperm cells present in the lumen of ST). Although there were fewer ST in PTU+H (20 ± 0.5) than control (25 ± 0.6; $P<0.05$), the diameter of the ST in PTU+H was greater than in the control (250-350 μm in PTU+H vs 200-300 μm in control; $P<0.05$). The diameter of the lumen was similar in both groups (150 ± 7.9; $P>0.1$). The total number of the cell layers in the ST of the two groups were variable (6 ± 0.9 layers of cells).

4.3.5. Testosterone concentration and pulse frequency

Average concentration of testosterone was similar in both groups throughout the experiment ($P>0.1$; Figure 13). A positive correlation existed between testosterone concentration and age of the lambs in the two groups ($r = + 0.74$ and $+ 0.87$ for control and PTU+H respectively) indicating effects of age on the testosterone concentration. The average number of testosterone pulse frequencies per 6-h sampling period were greater in PTU+H than control by one week from induction of hyperthyroidism through until 4 week following end of treatment, then again from 25 through 31 week of age ($P<0.01$; Figure

14). There were positive correlations between serum testosterone concentration and testosterone pulse frequencies (r = + 0.68 vs + 0.34 in control and PTU+H).

4.3.6. FSH concentration

FSH concentration in the two groups was different in week 6 through 9 of age only ($P<0.05$; Figure 15). Pattern of the FSH profile was the same in both groups and there was a positive correlation between FSH and age of the lambs in both groups (r = + 0.77 for PTU+H and + 0.81 for control). Likewise, there was positive correlations between FSH and testosterone concentration in both groups (r = + 0.55 and + 0.47 for PTU+H and control, respectively). Correlation coefficient for FSH and SC were + 0.74 in the PTU+H and + 0.72 in the control.

4.3.7. IGF-I concentration

In PTU+H lambs, IGF-I levels were lower than controls at week 10, 11, 12, and 13 of age ($P<0.05$) but were similar at other ages ($P>0.1$). The average IGF-I concentration at week 11 was 412.5 ± 19 and 930 ± 55 ng ml^{-1} for PTU+H and control, respectively. There was an effect of age on the IGF-I levels in both groups ($P<0.05$). In the control group, the increase in the IGF-I level was bi-phasic. IGF-I increased from 7 through 15 week of age followed by a decrease until week 27 and increased again at 27 through 34 week of age ($P<0.05$). In the PTU+H lambs, an increase in IGF-I levels started at week 8 through 10 followed by a decline in the hormone level from week 10 through week 12. From week 13

on, IGF-I pattern was similar between the groups (Figure 16). After week 13 to the end of experiment, a positive correlation existed between IGF-I, T_3 and T_4 ($r = +0.48$ vs 0.79 for T_3 and $+0.79$ vs $+0.93$ for T_4 in the PTU+H and control, respectively).

4.3.8. Concentrations and ratio of T_4 and T3

In the PTU+H group, serum T_4 and T_3 concentration were higher during the treatment period ($P<0.01$) while their concentration were similar thereafter ($P>0.1$). At week 6 (before treatment) levels of T_4 and T_3 were similar between the two groups; T_4 concentrations were 5.8 ± 0.2 vs 5.8 ± 0.3 $\mu g\ dl^{-1}$ for control and PTU+H and T_3 concentrations were 126.6 ± 5.2 and 105.6 ± 5.5 $ng\ dl^{-1}$ for control and PTU+H, respectively. At week 10 mean level of T_4 reached a maximum at 13.2 ± 0.4 $\mu g\ dl^{-1}$ for PTU+H while in the control it was 5.14 ± 0.5 $\mu g\ dl^{-1}$ (Figure 18). At week 9, in the PTU+H group, T_3 concentration reached a maximum at 544 ± 47.0 vs 157.3 ± 26 $ng\ dl^{-1}$ in the control group (Figure 17). The ratio of T_4/T_3 during treatment period was 38.9 ± 2.5 in control and 32.9 ± 6.6 in PTU+H while after treatment through the end of experiment it was 43.9 ± 2.4 vs 46.7 ± 2.6 for control and PTU+H. A negative correlation existed between the T_3 and age of the lambs ($r = -0.58$ for PTU+H and -0.8 for control). The Pearson product-moment for T_4 and age of the lambs were -0.65 and -0.78 for PTU+H and control, respectively indicating the effect of age on the level of thyroid hormones. After injection of the hormones were ended at 12 weeks of age, the serum level of the hormones in the PTU+H dropped dramatically (0.32 ± 0.15 $\mu g\ dl^{-1}$ for T_4 and 38.1

$\pm$ 4.9 ng dl^{-1} for T_3). Level of both hormones rose and was comparable to controls within the next two week.

4.4. DISCUSSION

In other studies, injection of T_4, at the rate of 13.15μg kg^{-1} BW d^{-1}, into adult sheep, did not produce catabolic effects seen in thyrotoxicosis although live weight was slightly reduced (Godden and Weekes, 1984). This amount of hormone is 5 times more than daily secretion of T_4 by thyroid gland in young lambs. Moreover, following T_3 injection at the rate of 5 μg kg^{-1} d^{-1} into young lambs, serum T_3 concentrations were not considered within the toxic range (Lynch *et al.*, 1983). In the present study, administration of 8 and 16 μg kg^{-1} BW d^{-1} of T_3 and T_4, respectively were somewhat higher than the aforementioned levels but T_4 dose was much less than the dose used by Martinoli and Pelletier (1989) who injected 80 μg kg^{-1} BW d^{-1} of T_4 in the rats. Following cessation of the PTU + hormone treatment, thyroid hormone concentrations returned to levels similar to control within two week. The depressed T_4 and T_3 levels in the first week after treatment were likely due to residual depressor effects of the treatment on thyroid function and on TSH and (or) TRH release (Hadley, 1992). The ratio of T_4 and T_3 was similar both during and after treatment implying the amounts injected corresponded to the normal physiological ratio.

Although ADF was not different, the RG of the PTU+H lambs, during the treatment period only, was slowed compared to control lambs. This is consistent with an increased metabolic rate, mobilization of energy stores, and (or) loss of BW and muscles

associated with hyperthyroidism (Hadley, 1992; Danforth and Burger, 1984). Up to 46% reduction in the BW of 100 d old TNHI rats has been reported (van Haaster *et al.*, 1993). Depression of RG in ram lambs was short lived and there were no other detrimental residual effects of the hyperthyroidism.

In spite of the initial lower RG, the transient hyperthyroidism did enhance testicular function and onset of puberty in these lambs. Hyperthyroid rats had reduced final testicular size (van Haaster *et al.*, 1993), whereas in hypothyroid rats testicular size at puberty was higher than controls (van Haaster *et al.*, 1992). Testicular enlargement was determined to be the result of hyperplasia and hypertrophy of Sertoli and Leydig cells (Cooke *et al.*, 1992). Cooke *et al.* (1994 b) indicated that T_3 directly inhibited mitogenesis of cultured neonatal rat Sertoli cells. Moreover, excess thyroid hormones in rats caused enhanced maturation and decreased proliferation time of the Sertoli cells. Therefore, one could have expected smaller testicles with lower number of Sertoli cells that are more differentiated in the PTU+H lambs than controls.

The difference in the effects of TNHI in sheep and rats may be due to: 1) species differences i.e. sheep is a precocious animal (Brzezinska-Slebodzinska and Krysin, 1990) and the hypothalamic-pituitary-thyroid axis is well developed at birth in sheep but not in rats (Dubois and Dussault, 1977). Moreover, the maximal proliferation of rat Sertoli cells occurs perinatally which coincides with the onset of neonatal thyroid function (Jannini *et al.*, 1990). On the other hand, in lambs thyroid activity starts *in utero* and Sertoli cell proliferation is maximal postnatally at an early age (Gondos and Berndston, 1993). 2) the time window and duration in which hyperthyroidism is effective on the gonadal function

may be different in the two species. The upper limit of this time window in rats is d 21 to d 24 (van Haaster *et al.*, 1993; Palmero *et al.*, 1989) but it is not known in lambs. 3) rate of proliferation of rat Sertoli cells might be faster than sheep. During the fast proliferation, labeling index of the Sertoli cells of rat was 20% and in calf it was 3 to 5% (Cooke *et al.*, 1994a). If the labeling index of sheep Sertoli cells are similar to calves, induction of hyperthyroidism should be of longer duration in lambs. Consequently, induced hyperthyroidism in our experimental lambs might enhance maturation of Sertoli cells and the subsequent enlargement of the testicles might be due the developmental processes which are concomitant with maturation of Sertoli cells. 4) Growth of the testicles in sheep is intermittent which is due to the seasonality of breeding while testicular growth in rats is uninterrupted. Sheep is a seasonal breeder and maximum size of the testis is attained at 3 years of age (Perry *et al.*, 1991; Land, 1978). Thus, in sheep, testicular growth is in three breeding season prior to reach maximum size but, in laboratory rats testicular growth is continuous and maximum size is attained at approximately 140 d of age.

If TNHI enhanced maturation of the testicles, it has displaced timing of the events leading to increase in the testicular size. Therefore, hyperthyroid lambs have testicles of more advanced stages of life. Testicular tissues in the PTU+H lambs had undergone either hyperplasia and (or) hypertrophy which increased the diameter of the ST and size of testicles. Moreover, treatment lambs produced viable sperm cells earlier than control lambs and the number of sperm cells were greater in PTU+H than contemporary controls, indicating that Sertoli cells of PTU+H lambs were in more advance stages of maturation to support and nourish large numbers of sperm cells. In other words, the increase in size of

the testicles of PTU+H lambs might be due to hypertrophy of Sertoli cells and hyperplasia of the germ cells. An eight-fold increase in Sertoli cell protoplasm was demonstrated during peripubertal period in the lamb (Monet-Kuntz *et al.*, 1984). Mature Sertoli cells produce ABP which binds to testosterone (Martin *et al.*, 1991), thereby, increasing testosterone concentration in the ST. The maintenance of high intra-testicular testosterone concentrations enhances spermatogenesis (Palmero *et al.*, 1989) and the nourishment of the developing germ cells (Mita *et al.*, 1982). Therefore, it may be speculated that, excess thyroid hormones enhanced maturation and growth of testicles and advanced the chronology of testicular growth in the PTU+H rams.

Testicular size, weight, daily sperm production, total number and size of Sertoli cells are positively correlated (Berndtson *et al.*, 1987, Monet-Kuntz *et al.*, 1984). Likewise, larger seminiferous tubules are more mature and have higher capacity of spermatogenesis (Berndtson *et al.*, 1987). Bigger testicles, larger seminiferous tubules, higher testosterone pulse frequency, higher sperm concentration, and enhanced production of high quality semen in the PTU+H lambs are all indications of advanced profile and chronology of the events that normally leads to puberty in the lambs.

There are no reports showing the relationship between TNHI and testosterone concentration. Renaville *et al.* (1993) defined puberty as a physiological step characterized by a pulsatile release of testosterone from the gonads which is followed by spermatogenesis. Consequently, increases in the testosterone pulses could be one of the reasons of enhancement of puberty in the PTU+H lambs. However, higher pulse frequency in the PTU+H lambs was not due to the changes in the FSH level because, at any

particular period, FSH concentration was not higher in the PTU+H than control lambs. Therefore, increase in the testosterone pulse frequency might be due to an increase in the pulses of GnRH and (or) LH (Sanford *et al.*, 1978).

Whereas low levels of FSH in the hyperthyroid lambs were transient, reduction of FSH was permanent in TNHI rats (van Haaster *et al.*, 1993). Moreover, FSH concentration was reduced in hypothyroid rats as well (Kirby *et al.*, 1992). Accordingly, any change in the thyroid state in neonatal animals may reduce level of FSH. In the cultured neonatal rat Sertoli cells, T_3 alone directly stimulated production of inhibin-β_b mRNA even in the absence of FSH and potentiated the actions of FSH on inhibin-β_b mRNA production (Cooke *et al.*, 1994b). If the mechanism of actions of thyroid hormones in rats and lambs are similar, it is possible that high levels of thyroid hormones increased inhibin production by the Sertoli cells of the PTU+H lambs which was followed by a reduction in FSH concentration. Inhibin regulates FSH secretion by a feedback mechanism (Martin *et al.*, 1991). However, van Haaster *et al.* (1992) concluded that changes in FSH levels in hypothyroid rats were as a result of direct effects of thyroid hormones on pituitary development and not because of an increase in the concentration of inhibin.

During the treatment period, IGF-I concentration was lower in the PTU+H group than control which is consistent with the results of Elsasser *et al.* (1991) in hyperthyroid steers. At physiological levels, thyroid hormones stimulate pituitary GH production maximally (Martinoli and Pelletier, 1989) which in turn stimulates IGF-I production in the liver and other tissues (McGuire *et al.*, 1992). Moreover, hypothyroidism reduced GH

mRNA production in the rat pituitary gland (Samuels *et al.*, 1989; Smith *et al.*, 1987). Therefore, it is conceivable that IGF-I concentration in the PTU+H lambs should be more than that of control lambs but, the opposite was the case during PTU+H treatment. High levels of thyroid hormones in the treatment lambs might produce adverse side effects seen in hyperthyroidism (Hadley, 1992) which deteriorated IGF-I production in the liver. Moreover, high concentrations of thyroid hormones causes extensive glucose uptake by somatic cells (Hall and Mita, 1984; Segal, 1989) accompanied by a reduction in the blood glucose which is followed by a reduction in IGF-I concentration (Breier *et al.*, 1986).

4.5. CONCLUSION

To our knowledge, this is the first report of TNHI in sheep and this report can contribute to the present knowledge about the effects of thyroid hormones during neonatal gonadal development which appear to be different than in rats. Our results show that TNHI advanced the sequence of events leading to the onset of puberty in hyperthyroid lambs as compared to the control lambs. In order to find the optimum dosage, duration and route of administration of PTU and thyroid hormones, and to answer some new questions, there is a need to conduct more research. The reason for higher testosterone pulse frequency, increase in the testicular size and function in the PTU+H lambs compared to controls is not clear. Likewise, the cause of lower IGF-I and FSH levels during the treatment period in the PTU+H lambs is not known. On the other hand, this experiment gives us an idea about the complexity of the neonatal gonadal development and involvement of thyroid hormones in this process. In the present experiment it was demonstrated that

modifications in the level of thyroid hormones of young lambs can change reproductive parameters of the peripubertal lamb.

Figure 10. Relative growth (weekly increase in the body weight as a percentage of body weight) in the PTU+H ■ and control □ lambs from 6 to 36 weeks of age. * indicates difference between the treatment groups at $P<0.05$. Treatment started at week 6 and ended at week 12 of age.

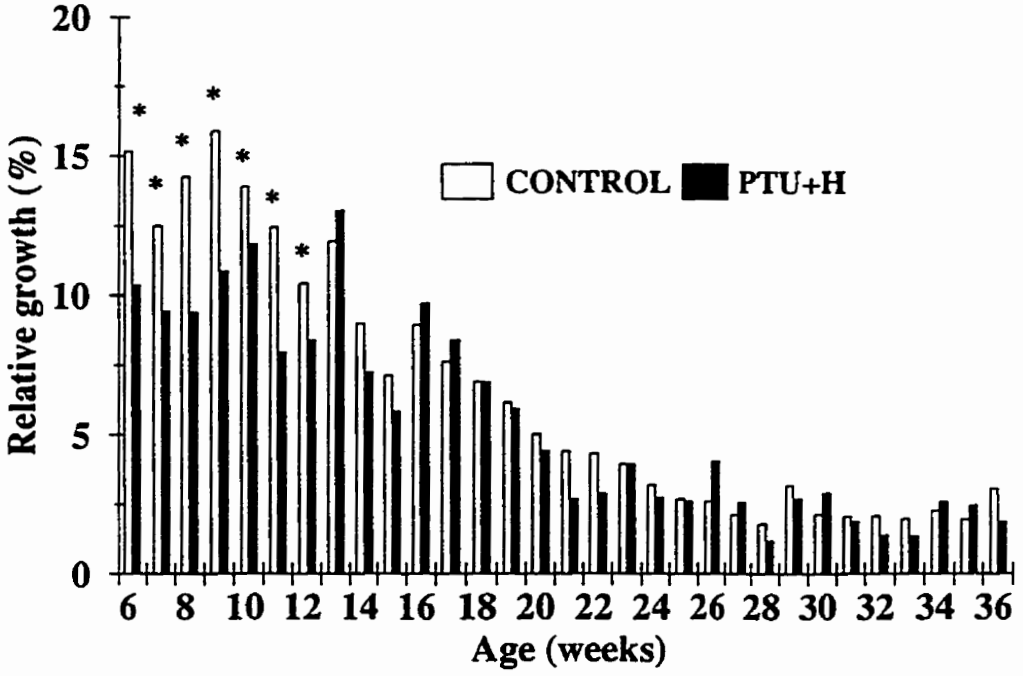

20
15
10
5
0
Relative growth (%)
CONTROL
PTU+H
*
6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
Age (weeks)

Figure 11. Scrotal circumference of the PTU+H ■ and control □ lambs from 22 to 36 weeks of age. ✳ indicates difference between the treatment groups at $P<0.05$. Treatment started at week 6 and ended at week 12 of age.

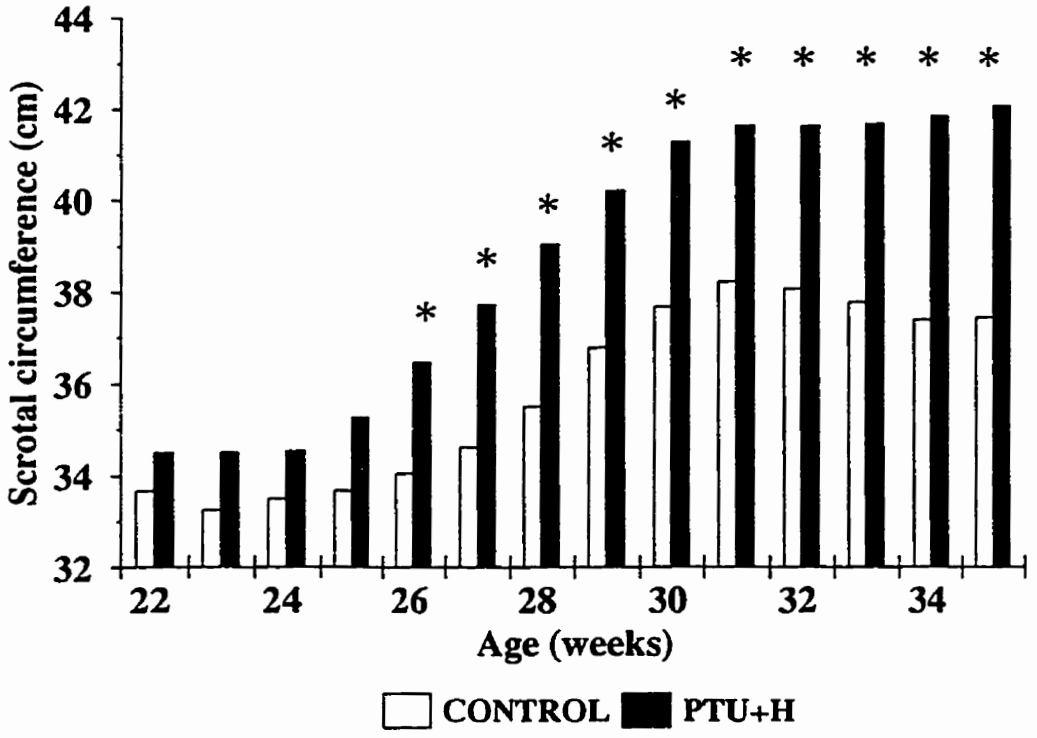

Scrotal circumference (cm)
44
42
40
38
36
34
32
22
24
26
28
30
32
34
Age (weeks)
CONTROL
PTU+H

Figure 12. Gonado-somatic index of the PTU+H ■ and control □ lambs throughout the experiment. Treatment started at week 6 and ended at week 12 of age.

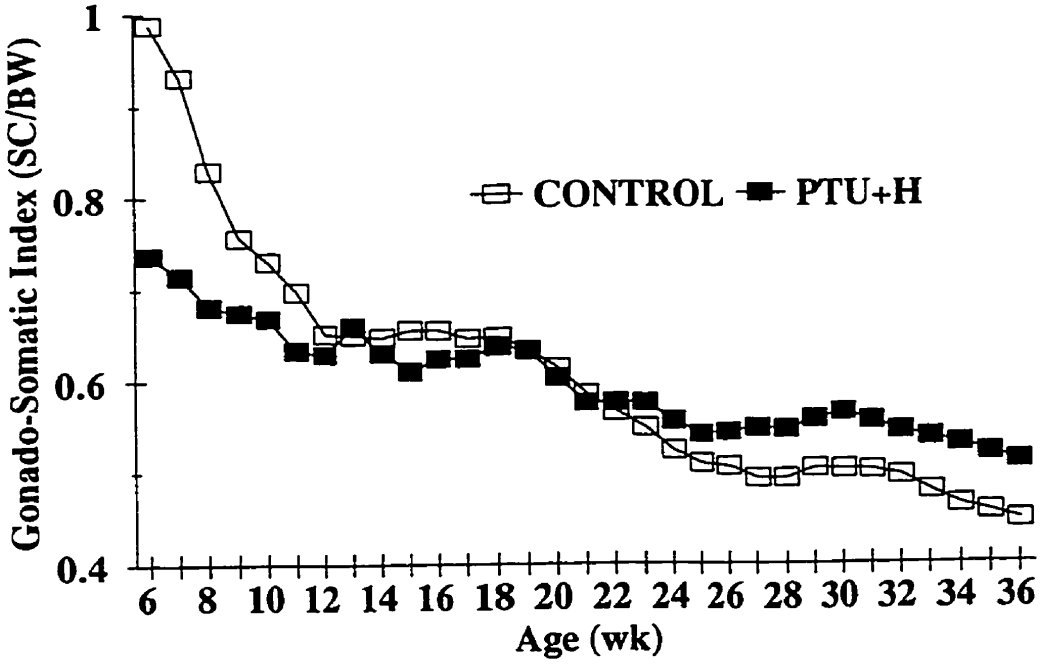

Gonado-Somatic Index (SC/BW)
1
0.8
0.6
0.4
CONTROL
PTU+H
6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
Age (wk)

Figure 13. Mean serum testosterone concentrations of the two groups throughout the experiment. No differences were detected between the PTU+H ■ and control □ lambs ($P>0.1$). Treatment started at week 6 and ended at week 12 of age.

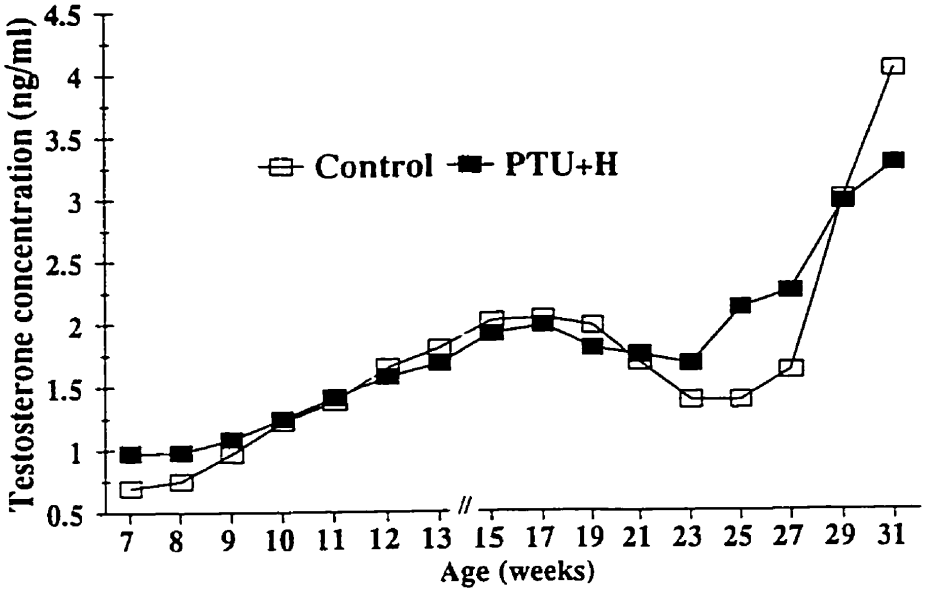

Testosterone concentration (ng/ml)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
Control
PTU+H
7 8 9 10 11 12 13 15 17 19 21 23 25 27 29 31
Age (weeks)

Figure 14. Testosterone pulse frequencies in 6-h period d^{-1} during the experiment in the two groups. The average pulse frequency were higher in the PTU+H ■ than control □ lambs * indicates difference between the treatment groups at $P<0.01$. Treatment started at week 6 and ended at week 12 of age.

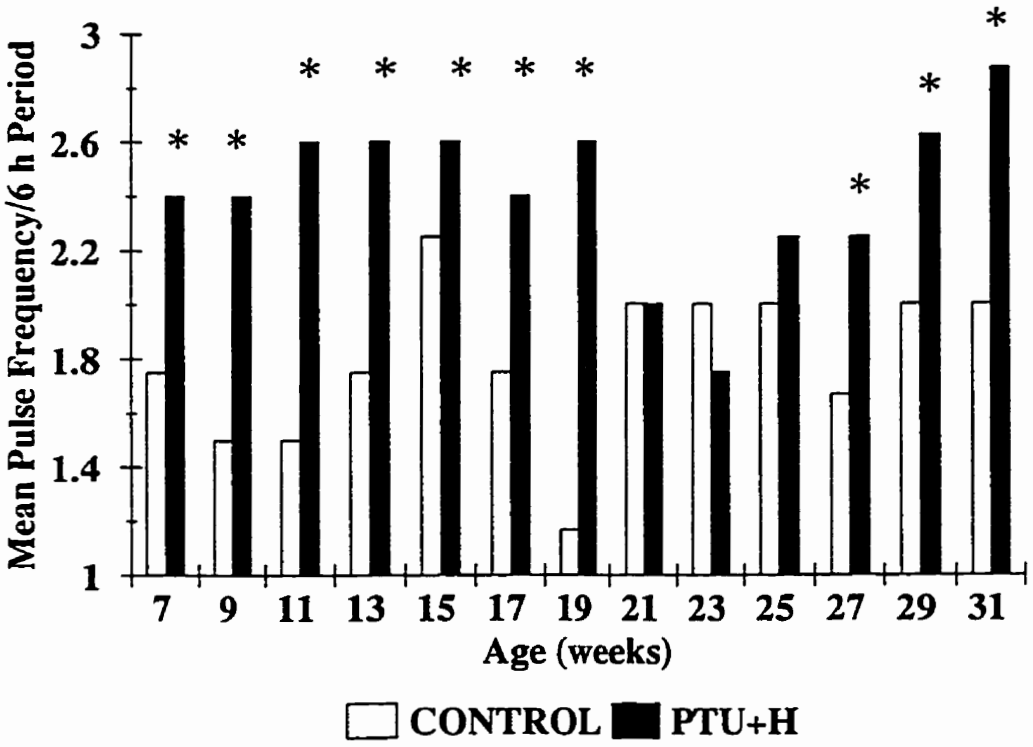

Mean Pulse Frequency/6 h Period
3
2.6
2.2
1.8
1.4
1
7
9
11
13
15
17
19
21
23
25
27
29
31
Age (weeks)
CONTROL
PTU+H

Figure 15. Serum FSH concentrations in the two groups during the experiment. FSH was lower at weeks 7, 8 and 9 in the PTU+H ■ than control ❑ lambs * indicates difference between the treatment groups at $P<0.05$. Treatment started at week 6 and ended at week 12 of age.

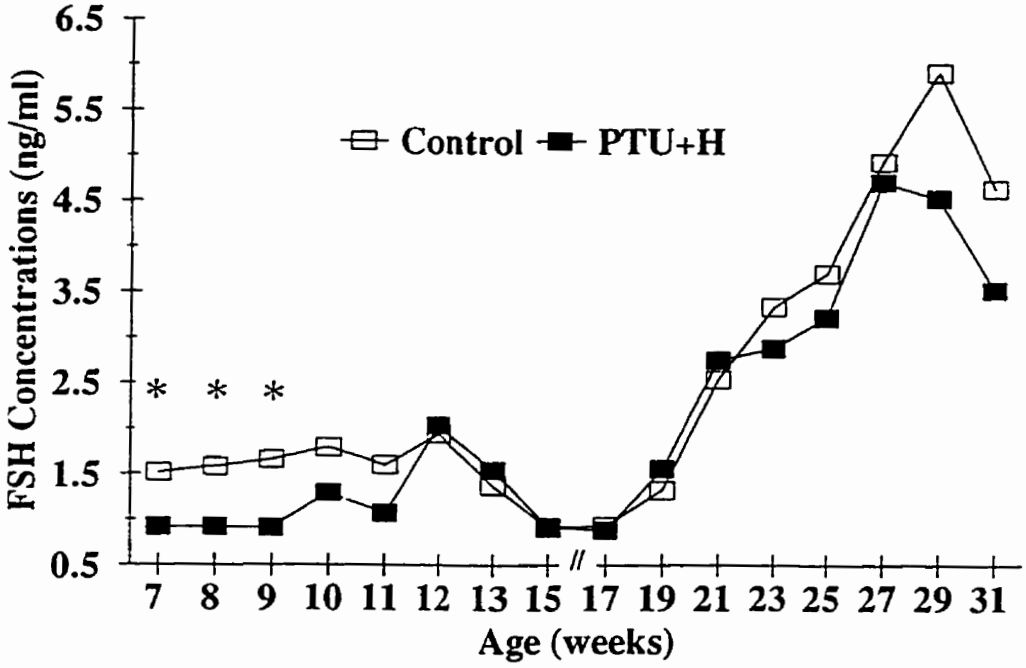

6.5
5.5
4.5
3.5
2.5
1.5
0.5
FSH Concentrations (ng/ml)
Control
PTU+H
*
*
*
7
8
9
10
11
12
13
15
17
19
21
23
25
27
29
31
Age (weeks)

Figure 16. Serum concentration of IGF-I during the sampling period. Average IGF-I levels were lower in the PTU+H ■ than control □ lambs at 10, 11, 12 and 13 weeks of age. ✳ indicates difference between the treatment groups at $P<0.05$. Treatment started at week 6 and ended at week 12 of age.

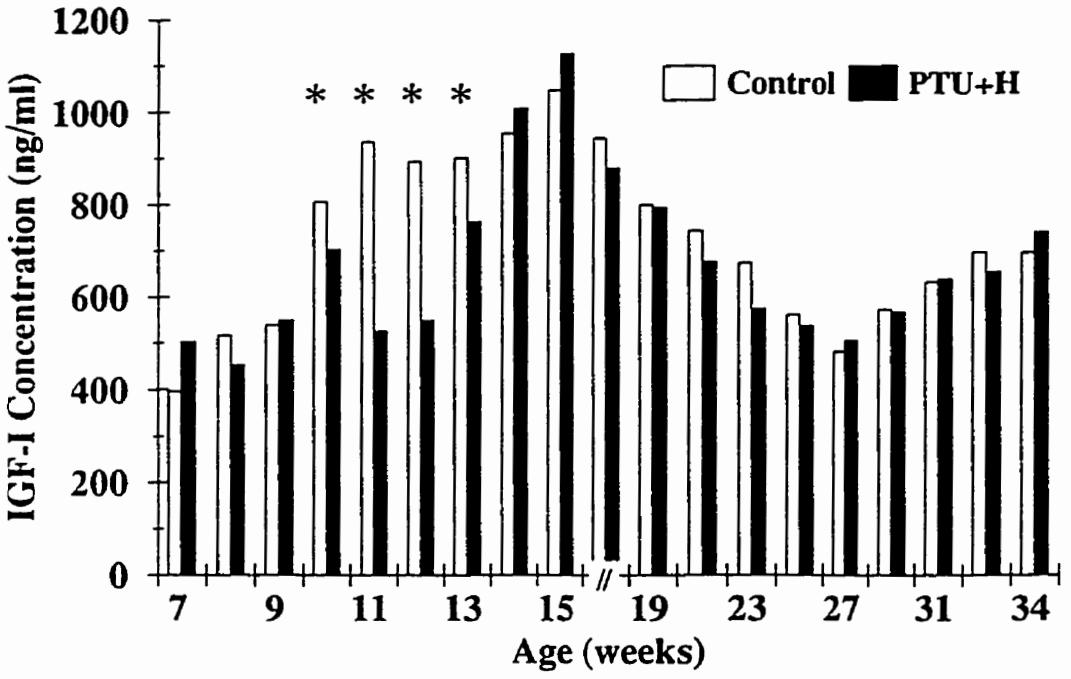

1200
1000
800
600
400
200
0
IGF-I Concentration (ng/ml)
* * * *
Control
PTU+H
7
9
11
13
15
19
23
27
31
34
Age (weeks)

Figure 17. Serum T_3 concentrations throughout the sampling period. During weeks 7 to 10, T_3 was higher in the PTU+H ■ than control □ lambs ($P<0.01$). At week 12, level of the hormone dropped in the PTU+H to nearly zero. * indicates difference between the treatment groups at $P<0.01$. Treatment started at week 6 and ended at week 12 of age.

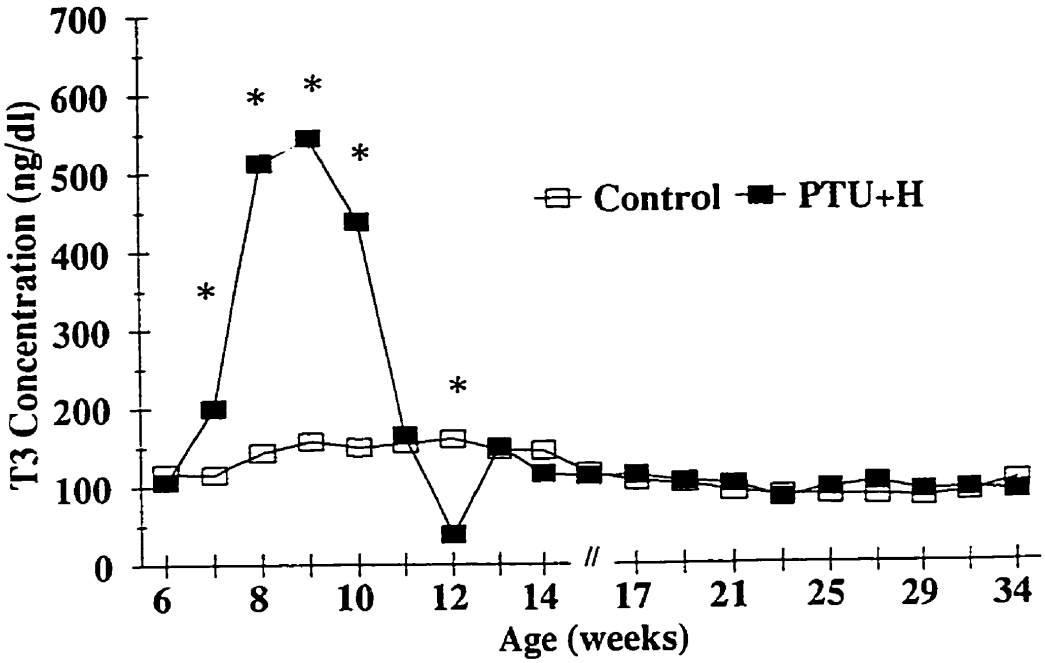
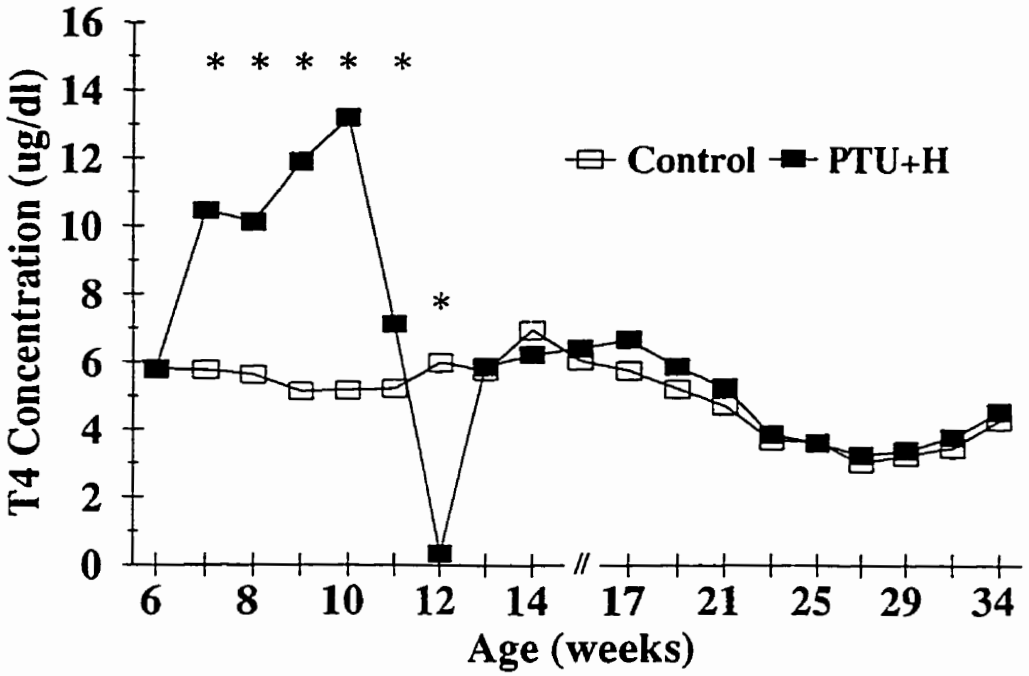

700
600
500
400
300
200
100
0
T3 Concentration (ng/dl)
*
*
*
*
*
Control
PTU+H
6
8
10
12
14
17
21
25
29
34
Age (weeks)

Figure 18. Serum T_4 concentrations throughout the experiment. During weeks 7 to 11, level of T_4 was higher in the PTU+H ■ than control □ lambs ($P<0.01$) but, it was lower in the PTU+H at week 12. * indicates difference between the treatment groups at $P<0.01$. Treatment started at week 6 and ended at week 12 of age.


16
14
12
10
8
6
4
2
0
T4 Concentration (ug/dl)
* * * * *
*
Control
PTU+H
6
8
10
12
14
17
21
25
29
34
Age (weeks)

5. Manuscript III

Transient Early Hypothyroidism Suppressed Testicular Function in Pubertal Suffolk Ram Lambs.

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ABSTRACT

Induction of transient neonatal hypothyroidism (TNH) in rat pups by 6-propyl-2-thiouracil (PTU) has been demonstrated to increase adult testicular size. As part of a research project studying thyroid function and puberty, twelve 6-week-old Suffolk ram lambs were divided into three groups and housed indoors in an artificially controlled environment. Treatments started at 6 weeks of age. To achieve a hypothyroid state, four lambs received PTU orally for 40 d (PTU40) and 4 lambs received PTU for 55 d (PTU55) at the rate of 15 mg kg^{-1} BW d^{-1}. Four control lambs received vehicle only. At weekly intervals, blood was collected every 20 minutes for 6 h. Treatment started at 6 weeks of age. Serum level of thyroxine (T_4), tri-iodothyronine (T_3), follicle stimulating hormone (FSH), insulin like growth factor-I (IGF-I) and testosterone (T) were determined. Other parameters of interest included: average daily dry matter consumption (DDM), average daily gain (ADG), rectal temperature, semen volume, sperm cells concentration and scrotal circumference (SC). From 16 to 36 week of age, semen was collected by electro ejaculation and evaluated. Mean SC, DDM and ADG were not different in the 3 groups throughout the sampling period ($P>0.05$). In the PTU40 and PTU55 lambs, spermatogenesis was delayed as compared with controls. Semen volumes were not different in the three groups at 36 weeks of age ($P>0.1$) but the sperm cell concentration in control was higher than PTU40 and PTU55 ($P<0.05$). At 36 weeks of age, histological sections showed that the diameter of the seminiferous tubules (ST) in the PTU40 and PTU55 were smaller than the control ($P<0.05$). During the treatment period only, T_4 and T_3 ($P<0.05$) levels were lower in PTU40 and PTU55 than the control. During the

treatment period, FSH was lower in both treatment groups and remained low in the PTU55 after the treatment period ended ($P<0.05$). Testosterone concentrations were similar ($P>0.1$) but testosterone pulse frequencies were different in the three groups ($P<0.05$). IGF-I concentration was similar in all three groups except at week 13 when it was lower in PTU55 than control. In conclusion, TNH suppressed testicular function and FSH secretion in PTU treated lambs at first breeding season.

5.1. INTRODUCTION

Spermatogenesis is highly dependent on interaction between Sertoli cells and germ cells. The endocrine regulation of spermatogenesis is the result of actions of sex hormones on Sertoli cells rather than germ cells. Therefore, Sertoli cell number is the major limiting factor for spermatogenesis (Orth *et al.*, 1988). The critical role of thyroid hormones in the development of the testes has been demonstrated in neonatal rats (Cooke and Meisami, 1991), mice (Joyce *et al.*, 1993) and hamsters (Kirby *et al.*, 1993). Specifically, transient neonatal hypothyroidism (TNH) resulted in extension of neonatal Sertoli cell proliferation. In the hypothyroid rats and mice, increased number of Sertoli cells resulted in greater testis size and sperm cells production at sexual maturity (Hess *et al.*, 1993, Joyce *et al.*, 1993). On the other hand, neonatal hyperthyroidism decreased proliferation, but enhanced differentiation rate of rat Sertoli cells (van Haaster *et al.*, 1993). As well, serum follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels were reduced significantly in both hyper- and hypothyroid rats (van Haaster *et al.*, 1993, 1992). Cooke and Meisami (1991), found no significant difference in serum testosterone concentration among TNH and control rats at any particular age. Conversely, manipulations in the thyroid state of neonatal rats changed concentrations of thyroid stimulating hormone (TSH), insulin like growth factor-I (IGF-I), inhibin, prolactin (PRL) and growth hormone (GH)(van Haaster *et al.*, 1991, 1993, Kirby *et al.*, 1992). Therefore, changes in the behavior of the Sertoli cells were not mediated through alterations in the level of FSH and (or) testosterone and are believed to be due to the direct actions of thyroid hormones on the Sertoli cells.

While TNH has modified testicular size and function in some rodents, its influence on other species has not been extensively tested. This study was designed to investigate the importance of neonatal thyroid function in ram lambs by studying the influence of TNH on the profiles of testosterone, FSH, testicular development and function at the onset of puberty.

5.2. MATERIALS AND METHODS

5.2.1. Animals, housing and environment

Twelve Suffolk ram lambs, selected from a large flock after weaning, were randomly assigned to one of three treatment groups at 40 ± 2 d (6 week) of age and weighing 14.42 ± 0.63 kg (mean ± SEM). Lambs were housed in a mechanically ventilated room in groups of 4 in 180 × 300-cm pens on a concrete floor covered by wood shavings. Room light duration was adjusted weekly to correspond to the natural photoperiod. Lambs had free access to fresh water, were fed chopped hay and a barley based concentrate *ad libitum* and were maintained in accordance with the Canadian Council on Animal Care guidelines (1993). The ration was formulated to meet 1985 NRC requirements for fast growing lambs. Room and rectal temperatures were recorded thrice weekly during the experiment.

5.2.2. Treatments

To suppress normal thyroid function, 6-propyl-2-thiouracil (PTU) prepared as a 0.1% (wt/vol) solution with 1% sugar added for palatability was administered to the treatment lambs at the rate of 15 mg kg^{-1} BW d^{-1}. Due to longer duration and slower proliferation

rate of Sertoli cells in sheep compared to rats, the time period of induction of hypothyroidism was chosen to be either 40 d (PTU40, n = 4) or 55 d (PTU55, n = 4) from 42 d of age (week 6). Control lambs (n = 4) were given placebo (1% sugar solution). The total daily dose was divided into two equal parts administered by drench at 0800 and 1600 h for 40 and 55 d for PTU40 and PTU55, respectively.

5.2.3. Blood sampling

To measure the concentration of thyroxine (T_4), tri-iodothyronine (T_3), FSH, IGF-I and testosterone, blood was collected by indwelling jugular catheters once a week, at 20-min intervals from 0900 to 1500 h. The catheters were put in place according to the procedure explained by Sakurai *et al.* (1993). In order to avoid any detrimental effects of frequent blood sampling, hematocrit was determined every two-h during the sampling days. Blood samples were stored up to 16-h at 4°C. Serum was collected after centrifugation for 20 min at 2000 g (Joan CR 3000 centrifuge, Vinchester, VA USA) and stored at –20°C.

5.2.4. Other parameters

On a weekly basis, scrotal circumference (SC) of the lambs was measured with a measuring tape while the lambs were restrained in a sitting position. Scrotal wool was clipped off and the testes were pulled fully into the scrotum before measurement. Weekly body weight (BW) and feed intake were recorded until 36 weeks of age. Average daily gain (ADG), gonadosomatic index (GSI = SC/BW), and average daily DM consumption (DDM) were calculated. Total body fleece was removed once in July and adjustments

made to calculations for the reduction in body weight. Rectal temperature was taken three times a week throughout the experiment.

5.2.5. Semen collection and evaluation

Semen was collected by electro ejaculation (Bailey Ejaculator, Western Instrument Company Denver, CO) twice a week from 16 to 36 weeks of age and evaluated. The volume of the ejaculate was recorded and a semen sample was examined immediately with a light microscope to evaluate the quality of the semen. The microscope slides were pre-warmed (+ 30°C) to reduce cold shock to sperm cells. To determine the sperm concentration a 20-µl sample of the ejaculate was fixed in acetic acid and the number of sperm cells counted on a hemocytometer (Reichert Scientific Instruments, Buffalo, NY). Percentage of dead sperm cells were 6 ± 3 (mean $\pm$ std).

5.2.6. Castration of the lambs and testicular histology

At 36 week of age, rams were castrated surgically, the testicles weighed and histologic sections prepared by hematoxilin and eosin staining method to examine the number and diameter of seminiferous tubules (ST) and number of cell layers in the ST. Diameter of ST was determined using a calibrated microscopic slide (Carl Ziess, Germany). Number of cell layers were determined by counting the average number of cells standing in a column from the basal membrane to the lumen of ST in four different directions. Due to the large diameter of the testicles, it was not possible to count the total number of ST in the cross section of the testis, therefore the number of the ST per view field of the microscope were

counted at × 100 magnification and at least 10 view fields were observed to obtain the average number and diameter of ST. The longitudinal sections were avoided as much as possible.

5.2.7. Hormone concentrations and pulse calculations

Mean hormone values for each sheep were calculated from either the 4 hourly samples (for T_4, T_3, FSH, and IGF-I) or from the total 19 samples per sampling d (for testosterone). Pulses of testosterone were calculated according to the method described by Veldhuis *et al.* (1984). Veldhuis *et al.* (1984) modified the algorithm used by Merriam and Wachter (1982) and based their detection of pulses on the measurements of the hormone that exceeded the preceding local nadir by a value that was fourfold greater than the intra-assay coefficient of variation (CV) of the RIA. This method allows elimination of the noises produced by the error of the assay and leaves less false positive pulses compared to other methods. The ratio of T_4/T_3 during and after treatment periods were calculated.

5.2.8. Testosterone RIA

Concentrations of testosterone, expressed as ng ml^{-1}, were determined in duplicate by RIA following the procedure of Sanford (1974). The sensitivity of the assay at 90% maximum binding was 12.5 pg testosterone 500 μl^{-1}. The mean intra-assay CV calculated according to the Wilson and Lapwood (1979) procedure for high, medium, and low reference pools of serum were 9.9%, 9.2%, and 12.7% respectively. Mean inter-assay CV was 14.2%.

5.2.9. T_3 and T_4 RIA

Total T_4 and T_3 were measured by RIA using Coat-A-Count® kits [total $T_4$1 1081 and total $T_3$1 501 for T_4 and T_3 respectively, Diagnostic Products Corp. (DPC) Los Angeles, CA]. These procedures have been validated by others for sheep (Wrutniak *et al.*, 1985; Milter and Albyl, 1985). Mean intra-assay CV were 4.8% and 4.4% for T_4 and T_3 while inter-assay CV for T_4 and T_3 were 8.5% and 9.0% respectively. The values of T_4 and T_3 are expressed as µg and ng dl^{-1}, respectively. The sensitivities of the assays were 0.25 µg dl^{-1} for T_4 and 7 ng dl^{-1} for T_3.

5.2.10. FSH RIA

Serum samples were assayed for FSH according to the method described by Evans *et al.* (1992). The average intra- and inter-assay CVs were 3.1% and 7.8% respectively. Sensitivity of the assay was 0.1 ng ml^{-1}.

5.2.11. IGF-I ELISA

IGF-I concentrations were determined by using human IGF-I ELISA kits [Diagnostic Systems Laboratories, Inc. (DSL) Texas]. This method involves acid ethanol extraction (AEE) which has been confirmed to be the most reliable method (Breier *et al.*, 1991, Daughaday *et al.*, 1989). This technique was considered reliable for sheep IGF-I since the amino acid sequence of IGF-I is identical in the human and ovine IGF-I except for amino acid number 66 (Francis *et al.*, 1989). In addition, purified ovine and human IGF-I are similar in biological activity (Breier *et al.*, 1991, Francis *et al.*, 1989, McNeil, 1989,

Wallace *et al.*, 1989). For this study, the mean intra-assay CV for IGF-I was 9.0% and inter-assay CV was 5.6%. The values of IGF-I were expressed as ng ml^{-1}. The sensitivity of the assay was 0.03 ng ml^{-1}.

5.2.12. Statistical analysis

The experiment was a completely randomized design (CRD) with each lamb randomly assigned to one of the three groups. Data are reported as mean ± SEM unless stated otherwise. Statistical analysis included analysis of variance (ANOVA) and Student's t-test. To compare the hormone values at different ages within a group (i.e. different sampling week), mean hormone levels of each sampling d of the lambs in the same group were analyzed by ANOVA in a CRD and, if the difference was significant, the means were compared by least significant difference (LSD) test (Steel and Torrie, 1960) corrected to hold experimental error at 0.05. Hematocrit measurements from 0900 and 1500-h were analyzed using a paired t-test assuming equal variances. Correlation coefficients (Pearson product-moment) were calculated by using a computer software (Quatro-pro, version 5, Borland international, USA). Due to changes that occurred in the level of T_4 and T_3 during treatment period, correlation coefficients were calculated from the end of treatment to the end of experiment. Mean testosterone pulse frequencies of the three groups were calculated and compared throughout the sampling period. If the difference was significant, the pulses during the 6-h sampling period were compared in the three groups by using a split plot design.

5.3. RESULTS

5.3.1. Growth performance

Hematocrits were not different between 0900 and 1500 h in any of the experimental lambs throughout the experiment (P>0.1). Mean initial BWs of the lambs were similar at 12.7 ± 0.4, 13.5 ± 1.4 and 14.7 ± 1.6 kg for control, PTU40 and PTU55, respectively. Similarly, final BW was not different at 85.4 ± 3 kg for control, 78.7 ± 1.6 kg for PTU40 and 86.6 ± 0.6 kg for PTU55. Body weight and relative growth (RG; daily gain as a percentage of the total BW) of the control and treatment groups were similar throughout the experiment (P>0.1, Figure 19). Dry matter intake kg^{-1} BW and ADG were similar (P>0.1) among groups and within the acceptable range for rapidly growing ram lambs (NRC, 1985). Average rectal temperatures of the lambs in the three groups were similar throughout the experiment (P>0.1).

5.3.2. Testicular growth and GSI

The initial average SCs of the three groups were similar (12.5 ± 0.3, 12.3 ± 0.7 and 12.1 ± 0.5 cm for the control, PTU40 and PTU55, respectively) and they remained similar throughout the experiment (P>0.1, Figure 20). Mean final SCs were 37.5 ± 2.8 cm for the control, 38 ± 0 cm for the PTU40 and 38.8 ± 2.7 cm for the PTU55. Likewise, GSIs in the three groups were similar (P>0.1). Testicular weight measured immediately after the rams were castrated. The mean weights of the testicles were also similar (P>0.1).

5.3.3. Semen quality

Semen collection started at 16 and ended at 36 weeks of age (Table 1). The mean semen volumes of the last three consecutive ejaculations in the three groups were similar (P>0.1; 1.0 ± 0.1, 1.1 ± 0.1 and 0.9 ± 0.1 ml for control, PTU40 and PTU55, respectively). However, sperm cells concentrations were lower in the PTU treated lambs as compared to the controls [(2.07 ± 0.06) × 10^9 for control, (1.23 ± 0.17) × 10^9 for PTU40 and (8.6 ± 0.05) × 10^8 ml^{-1} for PTU55] (P<0.05).

5.3.4. Histology of the testis

Comparison of the testicular histology showed that STs in the control lambs were more developed than in the treated lambs. The diameter of the ST in the control lambs was larger (P<0.05; 200 to 300 μm in control vs 180 to 250 μm in PTU40 and PTU55) but, the diameter of the lumen was not different in all three groups (P>0.1). The total number of cell layers in the ST of the three groups was variable perhaps because they were in different stages of the seminiferous cycle (6 ± 0.9 layers of cells).

5.3.5. Testosterone concentrations and pulse frequency

Average concentration of testosterone throughout the experiment was similar in all groups (P>0.1; Figure 21). There was a positive correlation between testosterone concentration and age of the lambs in all groups (r = + 0.74 for control, + 0.81 for PTU40 and + 0.88 for PTU55) indicating effects of age on the testosterone concentration. However, at week

9, testosterone pulses were higher in PTU55 than PTU40 and control (a; $P<0.05$) and at week 11 testosterone pulses were higher in PTU55 than control (b; $P<0.05$; figure 22). Mean testosterone pulse frequencies of the 3 groups during the 6-h sampling period were similar throughout the experiment ($P>0.1$, Figure 22). There were positive correlations between testosterone concentration and testosterone pulses ($r = + 0.68, + 0.21 + 0.26$ in control, PTU40 and PTU55 respectively).

5.3.6. FSH concentrations

FSH concentration was higher in control than the treatment groups from 7 to 12 and at 21 week of age (a; $P<0.05$). However, from week 23 to the end of experiment, FSH concentration was higher in control than PTU55 (b; $P<0.05$) only ($P>0.1$; Figure 23). The pattern of the FSH profile was the same in the three groups and there was a positive and significant correlation between FSH and the age of the lambs ($r = + 0.77, + 0.87$ and $+ 0.75$ for control PTU40 and PTU55 respectively), and between FSH and testosterone concentration ($r = + 0.56$ for control, $+ 0.84$ for PTU40 and $+ 0.79$ for PTU55).

5.3.7. IGF-I concentrations

Levels of IGF-I were similar among the groups except at week 13 when IGF-I was lower in PTU55 than control lambs (914 ± 25.5, 737 ± 17.6 and 497 ± 30 ng ml^{-1} for control, PTU40 and PTU55 respectively; $P<0.05$). The effect of age on IGF-I concentration was different in the treatment groups than control ($r = - 0.26$ for control, $+ 0.40$ for PTU40

and + 0.18 for PTU55, P<0.01; Figure 24). The pattern of changes in the level of IGF-I was bi-phasic and similar in the three groups.

5.3.8. Concentrations and ratio of T_4 and T3

Level of T_4 was higher in the control during the treatment period (from week 7 to week 11 and 14 in the PTU40 and PTU55, respectively; P<0.01; Figure 25). Concentrations of T_3 were higher (P<0.05) in control than PTU40 from week 8 to week 11 and from week 8 to week 12 of age in PTU55 (Figure 26). Levels of both hormones were similar at all other points (P>0.1). The ratio of T_4/T_3 during the treatment period was different in the 3 groups (P<0.05; 38.9 ± 2.5, 16.8 ± 4.5 and 14 ± 3.2 in control, PTU40 and PTU55, respectively). After treatment through to the end of the experiment the ratio was similar in the three groups (P>0.1) and were 43.9 ± 2.4 in control, 45.4 ± 2.4 in PTU40 and 47.9 ± 1.7 in PTU55. A negative correlation existed between thyroid hormones and age of the lambs from the end of treatment to the end of experiment ($r = -0.8$ in the control, -0.44 in the PTU40 and -0.76 in the PTU55 for T_3 and $r = -0.66$ in the control, -0.71 in the PTU40 and -0.2 in the PTU55 for T_4).

5.4. DISCUSSION

The transient depression of T_4 and T_3 during PTU treatment indicated successful suppression of thyroid function. However, similarity between ADG, DDM and RG of the three groups demonstrated that thyroid gland function was not suppressed to the point where animal performance deteriorated. In PTU treated hypothyroid rats, BW was reduced by 50% (Meisami *et al.*, 1992, Cooke and Meisami 1991) to 91% (Meisami *et al.*, 1994). Those rats had been treated with 175 mg of PTU kg^{-1} BW d^{-1} which suppressed thyroid function, BW and water intake. Later, it was demonstrated that 10.5 mg kg^{-1} BW d^{-1} could produce the same suppressive effects on the thyroid gland without suppressing animal performance (Cooke *et al.*, 1993). Rams in the present study were dosed with 15 mg kg^{-1} BW d^{-1} PTU.

Cessation of the PTU treatment was followed by return of the thyroid hormone concentrations to levels similar to control lambs. PTU was more effective in suppressing T_4 than T_3, so that during treatment, the ratio of T_4/T_3 was lower in the treatment groups than control in the following order control>PTU40>PTU55. This ratio was similar in the three groups at any other period which is consistent with the findings of Kirby *et al.* (1992).

Low levels of thyroid hormones might have increased the proliferation period and suppressed maturation of Sertoli cells of the treated lambs. This was the case in rats (Cooke *et al.*, 1992) and mice (Joyce *et al.*, 1993) which had a 180% increase in their testicular size. On the other hand, in the three groups of lambs, SC was similar throughout the experiment. Increase in the testicular size of rats was determined to be the result of a

higher number of Sertoli cells (van Haaster *et al.*, 1992, Cooke *et al.*, 1992). Labeling index of rat Sertoli cells during the fast proliferation period is 10 times more than that of the bull calf (Cooke *et al.*, 1994a) which may be similar to sheep. Therefore, the period of induction of TNH in the ruminants should be longer than in rats. The optimum time period for the induction of TNH in the rats were determined to be 20 d in length (from 4 to 24 d of age; Cooke and Meisami, 1991). In the present experiment, lambs were treated for 40 and 55 d (in the PTU40 and PTU55, respectively). Therefore, lack of change in the testicular size at the time of puberty in the hypothyroid lambs might be due to the short duration of treatment as well as low dose of PTU and (or) inappropriate time window for the PTU treatment. However, PTU treatment did suppress thyroid function and influenced some of the indices of testicular function.

Low levels of thyroid hormones during the treatment period were associated with low FSH production. Reduced FSH level was continued for 18 weeks after the treatment in the PTU55 group but, testosterone concentration was not different at any period in the three groups. Similar findings have been reported by Francavilla *et al.* (1991) and Cooke and Meisami (1991) in rats. It seems that treatment of the lambs for a longer duration in PTU55 group, was the major reason of suppression of FSH secretion. The fact that Sertoli cell proliferation is maximal postnatally at an early age in the ram (Gondos and Berndston, 1993) suggests that although treatment might have been started late, it was still within the proliferation and (or) maturation time window of Sertoli cells. Suppression of sperm cells production in the treated adult lambs may have been due to the direct effects of

hypothyroidism and (or) low levels of FSH (Orth and Christensen, 1978) which were also due to direct actions of hypothyroidism on the pituitary (Cooke *et al.*, 1994 b).

That hypothyroidism reduced sperm production of the postpubertal treated lambs was a reasonable indication of delayed gonadal maturation (Cooke and Meisami, 1991, Cooke *et al.*, 1991). Francavilla *et al.*(1991) also induced delayed maturation of ST and a permanently lowered level of FSH in TNH rats. To be able to support and nourish large numbers of sperm cells, Sertoli cells of the control lambs were likely more mature than those of the treated lambs. Although sperm cells concentration was reduced in both treatment groups, the reduction was greater in PTU55 than PTU40 lambs, a further indication that the induced transient hypothyroidism affected seminiferous tubular function.

Berndtson *et al.* (1987) demonstrated that the larger (and more mature) ST produce more sperm cells. Moreover, presence of a positive correlation between daily sperm production and maturity of Sertoli cells has been proven (Monet-Kuntz *et al.*, 1984). Therefore, larger ST and higher sperm cells concentrations in the control lambs were likely due to a higher state of maturity of the Sertoli cells in the control lambs. The results suggest that low T_3 and T_4 concentrations delay maturity of Sertoli cells which is consistent with the results of Russell *et al.*(1989) in hypothyroid rats.

Since both high (van Haaster *et al.*, 1993) and low (Kirby *et al.*, 1992) levels of T_3, early in the life, decreased FSH levels, it seems that any changes in the neonatal thyroid state of the animals modifies concentration of the gonadotropin. The mechanism of the reduction in FSH levels of the rats was not understood and is still unclear. Moreover,

it was not known if low levels of FSH was the result of modifications in the level of inhibin or a direct effect of T_3 on the higher brain centers. van Haaster *et al.* (1992) found that serum inhibin levels were also reduced up to d 30 in hypothyroid rats and conceivably, FSH levels should be higher. However, FSH levels were lower and they concluded that gonadotropin production was reduced as a result of a direct effect of hypothyroidism on pituitary function. Cooke *et al.* (1995) stated that reduction in the circulating gonadotropins appears to be due to either a reduced responsiveness to GnRH stimulation or an overall decrease in the synthetic capacity of gonadotropes in the pituitaries of treated males.

Although FSH concentration was reduced in the treated lambs, level of serum testosterone was not changed. Similar results were reported by Palmero *et al.* (1992), Hardy *et al.* (1993), Cooke *et al.* (1994 a), and Cooke and Meisami (1991) in the hypothyroid rats. Palmero *et al.* (1989) found that Sertoli cells of the more mature ST produced more androgen binding proteins which bound to testosterone and increased testosterone concentration in the ST micro environment. Therefore, it is possible that testosterone concentrations were reduced in the ST of the treatment lambs thereby, reducing spermatogenesis.

Although, during the treatment period, the level of IGF-I was generally lower in treated lambs, the difference was not significant throughout the rest of the experiment except at week 13 between control and PTU55. Samuels *et al.* (1989) reported a reduction in GH mRNA level in the pituitary gland of hypothyroid rats. It was also found that low levels of thyroid hormones caused a reduction in the GH enhanced IGF-I

production in the rat liver (Martinoli and Pelletier, 1989, McGuire *et al.*, 1992). Palmero *et al.* (1990, 1991) reported that TNH resulted in a severe impairment of IGF-I production by cultured rat Sertoli cells. Since at week 13, level of IGF-I was lower in the PTU55 lambs than controls, it can be suggested that IGF-I concentration was reduced due to direct (on GH) and (or) indirect effects (on IGF-I) of PTU and if PTU treatment was of longer duration, IGF-I concentration may have been affected more severely. Due to the fact that thyroid hormones are influential in the local production of IGF-I in the Sertoli cells (Palmero *et al.*, 1990, 1991), it is possible that the level of IGF-I was reduced in the Sertoli cells environs of the treated lambs. Therefore, these cells would have been deprived of the mitogenic effects of IGF-I which may be the cause of the lower spermatogenesis in treated lambs.

5.5. Conclusion

PTU treatment at the rate of 15 mg kg^{-1} BW d^{-1} in Suffolk ram lambs reduced thyroid hormone levels but, did not affect general body growth performance. This dose of PTU was enough to reduce the level of FSH indicating the suppressive effects of TNH on gonadotropin production. Despite the reduction in the level of FSH, TNH failed to change testosterone concentration but it changed testosterone pulse frequencies in the Suffolk ram lambs. However, hypothyroid lambs had lower sperm concentration than the contemporary control lambs. Low sperm cells counts were accompanied by histological changes in the testis of the treated lambs, indicating the negative effects of TNH on testicular function. Conversely these results implicate the importance of thyroid hormones

in normal gonadal development and subsequent adult testicular function. Changes in the chronology of the reproductive organ developments in the Suffolk ram lambs could be due to the direct effects of thyroid hormones on the Sertoli cells and (or) mediated through pituitary secretion of FSH. Although, during the treatment period, mean serum IGF-I concentration was changed slightly, the effects of TNH on the production of the IGF-I by Sertoli cells are not known at this time. These results help us to have a better understanding of the critical role of the thyroid hormones in early development of the male gonads.

Table 1. Observation of the first viable sperm cells in all the lambs of the three groups.

	First lamb	Second lamb	Third lamb	Fourth lamb
Control	July 21	August 18	August 25	September 26
PTU40	August 18	August 18	September 26	September 26
PTU55	July 21	July 25	August 4	August 18

Figure 19. Relative growth in the control □, PTU40 ■ and PTU55● lambs throughout the experiment. PTU treatment started at week 6 and stopped at week 12 or week 14 for PTU40 and PTU55, respectively. There were no differences in the relative growth among the three groups (P>0.1).

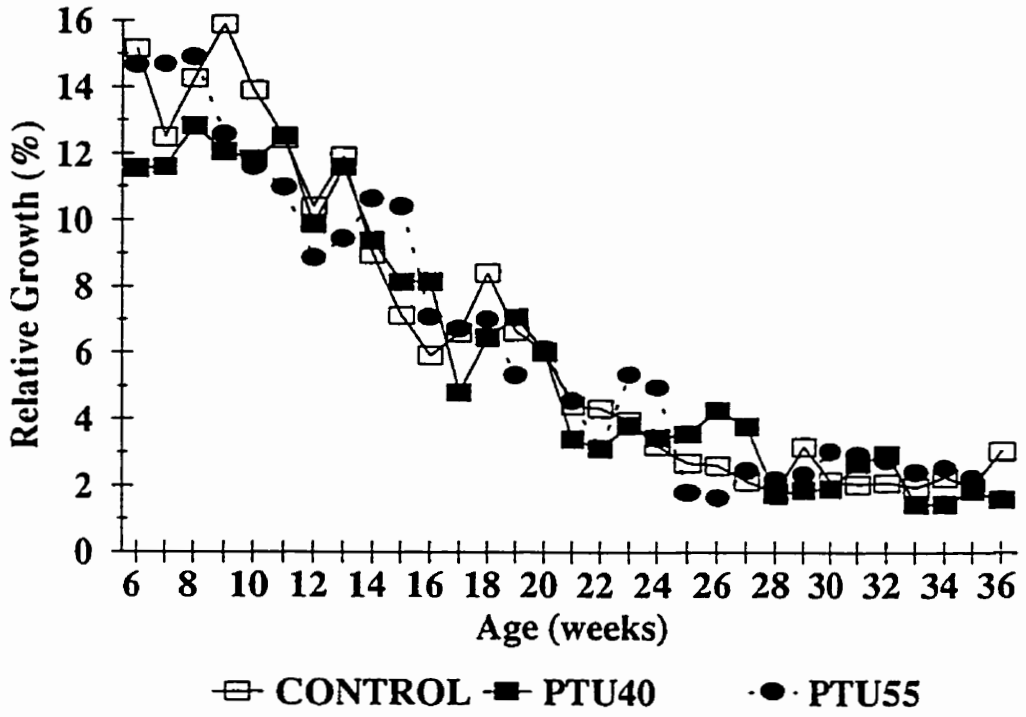

Relative Growth (%)
16
14
12
10
8
6
4
2
0
6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
Age (weeks)
CONTROL
PTU40
PTU55

Figure 20. Scrotal circumference in the three groups during the experiment. The growth of the scrotal circumference in the control □, PTU40 ■ and PTU55● lambs were not different at any period throughout the experiment (P>0.1). PTU treatment started at week 6 and stopped at week 12 or week 14 for PTU40 and PTU55, respectively.

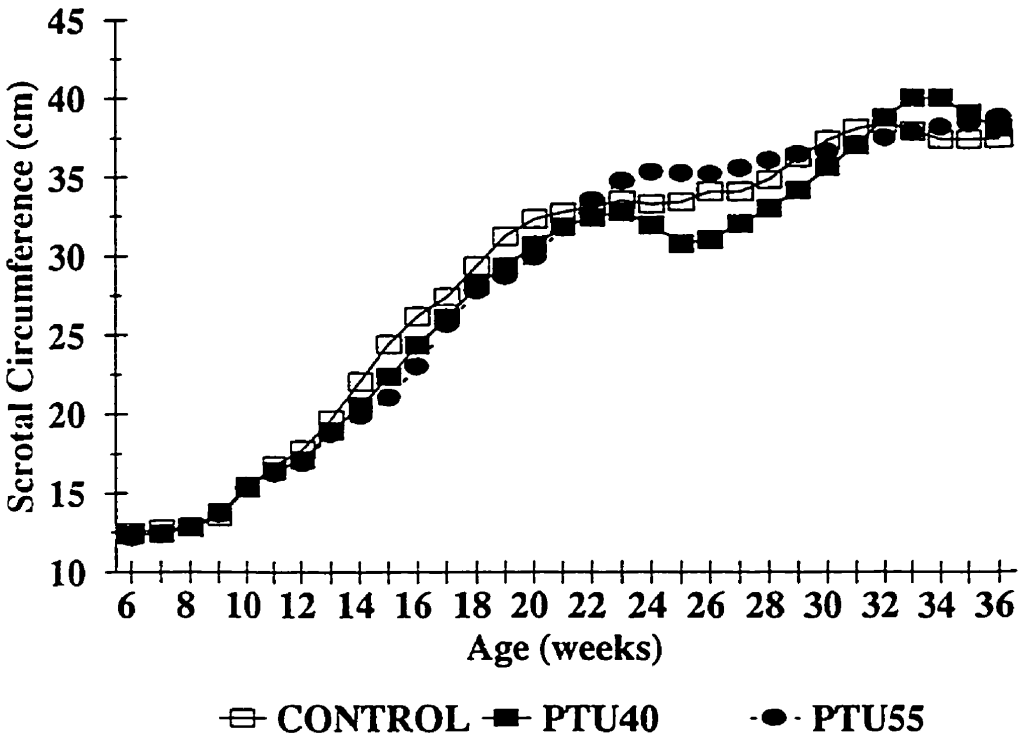

Scrotal Circumference (cm)
45
40
35
30
25
20
15
10
6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36
Age (weeks)
CONTROL
PTU40
PTU55

Figure 21. Mean serum testosterone concentration throughout the experiment. Average concentration of testosterone were similar in the control □, PTU40 ■and PTU55● lambs (P>0.1). PTU treatment started at week 6 and stopped at week 12 or week 14 of age for PTU40 and PTU55, respectively.

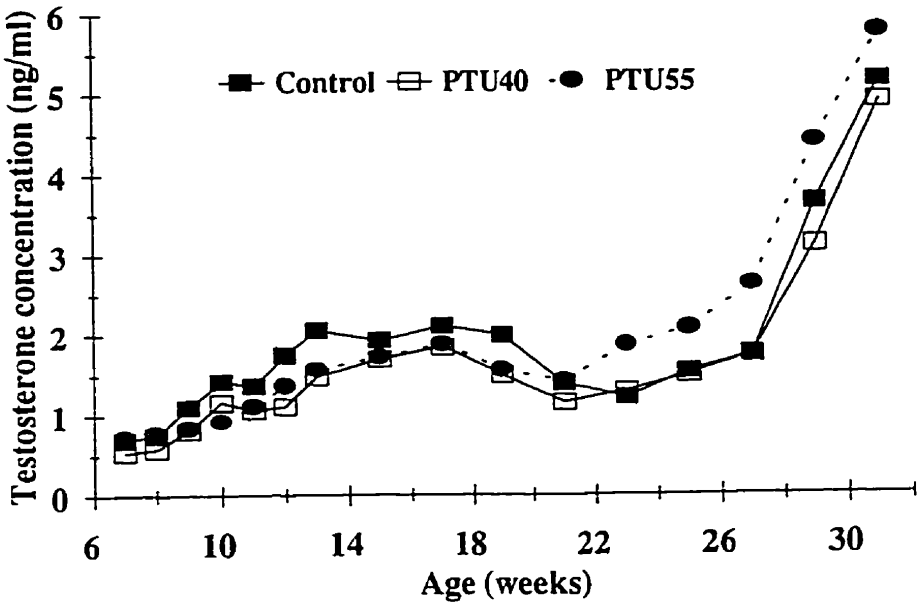

Testosterone concentration (ng/ml)
0
1
2
3
4
5
6
Control
PTU40
PTU55
6
10
14
18
22
26
30
Age (weeks)

Figure 22. Frequency of testosterone pulses in weekly 6-h sampling period d^{-1} during the experiment. At week 9 pulses were higher in PTU55 than PTU40 and control (a; $P<0.05$) and at week 11 pulses were higher in PTU55 than control (b; $P<0.05$). PTU treatment started at week 6 and stopped at week 12 or week 14 for PTU40 and PTU55, respectively.

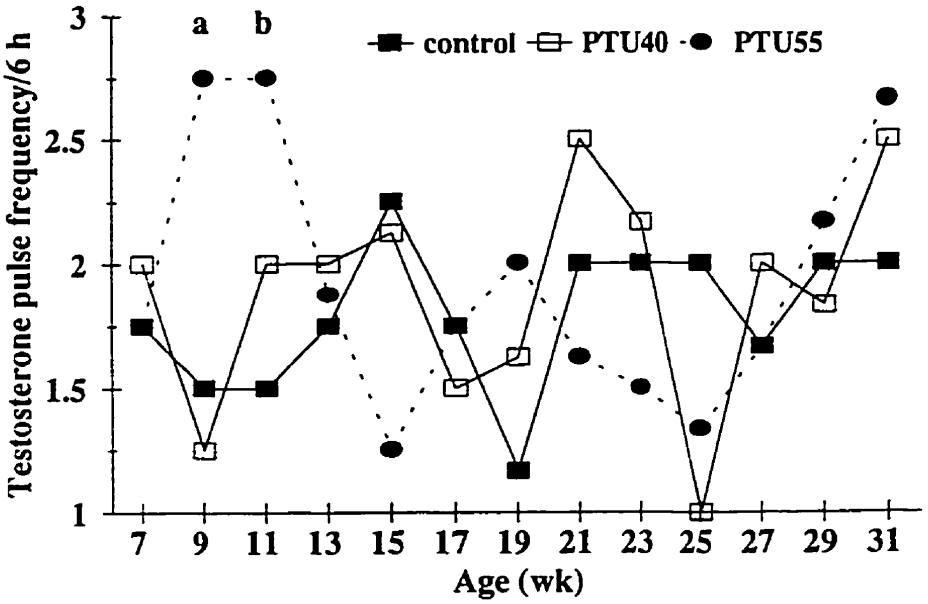

a
b
control
PTU40
PTU55
Testosterone pulse frequency/6 h
3
2.5
2
1.5
1
7
9
11
13
15
17
19
21
23
25
27
29
31
Age (wk)

Figure 23. Serum FSH concentration in the control □, PTU40 ■ and PTU55● lambs. FSH concentrations was higher in control than PTU40 and PTU55 (a = $P<0.05$) and higher in control than PTU55 (b = $P<0.05$). PTU treatment started at week 6 and stopped at week 12 or week 14 of age for PTU40 and PTU55, respectively.

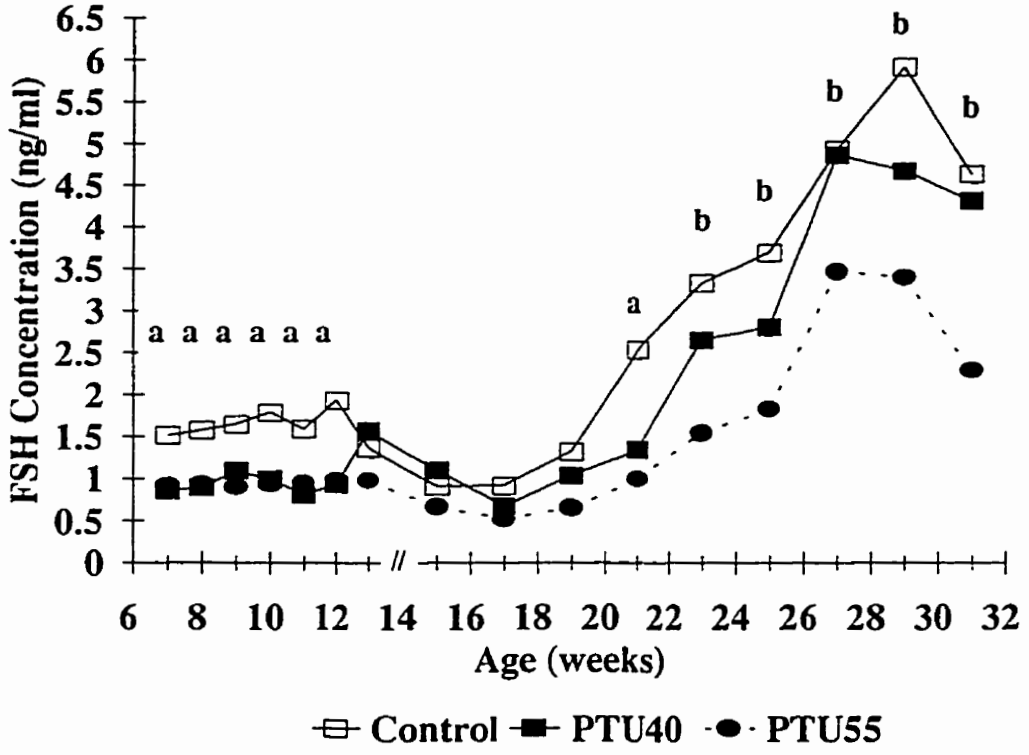

FSH Concentration (ng/ml)
6.5
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
a a a a a a
a
b
b
b
b
b
6 8 10 12 14 16 18 20 22 24 26 28 30 32
Age (weeks)
Control
PTU40
PTU55

Figure 24. Serum concentrations of IGF-I during the sampling period in the three groups. IGF-I concentration was similar in the control □ and PTU40 ■ lambs (P>0.1). Only at week 13 of age, was IGF-I level lower in the PTU55 ● than control lambs. ✳ indicates difference among the treatment groups at P<0.01. PTU treatment started at week 6 and stopped at week 12 or week 14 of age for PTU40 and PTU55, respectively.

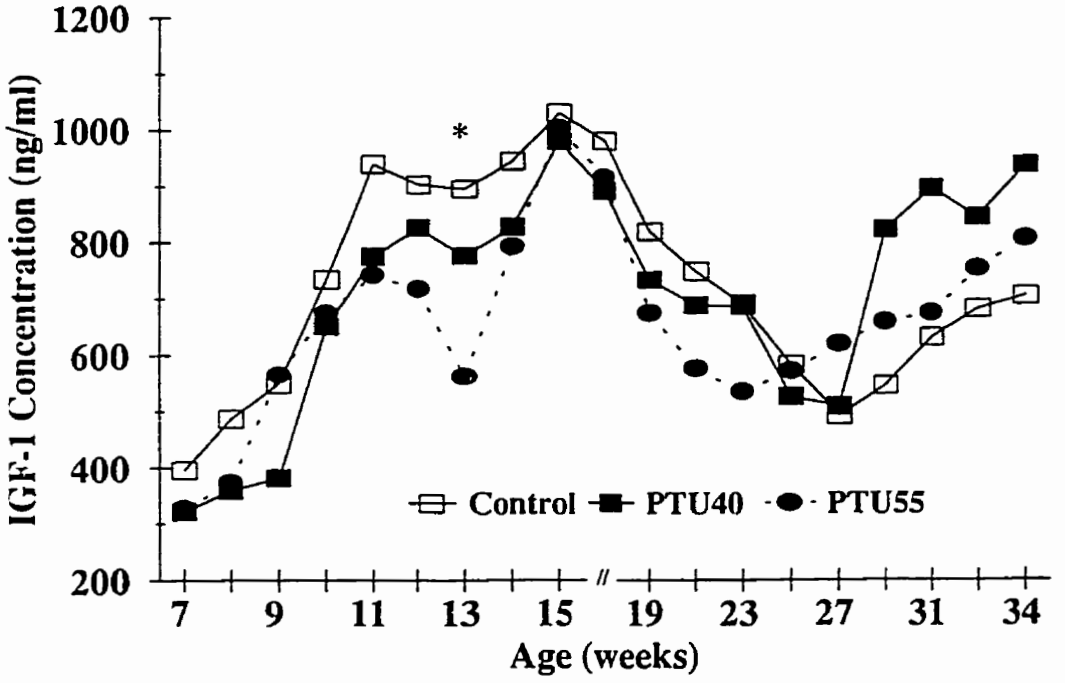

1200
1000
800
600
400
200
IGF-1 Concentration (ng/ml)
*
Control
PTU40
PTU55
7
9
11
13
15
19
23
27
31
34
Age (weeks)

Figure 25. Serum T_4 concentrations in the control □, PTU40 ■ and PTU55● lambs. T_4 levels were lower in the PTU40 and PTU55 than control lambs (a = $P<0.05$). At week 12, 13 and 14, T_4 was higher in control than PTU55 only (b = $P<0.05$). PTU treatment started at week 6 and stopped at week 12 or week 14 of age for PTU40 and PTU55, respectively.

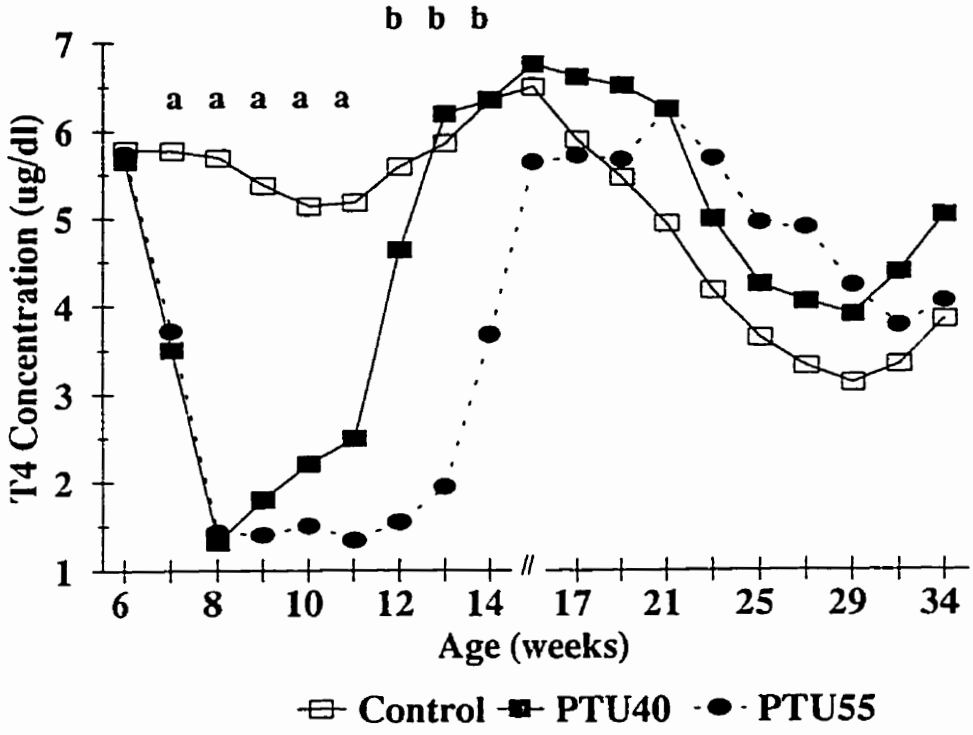
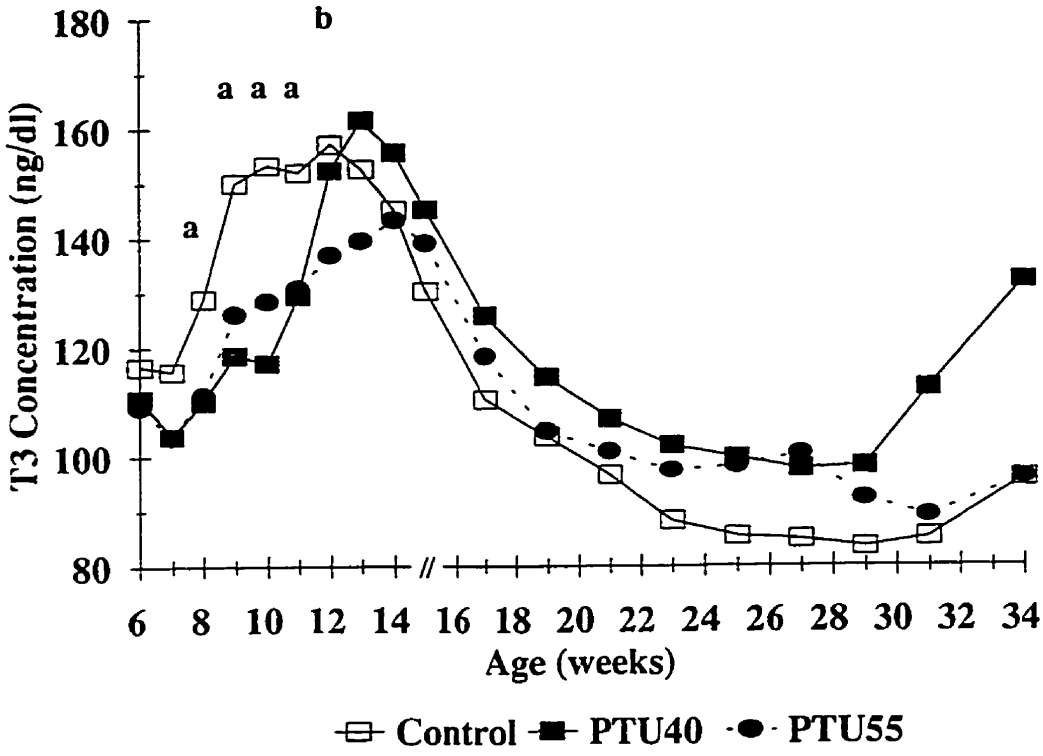

b b b
a a a a a
7
6
5
4
3
2
1
T4 Concentration (ug/dl)
6 8 10 12 14 17 21 25 29 34
Age (weeks)
Control PTU40 PTU55

Figure 26. Serum T_3 concentrations throughout the experiment in the control □, PTU40 ■ and PTU55● lambs. T_3 concentration was higher in the control than PTU40 and PTU55 (a = $P<0.05$). At week 12, T_3 was higher in control than PTU55 only (b = $P<0.05$). PTU treatment started at week 6 and stopped at week 12 or week 14 of age for PTU40 and PTU55, respectively.


180
160
140
120
100
80
T3 Concentration (ng/dl)
a
a a a
b
6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
Age (weeks)
Control
PTU40
PTU55

6. GENERAL DISCUSSION

Recently, considerable research effort has been directed towards determining the effects of thyroid hormones on reproductive processes. The role of thyroid hormones on the testicular physiology is far from clear (Cooke *et al.*, 1994 a). The direct or indirect effects of iodothyronines on the testis are highly dependent upon the age of the animal. During the perinatal period in many species the hypothalamic-pituitary-thyroid axis is more active than the hypothalamic-gonadal axis (Hadley, 1992). Moreover, manipulation of pubertal reproductive parameters by induction of transient neonatal hypo- and hyperthyroidism in male rats indicates a critical role of thyroid hormones in the development of the male gonads (Cooke *et al.*, 1994a). In the current research, results of the first experiment provided a clue to the possible relationship between thyroid hormones and testicular function in sheep. Higher T_3, T_4, IGF-I, testosterone concentration andtestosteronepulses concomitant with higher testicular function and higher prolificacy in the Out than in Sfk rams support the concept of interrelationships. Higher prolificacy is accompanied by greater testosterone pulse frequency (Sanford *et al.*, 1982) and concentrations of testosterone (Land, 1978) which may be the reason for greater testicular activity in Out which attained earlier pubertal testicular function than Sfk ram lambs. Direct effects of thyroid hormones on the production and secretion of GH (Casanova *et al.*, 1985) and FSH (Kirby *et al.*, 1992) by the pituitary gland and production of inhibin, ABP and IGF-I (Bunick *et al.*, 1994, Kirby *et al.*, 1992, Palmero *et al.*, 1989) in the testes during perinatal

and peripubertal life, suggest a role for thyroid hormones in male reproduction. There is considerable evidence for a critical role of the thyroid hormones in Sertoli cell proliferation and differentiation in neonatal rats (Meisami *et al.*, 1992), mice (Joyce *et al.*, 1993), and hamsters (Kirby *et al.*, 1993). Hepatic cytochrome P-450 enzymes, involved in steroid metabolism in males (Waxman *et al.*, 1989) inactivate testosterone. Opposing actions of thyroid hormones and GH on synthesis of these enzymes by hepatocytes is one way in which efficacy of the androgens are modified. GH increases hepatic production of P-450 enzymes while thyroid hormones suppress their production (Yamazoe *et al.*, 1990). Moreover, van Haaster *et al.* (1993) demonstrated that high levels of thyroid hormones, early in life, enhances Sertoli cell differentiation while Panno *et al.* (1994) found that transient neonatal hypothyroidism delays puberty by decreasing efficacy of androgens. In other words, high levels of thyroid hormones enhance the efficacy of androgens which might be the reason for enhanced puberty in Out rams. As indicated earlier, thyroid hormones enhance production of ABP which increases androgen concentration in the ST (Martin *et al.*, 1991) and provides the suitable environment necessary for spermatogenesis (Palmero *et al.*, 1989) and nourishment of the maturing germ cells (Mita *et al.*, 1982). Therefore, it may be postulated that in Out lambs Sertoli cells mature at a younger age than Sfks with consequent enhancement of spermatogenesis and puberty onset.

Reduction of FSH production by thyroid hormones is both direct, through actions on the gonadotrophs (Kirby *et al.*, 1992) as well as indirect by modification of inhibin production by the Sertoli cells (van Haaster *et al.*, 1992). However, Maruo *et al.* (1987) demonstrated that thyroid hormones amplify the actions of FSH on cultured porcine

granulosa cells which adds to the complexity of interactions between pituitary, thyroid and gonads.

A rise in the sex steroids is accompanied by a discharge of GH (Wilson, 1986; Copeland *et al.*, 1985) whereas low levels of thyroid hormones in rats reduces GH production (Samuels *et al.*, 1989). Hence, in Out ram lambs with highertestosteroneand thyroid hormones, higher concentrations of GH would be expected. In our experiment IGF-I which is the main mediator of GH on somatic cells was measured. However, production and secretion of IGF-I in Sertoli cells is controlled by thyroid hormones (Smith *et al.*, 1987). For earlier maturation of Sertoli cells in the Out than the Sfk lambs three major factors could be involved. 1) By considering the mitogenic effects of IGF-I, it may be speculated that high levels of thyroid hormones promote spermatogenesis by increasing IGF-I concentrations in the seminiferous tubule micro environment. In our study, Out lambs had higher IGF-I and sperm concentrations than Sfk ram lambs. 2) Higher concentrations of thyroid hormones enhance production of ABP, thereby increasingtestosteroneconcentration in the ST. Testosterone stimulates spermatogenesis by direct actions on the germ cells (Ashdown and Hafez, 1993). 3) Direct effects of thyroid hormones on the Sertoli cell proliferation and differentiation enhances spermatogenesis (Cooke *et al.*, 1994a). Therefore, due to a combination of these reasons, Out ram lambs initiated spermatogenesis earlier than Sfk.

Secretion of GH is pulsatile in nature (Hadley, 1992) but due to the presence of high levels of binding proteins (Butler and Gluckman, 1986) and a long biological half-life (Hall and Sara 1984), the IGF-I level is relatively constant with no obvious diurnal rhythm

in humans (McGuire *et al.*, 1992). Circulating concentrations of IGF-I are variable in ruminants (McGuire *et al.*, 1992) but not pulsatile in young steers (Breier *et al.*, 1986, 1988), pigs (Sillence and Etherton, 1986) and rats (Donaghue *et al.*,1990). Use of a moving average as suggested by Merriam and Wachter (1982) could reduce effects of the assay error but the data would not represent real concentrations of the hormone, therefore, it was not used in our analysis. At first glance, high fluctuations of IGF-I during the 6 h sampling period resembled real pulses. Consequently, these data were analyzed using the methodology described by Veldhuis *et al.* (1984). Frequent serum samples (every 20 min for 6 h) from Out and Sfk lambs with either high and (or) low quality semen revealed no episodic release of IGF-I during the peripubertal periods.

In the second experiment (manuscripts II and III), effects of hypo- and hyperthyroidism were examined. In both hypothyroid groups, during the treatment period, the levels of thyroid hormones were significantly lower than in the control, indicating the dose of PTU was sufficient and lambs were hypothyroid. It is conceivable that the minimum dose (10.5 mg $kg^{-1}d^{-1}$) suggested by Cooke *et al.* (1993) is safer than the high dose (175 mg $kg^{-1}d^{-1}$) which is routinely used in rats because it has fewer negative side effects. In hypothyroid lambs, during the treatment period, lower T_4/T_3 ratio was an indication of more suppressive effects of the goitrogen on T_4 production than T_3, which should be considered in the future experiments. In the hyperthyroid group, simultaneous use of T_4 and T_3 maintained the T_4/T_3 ratio in an acceptable range but in the first week after treatment ended, T_4 and T_3 levels were depressed likely due to residual depressor effects of the treatment on thyroid function and on TSH and (or) TRH release (Hadley,

1992). Normal T_4/T_3 ratio in man is approximately 50 (Hadley, 1992) and in the hypo- and hyperthyroid lambs, after the treatment period the ratio was between 48 and 44.

Although the RG tended to be slower in the PTU+H than control lambs, there were no significant differences in RG, ADG between the groups, meaning that unlike to findings with rats (Cooke and Meisami, 1991), PTU treatment did not affect body performance negatively. Reduction of BW in rats was due to negative side effects of high dose of PTU treatment (175 mg kg^{-1} BW d^{-1}). Rams in the present study were dosed with 15 mg kg^{-1} BW d^{-1} PTU. In the PTU55 (experiment 3), the level of IGF-I at week 13 was lower than control but not at any other week. This did not have any impact on body performance but was an indication of the side effects of PTU treatment on the GH-IGF-I axis. The IGF-I level returned to normal immediately after cessation of treatment but if this period was longer there might be a decrease in ADG and (or) BW similar to what has been reported in hypothyroid rats (Meisami *et al.*, 1992, Cooke and Meisami, 1991). Because of the reduction of IGF-I in the PTU+H and PTU55 during the treatment period, it seems that any change in the natural balance of thyroid hormones has a negative impact on IGF-I concentrations.

In spite of the fact that testicular function was altered to some degree, there was no change in the size of the testes in pubertal hypothyroid sheep. Why the testicular size did not change in the hypothyroid lambs is not clear. Perhaps the dose or duration of the treatment was insufficient. The PTU dose was sufficient to suppress thyroid gland because the level of T_4 was 4 times less than the control but PTU treatment did not completely abolish thyroid function. If the duration of treatment were to be longer than 55 days it

would be better to start treatment at a younger age because in sheep Sertoli cell proliferation begins during the perinatal period and reaches a maximum early in postnatal life (Gondos and Berndston, 1993). However, further experiments are needed to find the optimum window for PTU treatment in sheep. If the rate of sheep Sertoli cell multiplication is lower than rats, then the duration of the treatment period should be comparatively longer in sheep. There are many problems associated with prenatal PTU treatment in sheep. Therefore, to affect maximum number of Sertoli cells and increase their final number, it seems that treating lambs immediately after birth is a better option. Although hypothyroidism did not change testicular size in the experimental lambs, transient hyperthyroidism increased testicular size and enhanced testicular function. These results are contrary to those in rats (van Haaster *et al.*, 1993 and 1992). In the hyperthyroid lambs, as in rats, intensification and augmentation of the testicular function was obvious by higher concentrations of sperm and higher testosterone pulses. However, in hyperthyroid rats the size of the testicles were reduced (van Haaster *et al.*, 1993). It could be assumed that the number of Sertoli cells were not changed in the hypothyroid lambs.

Higher sperm counts in the hyperthyroid lambs, indicates that the treatments enhanced maturation of Sertoli cells in the hyperthyroid ram lambs. conversely, lower sperm counts in the hypothyroid rams suggest delayed differentiation of Sertoli cells. Conceivably, Sertoli cells of PTU+H lambs were in more advanced stages of maturation to support and nourish large numbers of sperm cells than the controls, PTU40 and PTU55 lambs. As far as ST development is concerned, ST of the PTU55 were the least

developed, exhibiting effects of prolonged thyroid suppression on testicular development early in life. Maturation of Sertoli cells in the ST environs is the key to the onset of male puberty while IGF-I (Palmero *et al.*, 1990, 1991), ABP (Martin *et al.*, 1991),testosteroneconcentration andtestosteronepulses (Renaville *et al.*, 1993) are the combinations of the lock. During the treatment period, in both hyper- and hypothyroid lambs, serum concentrations of IGF-I were lower but, testicular levels of the somatomedin are unknown. Thyroid hormones could increase IGF-I production in the rat Sertoli cell culture (Palmero *et al.*, 1992).

Testosterone concentrations were not different at any period in either hypo- or hyperthyroid lambs. No change was also found in hypothyroid rats (Francavilla *et al.*, 1991, Cooke and Meisami, 1991). Testicular concentrations of testosterone are unknown. However, higher testosterone concentrations in the ST (Martin *et al.*, 1991) could enhance spermatogenesis (Palmero *et al.*, 1989; Mita *et al.*, 1982). Similarly, higher testosterone pulse frequency might have contributed to the advanced maturation in the hyperthyroid lambs. However, early in life, Sertoli cells are the main cells in the testis possessing thyroid hormone receptors (Palmero 1988, 1989; Ulisse, 1992). Therefore, it is considered that testosterone production by Leydig cells is not changed by alterations in the thyroid state (Palmero *et al.*, 1992, Hardy *et al.*, 1993, Cooke *et al.*, 1994 a).

The main gonadotropin affecting Sertoli cells is FSH (Orth and Christensen, 1978). Although FSH is required for the onset of spermatogenesis, in the adult animal much of the role of FSH is transferred to testosterone. Both of these hormones exert their effects on spermatogenesis by acting on their Sertoli cell receptors. There is no reported proof for

the presence of androgen receptors in germ cells (for review refer to Griswold, 1995). Transient low levels of FSH in the hyper- and hypothyroid lambs is consistent with the results of van Haaster *et al.* (1993) in hyper- and Cooke *et al.* (1994 b) in hypothyroid rats except that it remained permanently low in the rats. Accordingly, any change in the neonatal thyroid state may reduce FSH concentrations. Therefore, enhancement in the maturation of the ST was unlikely to have been regulated by alterations in the FSH concentrations and were due to the direct effects of thyroid hormones on the Sertoli cells. However, van Haaster *et al.* (1992) concluded that changes in FSH levels in hypothyroid rats were due to the direct effects of thyroid hormones on the pituitary gland. In the PTU55 lambs, FSH concentration was lower than in controls even weeks after treatment was over which is similar to the findings in rats (Kirby *et al.*, 1992). Both the hypothalamus and the pituitary may be involved in the mechanisms by which production of FSH is altered. Although FSH concentration was lower in PTU55 than the other three groups, it did not affect testosterone concentration.

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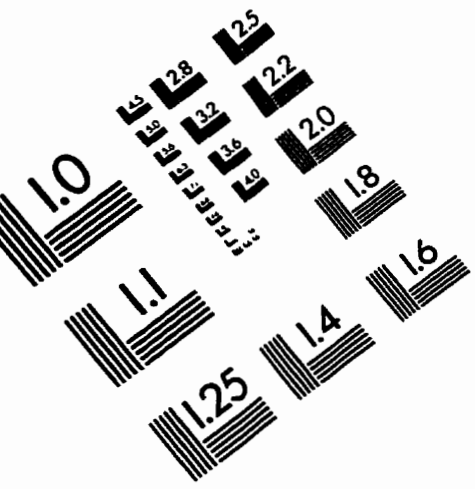
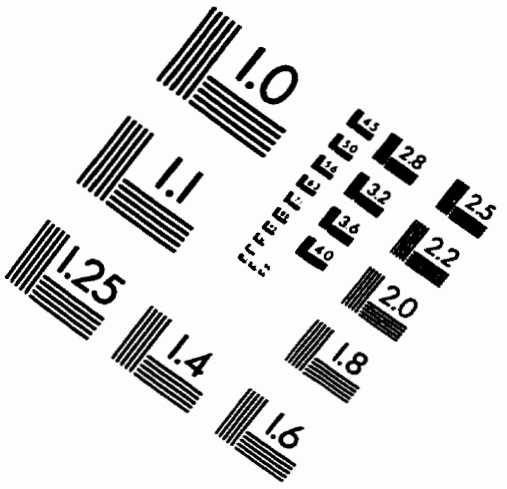
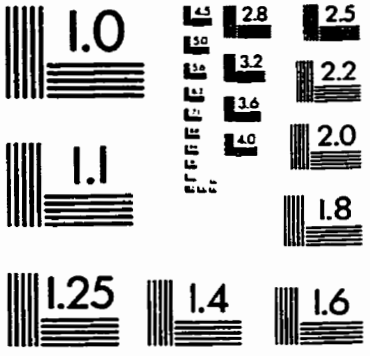
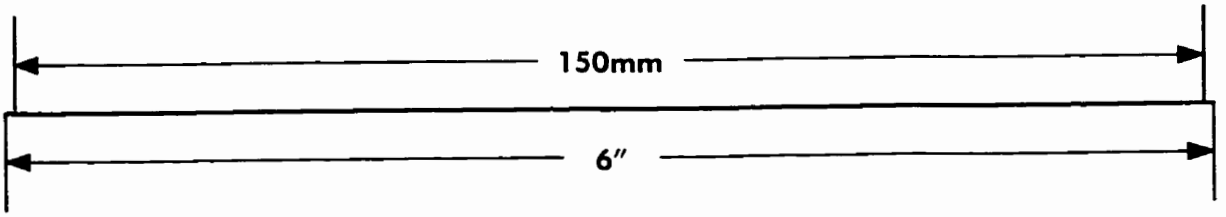
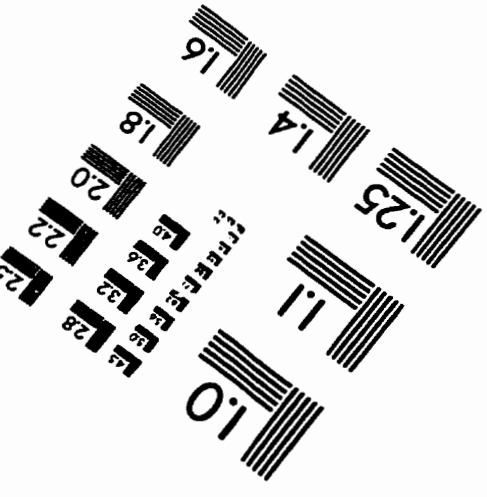
IMAGE EVALUATION
TEST TARGET (QA-3)

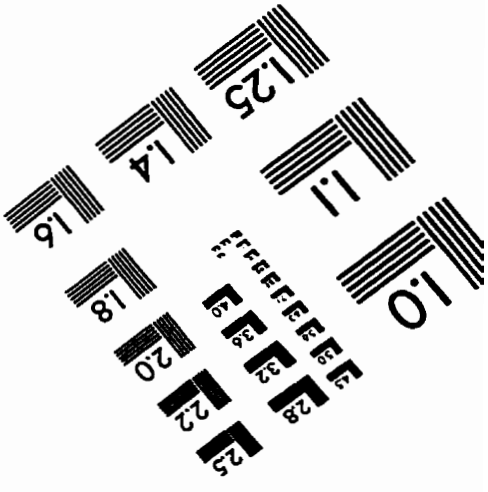












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