Determinants of Testicular Echotexture in the Sexually Immature Ram Lamb

by

Jennifer Lynn Giffin

A Thesis

presented to

The Faculty of Graduate Studies

of

The University of Guelph

In partial fulfilment of requirements

for the degree of

Doctor of Philosophy

in

Biomedical Sciences

Guelph, Ontario, Canada

© Jennifer Lynn Giffin, November, 2014
ABSTRACT

DETERMINANTS OF TESTICULAR ECHOTEXTURE IN THE SEXUALLY IMMATURE RAM LAMB

Jennifer Lynn Giffin
University of Guelph, 2014

Advisor:
Dr. P. M. Bartleewski

Throughout sexual maturation, dynamic changes in testicular macro- and microstructure and reproductive hormone levels occur. Future adult reproductive capability is critically dependent on these changes; therefore, regular monitoring of pubertal testicular development is desirable. However, conventional methods of assessment do not permit the frequent and non-invasive examination of testicular function. Recently, scrotal ultrasonography in conjunction with computer-assisted image analysis has emerged as a potential non-invasive alternative for male reproductive assessment. In this procedure, testicular echotexture, or the appearance of the ultrasonogram, is objectively quantified on the basis of brightness or intensity of the minute picture elements, or pixels, comprising the image. In general, testicular pixel intensity increases with age throughout sexual maturation; however, periodic fluctuations occur. Changes in testicular echotexture are related to microstructural attributes of the testes and reproductive hormone secretion, but reports on these relationships have been inconsistent. Therefore, the overall objective of the studies presented in this thesis was to investigate how testicular echotexture and its associations with testicular histomorphology and endocrine profiles may be influenced by various factors including: i) scrotal/testicular integument; ii) blood flow/content; iii) stage of development; and iv) altered spermatogenic onset.

Serial scrotal ultrasonograms, testicular biopsies or castrations and blood samples were collected from early postnatal or peripubescent ram lambs and analyzed to obtain echotextural, histomorphological and endocrine data, respectively. Testicular echotextural attributes were influenced by the presence of the scrotal and testicular integument, the stage of spermatogenic development (mitotic vs. post-mitotic) and the amount of germ cell heterogeneity present with or without retinoic acid exposure, but not by blood flow or content. Tubular and luminal size of the seminiferous tubules could be predicted from testicular pixel intensity using a simple linear regression model and demonstrated a moderate strength correlation with testicular echotextural parameters, as did a number of cellular and sub-cellular histomorphological attributes and reproductive hormone levels. These results clearly demonstrate the ability of testicular echotexture to detect experimentally and clinically relevant markers of testicular function. Computerized image analysis of scrotal ultrasonograms holds promise as a non-invasive diagnostic and prognostic tool and novel monitoring device of testicular histophysiology.
ACKNOWLEDGEMENTS

My heartfelt appreciation and gratitude go to Dr. Pawel Bartlewski for his guidance and support throughout my graduate studies and research involvement in the Department of Biomedical Sciences. I could not have had a more patient, kind and understanding advisor than you. Thank you for encouraging and inspiring me to reach for my fullest potential. It has been an honour and a delight working with you.

Most sincere appreciation is also expressed to Dr. Ann Hahnel for sharing her vast knowledge and expertise, and for her unending interest and support in all my endeavors.

The generous investment of time and effort by my advisory committee members, Dr. Neil MacLusky, Dr. Jim Petrik and Dr. Tracey Chenier, as well as my examination committee, is gratefully acknowledged. Your contributions have been essential to the successful completion of my degree and are greatly valued by me.

My graduate study experience would not have been the same without the hard work, dedication and willingness to help of the faculty, staff and students in the Department of Biomedical Sciences and the wider University of Guelph community. In particular, I would like to thank Pam Hasson and Jeff McFarlane from the Ponsonby Sheep Research Station for attending to the needs of the experimental animals, Kevin Hogg for his technical assistance capturing ultrasound images, Helen Coates for her skilled help in performing histological and histochemical techniques, Kanwal Minhas for her laboratory assistance in processing tissue samples and previous members of the Bartlewski and Hahnel labs: Sabrina Sangupta, Stephanie Wilson, Sean Kamani, Karan Dhir, Vicki Watts, Andrew Bertolini, Bret McLeod, Kamila Skalski, Taylor VanDuzer and Longfei Gao for their participation in various research activities. Thanks also to those who were not directly involved with my research, but nonetheless contributed to the supportive learning environment I encountered.

Financial support for the completion of this degree was generously provided by the Ontario Ministry of Agriculture, Food and Rural Affairs - University of Guelph Highly Qualified Personnel (OMAFRA-U of G HQP) Graduate Scholarship Program and funding of studies by OMAFRA and the Natural Sciences and Engineering Research Council of Canada is gratefully acknowledged.

I have truly been touched by the love and support that my family has given to me over the years. Thanks especially to my parents, Don and Jean, for always believing in me and encouraging me to aspire to my dreams.

Finally, I would like to thank God who has wonderfully orchestrated all the details and events encompassing the fulfillment of this degree and has enabled me to achieve far more than I could have ever imagined.
# TABLE OF CONTENTS

ABSTRACT ........................................................................................................................................... ii

ACKNOWLEDGEMENTS .................................................................................................................... iii

TABLE OF CONTENTS ........................................................................................................................ iv

DECLARATION OF THE WORK PERFORMED .................................................................................. viii

LIST OF TABLES .................................................................................................................................. ix

LIST OF FIGURES .............................................................................................................................. x

LIST OF ABBREVIATIONS .................................................................................................................. xii

1. INTRODUCTION ............................................................................................................................. 1

1.1 Testicular Anatomy and Physiology in the Sexually Mature Ram .............................................. 2

1.1.1 Testicular Macrostructure ....................................................................................................... 3

1.1.2 Testicular Microstructure ....................................................................................................... 6

1.1.3 Spermatogenesis ...................................................................................................................... 8

1.1.4 Steroidogenesis ......................................................................................................................... 13

1.1.5 Endocrine Regulation ............................................................................................................... 14

1.2 Stages of Testicular Development in the Sexually Immature Ram Lamb .................................. 16

1.2.1 Prenatal Testicular Development ............................................................................................ 16

1.2.2 Early Postnatal Testicular Development ............................................................................... 18

1.2.3 Prepubertal Testicular Development .................................................................................... 20

1.2.4 Postpubertal Testicular Development .................................................................................... 24

1.3 Scrotal Ultrasonography ............................................................................................................. 25

1.3.1 Image Formation ..................................................................................................................... 25

1.3.2 Image Analysis ........................................................................................................................ 28

1.3.3 Testicular Echotextural Trends ............................................................................................... 30
2. RATIONALE, OBJECTIVES AND HYPOTHESES ................................................................. 32

3. A STUDY OF INTEGUMENTARY, HISTOMORPHOLOGICAL AND HAEMODYNAMIC DETERMINANTS OF TESTICULAR ECHOTEXTURE IN RAM LAMBS .............................. 35

3.1 Abstract ......................................................................................................................... 36

3.2 Introduction .................................................................................................................. 37

3.3 Materials and Methods ............................................................................................. 39

3.3.1 Animals and Experimental Design ........................................................................ 39

3.3.2 Ultrasonography ..................................................................................................... 40

3.3.3 Tissue Processing and Histology .......................................................................... 41

3.3.4 Statistical Analyses ............................................................................................... 42

3.4 Results ........................................................................................................................ 44

3.4.1 General Results ...................................................................................................... 44

3.4.2 Testicular Echotexture .......................................................................................... 44

3.4.3 Linear Regression Models ...................................................................................... 46

3.5 Discussion .................................................................................................................... 50

3.6 Acknowledgements .................................................................................................... 53

4. CORRELATIONS AMONG ULTRASONOGRAPHIC AND MICROSCOPIC CHARACTERISTICS OF PREPUBESCENT RAM LAMB TESTES ......................................................... 54

4.1 Abstract ......................................................................................................................... 55

4.2 Introduction .................................................................................................................. 56

4.3 Materials and Methods ............................................................................................. 58

4.3.1 Animals and Experimental Design ........................................................................ 58

4.3.2 Ultrasonographic Examinations .......................................................................... 60

4.3.3 Histology and Immunohistochemistry .................................................................. 61

4.3.4 Statistical Analyses ............................................................................................... 64

4.4 Results ........................................................................................................................ 64
6. SUMMARY DISCUSSION AND FUTURE RESEARCH APPLICATIONS ............... 114

6.1 Summary Discussion .................................................................................... 114

6.2 Future Research Applications ...................................................................... 123

LIST OF PUBLICATIONS ................................................................................ 127

REFERENCES .................................................................................................... 128
DECLARATION OF THE WORK PERFORMED

I declare that with the exception of the items indicated below, all work reported in the body of this thesis was performed by me.

Dr. Ann Hahnel and members of her lab collected and processed testicular tissue obtained in Chapters 3 and 4, including immunohistochemical staining. They also took testicular volume measurements and completed a portion of the histomorphological analyses reported in Chapter 4, including tubular and luminal seminiferous tubule diameter measurements, staging of the seminiferous tubules according to the most advanced germ cell type, and counting the number of ubiquitin C-terminal hydrolase L-1 (UCHL-1)\(^+\) cells per seminiferous tubule cross-section.

Dr. Pawel Bartlewski performed all ultrasound scanning in Chapters 3 and 4 and a portion in Chapter 5. He also performed statistical analysis of the results and compiled the graphs presented in Chapter 3.

S. Elizabeth Franks assisted with data interpretation and editing a previous version of the contents presented in Chapter 3.

Castrations were performed by Dr. Jose Rafael Rodriguez-Sosa (Chapter 3) and by Ruminant Field Services, Ontario Veterinary College, University of Guelph (Chapter 5).

Prairie Diagnostic Services, Western College of Veterinary Medicine, University of Saskatchewan performed all radioimmunoassays of blood samples for follicle-stimulating hormone, testosterone and estradiol concentrations reported in Chapter 5.
LIST OF TABLES

Table 1.1 Typical ultrasound properties at 5–10-MHz. ................................................................. 27

Table 4.1 A summary of Pearson product moment correlations among echotextural and histomorphological attributes in the testes of twenty-two 10 to 19 week old Rideau-Arcott x Polled Dorset ram lambs at different phases of spermatogenic onset. ............................................. 74

Table 5.1 Pearson product moment correlation coefficients among echotextural, histomorphological and endocrine variables in control ($n = 8$) and retinoic acid-treated ($n = 9$) Dorset x Rideau-Arcott x Canadian Arcott ram lambs from 8 to 10.5 weeks of age. ................. 104
LIST OF FIGURES

Figure 1.1 Longitudinal section of a ram testis and associated structures .......................... 3
Figure 1.2 Schematic diagram of the scrotum and testicular capsule ............................... 5
Figure 1.3 Histological cross-section through an adult ram testis ................................. 7
Figure 1.4 Hypothalamo-pituitary-gonadal axis in the male ........................................... 15
Figure 1.5 Histological cross-section through an early postnatal ram lamb testis ............. 19
Figure 1.6 Histological cross-section through a prepubertal ram lamb testis .................. 22
Figure 1.7 Scrotal ultrasonograms of ram lambs in longitudinal and transverse views ...... 29

Figure 3.1 Testicular echotextural and histomorphological analysis of selected tissue regions and the abnormal histological appearance of a testicle removed from statistical analyses .......... 43

Figure 3.2 Mean (± SE) numerical pixel values and pixel heterogeneity for the testicular parenchyma determined by computer-assisted image analyses of testicular ultrasonograms obtained during castration in seven Rideau-Arcott x Polled Dorset ram lambs .................. 45

Figure 3.3 (above, page 47) Scatter plots, regression lines and equations, and squared correlation coefficients of the relationship between numerical pixel values and tubular or luminal seminiferous tubule areas of testicles scanned at various time points during surgical castration of seven Rideau-Arcott x Polled Dorset ram lambs .............................................. 48

Figure 3.4 (below, page 49) Scatter plots, regression lines and equations, and squared correlation coefficients of the relationship between pixel heterogeneity values and tubular or luminal seminiferous tubule areas of testicles scanned at various time points during surgical castration of seven Rideau-Arcott x Polled Dorset ram lambs .............................................. 48

Figure 4.1 Prepubertal ram lamb testicular micrographs representing four different stages of germ cell development and the general appearance of ubiquitin C-terminal hydrolase L-1 immunohistochemical staining ................................................................. 63

Figure 4.2 Mean (± SE) body weight and testicular volume in prepubescent ram lambs with respect to weeks of age and weeks relative to the first detection of elongated spermatids .... 66

Figure 4.3 Mean (± SE) numerical pixel values and pixel standard deviation from testicular ultrasonograms taken at twice weekly intervals in prepubescent ram lambs, aligned to chronological age of animals or weeks relative to the first detection of elongated spermatids .... 67
**Figure 4.6** Mean (± SE) percentages of seminiferous tubule cross-sections with pre-spermatogonia and/or spermatogonia, spermatocytes, round spermatids or elongated spermatids (ESt) as the most mature germ cell type present in ram lamb testes from 10 to 19 weeks of age or from −6 to 2 weeks relative to the first detection of ESt .................................................. 72

**Figure 5.1** Experimental design and sample sizes ................................................................................................................. 91

**Figure 5.2** Testicular micrographs of retinoic acid-treated and control ram lambs at 8.2 and 10.5 weeks of age .................................................................................................................................................. 97

**Figure 5.3** Mean (± SE) percentages of seminiferous tubule (ST) cross-sections (XS) with no germ cells present or with pre-spermatogonia I, pre-spermatogonia II, spermatogonia or spermatocytes as the most mature germ cell type present, tubular and luminal ST area, total number of cells/ST XS, nuclear : ST area and the number of degenerating cells/ST XS in treated and control ram lambs at 8.2 and 10.5 weeks of age .................................................................................................. 98

**Figure 5.4** Mean (± SE) serum concentrations of follicle-stimulating hormone, testosterone and estradiol in retinoic acid-treated and control ram lambs from 8 to 10.5 weeks of age .......... 100

**Figure 5.5** Mean (± SE) numerical pixel values and pixel standard deviation/heterogeneity in retinoic acid-treated and control ram lambs from 8 to 10.5 weeks of age ................................. 102
LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abb</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>DHT</td>
<td>Dihydrotestosterone</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DVD</td>
<td>Digital Versatile Disk</td>
</tr>
<tr>
<td>E₂</td>
<td>Estradiol</td>
</tr>
<tr>
<td>ESt</td>
<td>Elongated spermatid</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle-stimulating hormone</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>HPG</td>
<td>Hypothalamo-pituitary-gonadal</td>
</tr>
<tr>
<td>i.m.</td>
<td>Intramuscular</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravascular</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>MHz</td>
<td>Megahertz</td>
</tr>
<tr>
<td>NPV</td>
<td>Numerical pixel value</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-GS</td>
<td>5% normal goat serum in PBS</td>
</tr>
<tr>
<td>Pre-Sg I</td>
<td>Pre-spermatogonia I</td>
</tr>
<tr>
<td>Pre-Sg II</td>
<td>Pre-spermatogonia II</td>
</tr>
<tr>
<td>PSD</td>
<td>Pixel standard deviation</td>
</tr>
<tr>
<td>r</td>
<td>Correlation coefficient</td>
</tr>
<tr>
<td>r²</td>
<td>Squared correlation coefficient; coefficient of determination</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic acid</td>
</tr>
<tr>
<td>RSt</td>
<td>Round spermatid</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>Sc</td>
<td>Spermatocyte</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Sg</td>
<td>Spermatogonia</td>
</tr>
<tr>
<td>SSC</td>
<td>Spermatogonial stem cell</td>
</tr>
<tr>
<td>ST</td>
<td>Seminiferous tubule</td>
</tr>
<tr>
<td>T</td>
<td>Testosterone</td>
</tr>
<tr>
<td>UCHL-1</td>
<td>Ubiquitin C-terminal hydrolase L-1</td>
</tr>
<tr>
<td>XS</td>
<td>Cross-section</td>
</tr>
</tbody>
</table>
Chapter 1

INTRODUCTION

Breakthrough discoveries in male reproductive biology, as in other fields of science, typically follow major technological or methodological advances (Zirkin and Robaire, 2000). The discovery of sperm in 1677 by Antonie van Leeuwenhoek was achieved through his development of the simple microscope (Birkhead and Montgomerie, 2009) and continued improvements to this instrument over the next 200 years led to an understanding of the basic principles of spermatogenesis including the origin of sperm from cells constituting the seminiferous tubules (Kerr, 1995), the proper division of genetic material in progenitor sperm cells (i.e., mitosis, meiosis; Sumner, 2003) and the existence of “branched cells” which likely assisted in the formation of, but did not become sperm (i.e., Sertoli cells; Hess and de França, 2005). Leydig cells were first observed in 1850; however, it took 50 years before their role as a secretory cell was first suggested, and another 60–80 years before testosterone biosynthesis by specific organelles in Leydig cells was confirmed, following the introduction of biochemical and molecular biology methods (Zirkin and Robaire, 2000).

Ultrasound is one of the most prevalent imaging modalities worldwide. It accounts for nearly 25% of all medical imaging procedures (Goldberg, 2003) and is second in frequency of use only to X-ray imaging (Shung and Thieme, 1993). Gray-scale (B-mode), real-time ultrasonography has had a major impact on the field of animal research and clinical reproduction (Ginther, 1995). In the testes, as in other organs, ultrasonography is commonly used for the non-invasive assessment of internal, macroscopic structures. However, computer-assisted image
analysis of scrotal ultrasonograms may provide an indication of microscopic structures as well as reproductive hormone concentrations (Evans et al., 1996; Brito et al., 2012), based on the gray-scale appearance of the ultrasonogram, or echotexture. During sexual maturation, dynamic changes in testicular morphology and reproductive hormone secretion occur, which appear to be reflected in the unique echotextural trends observed in the testes. Ram lambs, defined as male sheep less than one year of age (Ekarius, 2008) are a suitable model to study the ultrasonographic characteristics of developing testes as they have distinct, well-characterized developmental stages throughout sexual maturation. Therefore, the objectives of this chapter are to provide an overview of the anatomy and physiology of the testes in both the sexually mature ram and throughout each stage of development in the sexually immature ram lamb, to outline the basic principles of ultrasound image formation, and to describe computer-assisted image analysis as a method for analyzing scrotal ultrasonograms and the research findings generated using this technique to date.

1.1 Testicular Anatomy and Physiology in the Sexually Mature Ram

In mammals, the testis is divided into two major sections, both structurally and functionally. The tubular compartment, which occupies the majority of the testicular parenchyma, contains seminiferous tubules in which spermatogenesis, or the production of mature male gametes/sperm, occurs. The interstitial compartment, situated between the seminiferous tubules, contains Leydig cells, which produce androgens and other steroid hormones. Together these two compartments are regulated by hormones produced by the hypothalamus, pituitary gland and testis in an integrated system known as the hypothalamo-pituitary-gonadal (HPG) axis.
1.1.1 Testicular Macrostructure

In adult rams, the paired testes are approximately oval-shaped, weighing between 200–250 g each (Constantinescu, 2008), with a scrotal circumference of 30–40 cm (Sargison, 2008). The testicular parenchyma is largely composed of seminiferous tubules, which measure 3,000 m in length in rams (Hochereau-de Reviers et al., 1993), and are highly convoluted except for a short region referred to as the *tubulus rectus*, in contact with the rete testis, an anastomosing network of channels that facilitates the passage of sperm out of the testis. In rams, the rete testis is located centrally and extends up to approximately three quarters of the length of the testis (Figure 1.1; Setchell and Breed, 2006).

![Figure 1.1](image.png)

**Figure 1.1** Longitudinal section of a ram testis and associated structures. T, testicular parenchyma; R, rete testis; E<sub>H</sub>, head of the epididymis; E<sub>T</sub>, tail of the epididymis; S, spermatic cord. Modified from Setchell and Breed (2006).
Closely apposed to the testis along the caudomedial border is a highly convoluted structure known as the epididymis (Constantinescu, 2008). The head (caput) of the epididymis is attached to the dorsal pole of the testis through the efferent ductules, a multi-numbered extension of the rete testis (~17–20 in rams; Hemeida et al., 1978), which joins together to form a single epididymal duct, through which sperm are transported to the body (corpus) and finally the tail (cauda) of the epididymis. During the 16 day transit through the 50 m long epididymis in rams (Amann et al., 1976; Jones, 1999), sperm gradually acquire the ability to fertilize an oocyte (Jones, 1999). The cauda epididymis, firmly attached to the ventral pole of the testis by a testicular and epididymal ligament (Constantinescu, 2008), appears to be especially important for storing sperm prior to ejaculation (Jones, 1999), when the sperm are released into the ductus deferens and then the urethra.

The testes and epididymides are vertically suspended from the abdomen in rams by the spermatic cord. Measuring 10 cm in length on average in rams (Setchell et al., 1966), the spermatic cord contains blood and lymphatic vessels, nerves and the ductus deferens (Liguori et al., 2012). Surrounding the spermatic cord, testes and epididymides is a sac-like structure known as the scrotum. The scrotum is composed of seven layers (Figure 1.2; Davis et al., 1970); namely i) the skin; ii) the dartos muscle, which regulates scrotal surface area (Kumar and De, 2013); iii) the superficial perineal fascia, which forms an incomplete scrotal septum to divide the scrotal contents between left and right (Wittenberg et al., 2006); iv) the external spermatic fascia; v) the cremasteric fascia containing the cremaster muscle, which regulates the position of the scrotum relative to the body (Kumar and De, 2013); vi) the internal spermatic fascia; and vii) the tunica vaginalis parietalis, from most superficial to deepest layers (Davis et al., 1970).
A small fluid-filled cavity separates the scrotum from the testicular capsule (Figure 1.2; Davis et al., 1970). Three distinct layers comprise the testicular capsule including: i) the tunica vaginalis visceralis, a thin serous membrane forming the outermost layer of the capsule (Davis et al., 1970); ii) the tunica albuginea, a thick fibrous membrane comprising the bulk of the testicular capsule (Davis et al., 1970); and iii) the tunica vasculosa, sometimes referred to as the inner layer of the tunica albuginea (Arenas et al., 1997), consisting of a loose connective tissue rich in blood vessels (Jequier, 2000), which is particularly prominent in bulls (Ekstedt et al., 1986) and likely in other ruminant species. The tunica albuginea enters the testis along its anterior border and forms a loose connective tissue called the mediastinum testis, which
encompasses the rete testis and sends out radiating sheets of connective tissue septa, called the *septula testis* (Wrobel, 1998), effectively partitioning the testis into lobules (Constantinescu, 2008), each containing 1–4 convoluted seminiferous tubules (Wrobel, 1998). While collagen is the most prevalent component of the *tunica albuginea* (Davis *et al.*, 1970), it is penetrated by the testicular artery along the posterior surface of the testis, which branches and forms the terminal vascular system within the *tunica vasculosa* (Liguori *et al.*, 2012), and is also interspersed with smooth muscle cells (Chacon-Arellano and Woolley, 1980). The smooth muscle cells within the *tunica albuginea* allow for spontaneous or induced autorhythmic contractions (Davis *et al.*, 1970; Mitchell and Seeley, 1977; Setchell and Breed, 2006) to aid in the expulsion of sperm from the testis to the epididymis and in maintaining the interstitial tissue pressure of the testis (Setchell and Breed, 2006). Depending on the abundance of smooth muscle cells, blood vessel size and proximity to the *rete testis*, the thickness of the testicular capsule has been shown to vary across the surface of the testis in several species (Davis *et al.*, 1970; Aire and Ozegbe, 2007).

### 1.1.2 Testicular Microstructure

The cellular composition of the mammalian testis is complex and heterogeneous, with over 30 different cell types (Rodríguez-Casuriaga *et al.*, 2013; Figure 1.3). The tubular compartment of the testis contains the majority of cells including germ or spermatogenic cells, which become sperm, and Sertoli or nurse cells, which support the germ cells. Collectively, these two cell types form the seminiferous epithelium which lines the seminiferous tubules and surrounds a fluid-filled central lumen. Sertoli cells extend from the base of the seminiferous tubule to the lumen while germ cells are stratified within the seminiferous epithelium according to their stage of differentiation. The seminiferous epithelium is divided by an inter-Sertoli cell...
tight junctional complex into a basal compartment containing spermatogonia and early spermatocytes and an adluminal compartment containing late spermatocytes and spermatids; as the germ cells mature they move from a basal to an adluminal position. In adult rams, the seminiferous tubules have a diameter of 275 µm and occupy about 83% of the testicular parenchyma (Wrobel et al., 1995).

**Figure 1.3** Histological cross-section through an adult ram testis. Sg, spermatogonia; Sc, spermatocytes; RSt, round spermatids; ESt, elongated spermatids; SC, Sertoli cells; LC, Leydig cells; PTM, peritubular myoid cells BV, blood vessel; LV, lymphatic vessel. Image taken at 200x image magnification. Obtained with permission from Dr. J. R. Rodriguez-Sosa.

A diverse array of cells are located between the seminiferous tubules in the interstitial tissue, including a layer of modified smooth muscle cells that surround the seminiferous tubules,
known as peritubular myoid cells (Kerr et al., 2006), blood and lymphatic vessel endothelial cells, nerves, a host of immune system cells including macrophages, lymphocytes and mastocytes, fibroblasts and Leydig cells (Geisinger, 2007). In rams, these cells are enmeshed in a loose connective tissue with a centrally located lymphatic vessel and more peripherally positioned blood vessels (Fawcett et al., 1973). Leydig cells, the principle testosterone-secreting cell in males, comprise about 7.5% of the interstitial tissue or 1% of the entire testis in rams (Setchell, 1991), and are found singly or in small clusters throughout the ovine connective tissue stroma (Fawcett et al., 1973).

1.1.3 Spermatogenesis

Spermatogenesis is a highly organized process that involves the proliferation and differentiation of germ cells to form mature sperm. It is typically divided into three main phases (Sharpe, 1994). First, in the spermatogonial or proliferative phase, spermatogonia (the most primitive germ cells) divide by mitosis as self-renewing stem cells and/or progenitor spermatogonia committed to differentiate into more advanced germ cell types (Oatley and Brinster, 2012). The spermatogonial stem cells (SSCs), from which all further spermatogenic cells arise, are at least partly comprised of A\textsubscript{single} (A\textsubscript{s}) spermatogonia, which divide into A\textsubscript{paired} (A\textsubscript{p}) spermatogonia in chains of 2, and finally A\textsubscript{aligned} (A\textsubscript{al}) spermatogonia in chains of 4, 8 and 16 in the ram (Lok et al., 1982; Nakagawa et al., 2010); together, these three spermatogonial types are categorized as undifferentiated spermatogonia. Transformation of the undifferentiated spermatogonia into A\textsubscript{1} spermatogonia, the first differentiated spermatogonial cell type, is an irreversible step and is thought to involve retinoic acid (Griswold and Oatley, 2013), the primary active metabolite of vitamin A (Griswold et al., 2012). Subsequently, the A\textsubscript{1} spermatogonia
resume proliferation into the remaining differentiated spermatogonial cell types: A₂ and A₃ spermatogonia, intermediate (In) spermatogonia, B₁ and finally B₂ spermatogonia (Lok et al., 1982). With up to ten proliferation steps in rams, the spermatogonial population undergoes rapid exponential growth, theoretically resulting in the production of 1024 primary spermatocytes from one SSC.

Spermatogonia have a distinct morphology and are readily distinguished from other types of germ cells. They are found only in the basal compartment of the seminiferous epithelium, in contact with the basement membrane (Wrobel et al., 1995). Throughout spermatogonial differentiation, there is an increase in the amount of heterochromatin present in the nuclei (Chiarini-Garcia and Meistrich, 2008; Hermo et al., 2010); this formed the basis of the spermatogonial nomenclature system (de Rooij and Russell, 2000), with type A, In, and type B spermatogonia having a lack of, intermediate amount, or large amount of heterochromatin, respectively (de Rooij and Russell, 2000).

The meiotic phase of spermatogenesis is initiated following the division of B₂ spermatogonia into two preleptotene spermatocytes. The preleptotene spermatocytes undergo an extended period of chromosomal synthesis (Handel and Schimenti, 2010) before entering a prolonged meiotic prophase I, which is categorized into 4 different substages based on chromosome configurations and structure (Handel and Schimenti, 2010): i) leptonema, in which homologous (maternal and paternal) chromosomes start to align; ii) zygonema, in which homologous chromosomes begin to pair and intimately associate (synapse) together; iii) pachynema, the lengthiest substage (Cobb and Handel, 1998), in which the homologous chromosomes undergo recombination, or the exchange of genetic material that is important for generating diversity as well as situating the chromosomes in the proper alignment for their
eventual disjunction; and iv) diplonema, in which chromosomes undergo desynapsis and markedly condense (Handel and Schimenti, 2010). The germ cells undergoing these changes are referred to as leptotene, zygotene, pachytene and diplotene spermatocytes, respectively. Following the separation of homologous chromosomes by a reductional division in meiosis I (Handel and Schimenti, 2010), two secondary spermatocytes are formed and quickly enter meiosis II (Wrobel et al., 1995), during which the sister chromatids are separated in an equational division (Handel and Schimenti, 2010) to form two spermatids each. Thus, one diploid primary spermatocyte may theoretically give rise to four haploid secondary spermatocytes, bringing the total potential number of daughter cells from one SSC to 4096 spermatids.

As a group, spermatocytes are easily recognizable by the pronounced thread- or patch-like appearance of chromatin within the nucleus. However, there is considerable variation in size and location of the spermatocytes throughout the meiotic phase of spermatogenesis. From the preleptotene to diplotene stages, there is nearly a 5-fold increase in cellular volume and a 3-fold increase in nuclear volume in rams, making diplotene spermatocytes the largest members of the germ cell line (Wrobel et al., 1995). The nuclear and cellular volumes are approximately halved with each meiotic division (Wrobel et al., 1995). Since germ cells that have completed or are in the process of completing meiosis are highly autoantigenic (Cheng and Mruk, 2012), it is necessary for the developing spermatocytes to traverse the blood-testis barrier, which also acts as an immunological barrier. In rams, this process is started in the preleptotene stage and is completed by the zygotene stage (Wrobel et al., 1995).

In the final stage of spermatogenesis, there are no germ cell divisions but rather an extensive phase of differentiation known as spermiogenesis, in which spermatids, initially round
in shape, undergo extensive nuclear and cytoplasmic modifications to form mature elongated sperm. Five major morphological events occur during spermiogenesis (Kerr et al., 2006): i) the formation of an acrosome, a cap-like structure that spreads over the nuclear surface of the spermatid and contains a variety of enzymes involved in fertilization; ii) maximal nuclear condensation and movement of the nucleus from a central to an eccentric position; iii) development of a tail or flagellum; iv) the shedding of excess cytoplasm as residual bodies; and v) the formation of specialized Sertoli cell-spermatid junctions to orient the spermatid heads basally with the developing flagella projecting into the lumen (Kerr et al., 2006). Whereas round spermatids occupy between 34% to over 40% of the seminiferous epithelium in rams (Wrobel et al., 1995), elongated spermatids occupy considerably less space, being roughly one quarter the size of earlier stage spermatids in the water buffalo (Singh Pawar and Wrobel, 1991). With the completion of spermiogenesis, the elongated spermatids are released into the lumen through the process of spermiation, and are thereafter referred to as spermatozoa or sperm. Newly-formed sperm are transported to the rete testis by the flow of fluid in the seminiferous tubule lumen and by smooth muscle contractions of the peritubular myoid cells and the testicular capsule (Hinton and Setchell, 1993).

With the exception of some seasonally breeding mammals, spermatogenesis is a continuous process. While influenced by photoperiod, spermatogenesis occurs year-round in rams (Rosa and Bryant, 2003). As the total duration of spermatogenesis is approximately 50 days in rams (Wrobel, 1998), a number of different rounds of spermatogenesis must occur simultaneously within the seminiferous epithelium to avoid the periodic release of sperm. During the differentiation of A_s spermatogonia into A_pr spermatogonia, the two daughter cells remain connected via cytoplasmic intercellular junctions which form after every subsequent division,
resulting in a large cohort of interconnected germ cells that differentiate synchronously (Hamer et al., 2003). Multiple germ cell cohorts at specific stages of development tend to associate together and are referred to as stages. Eight stages of the seminiferous epithelium have been described in rams with four different cohorts in each (Wrobel, 1998; Zeng et al., 2006). The complete progression throughout each of the stages is known as the cycle of the seminiferous epithelium (Hogarth and Griswold, 2010), which itself is typically comprised of 4.5 cycles, each with a duration of 10.4–10.6 days in rams (Amann and Schanbacher, 1983; França et al., 2005), in which a new cohort of undifferentiated spermatogonia enters the cycle while another cohort of elongated spermatids leaves the cycle (Hogarth and Griswold, 2010). The stages are arranged sequentially along the length of the seminiferous tubule, referred to as the spermatogenic wave (Hogarth and Griswold, 2010). A complete set of stages measures approximately 10 mm in bulls; however, variations in stage order (e.g., repetitions, inversions) occur frequently (Wrobel, 1998).

Spermatogenesis is an exceptionally productive process, with approximately 2–4 billion sperm produced per testis each day in rams, depending on season and breed (Dacheux et al., 1981). However, this is only a fraction of the total theoretical spermatogenic yield that could be generated. In reality, approximately 75% of all germ cells produced in the testis degenerate through apoptosis (Shaha et al., 2010). The majority of cells that undergo apoptosis are spermatogonia and spermatocytes (Royere et al., 2004), although differences exist in the reason for cell death in each germ cell type. Spermatogonia, particularly type A2 through type B spermatogonia, degenerate through density-dependent regulation, in which the number of germ cells is reduced to accommodate the supportive capacity of Sertoli cells (de Rooij and Russell, 2000), which are capable of supporting only a limited number of germ cells (Orth et al., 1988). By contrast, spermatocytes are believed to die primarily due to chromosomal damage (Hess and
de Franca, 2008). Degenerating germ cells display many of the classical morphological features of apoptosis, including dense and fragmented chromatin and compact and eosinophilic cytoplasm (Gobé and Harmon, 2008), and are phagocytosed by Sertoli cells within 24 hours for single cells or longer if a multinucleate syncytium is formed, as commonly occurs for round spermatids and some spermatocytes and elongated spermatids (Lanning et al., 2002); alternatively, they may be sloughed off into the seminiferous tubule lumen (Wrobel et al., 1994). However, not all degenerating germ cells demonstrate the same apoptotic features (Print and Loveland, 2000; Lanning et al., 2002), which may be attributed to the distinct nuclear morphology, particularly of spermatocytes and elongated spermatids (Print and Loveland, 2000), and/or the use of multiple different apoptotic pathways that are known to exist in the testis (Ruwanpura et al., 2010).

1.1.4 Steroidogenesis

More than 90% of all androgens, the primary male sex hormones, are produced in the testes (Wrobel, 1998). In addition to dehydroepiandrosterone (DHEA) and androstendione, which are also produced in the zona reticularis of the adrenal glands (Meaden and Chedrese, 2009) the testes synthesize testosterone de novo from the common steroid precursor cholesterol in Leydig cells, the only cells with all the required enzymes to do so (Payne, 2007). Testosterone is the most abundant androgen produced by the testes (Meaden and Chedrese, 2009) with a daily production rate of approximately 6 mg in rams (Schanbacher et al., 1987). Further metabolism of testosterone into dihydrotestosterone (DHT), a more potent androgen, or estradiol (E₂) by the cytochrome P450 aromatase enzyme occurs in the testes as well as in peripheral tissues (Henley et al., 2005; Meaden and Chedrese, 2009).
1.1.5 Endocrine Regulation

The hypothalamo-pituitary-gonadal (HPG) axis is an intercommunicating set of neural and endocrine tissues (Bliss et al., 2010) that allows for the coordinated release of hormones among the hypothalamus, pituitary gland and testes. As depicted in Figure 1.4, the HPG axis is activated when gonadotropin-releasing hormone (GnRH) is secreted in a pulsatile manner by peptidergic neurons in the hypothalamus and released into the pituitary portal vasculature (Krsmanovic et al., 2009). Depending on the frequency and amplitude of the GnRH pulses, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), glycoprotein hormones collectively known as the gonadotropins, are synthesized by gonadotropes in the anterior pituitary gland (Shimon and Melmed, 2005) and released episodically into the circulation (Bliss et al., 2010). LH release, measured in peripheral circulation, is more pulsatile compared to that of FSH, which may be related to its shorter half-life (Bliss et al., 2010). In the testis, LH and FSH bind to receptors on Leydig and Sertoli cells, respectively (Walker and Cheng, 2005; Payne, 2007). Testosterone and its metabolites, estradiol and DHT, are produced by Leydig cells in response to LH stimulation, and bind to androgen receptors on Leydig, Sertoli and peritubular cells (Walker and Cheng, 2005) or estrogen receptors found on most types of cells within the testis (Akingbemi, 2005). FSH and testosterone are considered the two major hormonal regulators of spermatogenesis (Sofikitis et al., 2008; Ruwanpura et al., 2010) and primarily regulate spermatogonia or spermatid development, respectively; both hormones act in concert to support spermatocyte development (Ruwanpura et al., 2010). Notably, FSH and testosterone do not directly interact with germ cells, but rather indirectly through Sertoli cells.
Figure 1.4 Hypothalamo-pituitary-gonadal axis in the male. GnRH, gonadotrophin-releasing hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; T, testosterone; E₂, estradiol; DHT, dihydrotestosterone.

The HPG axis is regulated at the level of the hypothalamus and pituitary gland through negative feedback inhibition from testicular steroids and the glycoprotein hormone inhibin, produced by Sertoli cells in response to FSH (Tilbrook and Clark, 2001). LH secretion is regulated mainly by testosterone, estradiol and DHT, which decrease GnRH secretion in the hypothalamus (Tilbrook et al., 1991). In contrast, FSH is regulated exclusively at the level of the pituitary gland, predominantly by inhibin through an unknown mechanism; however, testosterone and the interaction between inhibin and testosterone also regulate FSH secretion in rams (Tilbrook et al., 1993; Tilbrook and Clark, 2001).
1.2 Stages of Testicular Development in the Sexually Immature Ram Lamb

Testicular development in the sexually immature ram lamb can be divided into four main stages: prenatal, early postnatal, prepubertal and postpubertal; the pre- and postpubertal periods are sometimes denoted as the peripubertal stage. Following birth, the rate of testicular growth follows a sigmoid-shaped curve (Courot et al., 1970), with gradually increasing testicular size followed by a period of rapid testicular growth beginning at 60–70 days of age in ram lambs (~8.5–10 weeks of age; Kilgour et al., 1998), encompassing the onset of spermatogenesis (Courot et al., 1970), followed by a return to a more slowed developmental pace (Courot et al., 1970). These three developmental periods correspond with the early postnatal, prepubertal and postpubertal stages, respectively. Distinct histophysiologial characteristics are associated with each stage.

1.2.1 Prenatal Testicular Development

During prenatal development, the testes derive from a bipotential or indifferent gonad, referred to as the genital ridge, on the ventrolateral surface of the mesonephros (Kousta et al., 2010), which functions as the embryonic kidney in sheep (McLauren, 1998). Testis formation takes place from around 30–32 to 35 days after breeding in sheep (O’Shaughnessy and Fowler, 2011) and involves four main steps which overlap to some degree (Sharpe, 2006). First, primordial germ cells, which originate from the extra-embryonic ectoderm, migrate while proliferating through the yolk sac and along the dorsal mesentery of the hindgut to the genital ridge (Kousta et al., 2010). Secondly, precursor Sertoli cells migrate from the coelomic epithelium, which covers the genital ridge, to the developing gonad and differentiate through the expression of sex-determining region Y (Sry) gene, which is marked by the polarization of
Sertoli cells to surround clusters of primordial germ cells (Wilhelm et al., 2007). Thirdly, mesonephric endothelial cells migrate into the testis and begin partitioning the germ/Sertoli cell aggregates into seminiferous cords while establishing the vasculature (Combes et al., 2009) and a layer of peritubular myoid cells, likely induced from within the testis (Cool et al., 2008), encircle the Sertoli cells and work cooperatively with them in depositing a basal lamina around the seminiferous cords (Skinner et al., 1985). Upon seminiferous cord formation, primordial germ cells are referred to as gonocytes (Oatley and Brinster, 2008). Lastly, the interstitial tissue between the seminiferous cords is colonized by fetal Leydig cells and connective tissue elements, which derive mainly from the mesonephric mesoderm (Rey et al., 2009).

Following testis formation, testis mass and the total number of germ and somatic cells increase curvilinearly with fetal age (Hochereau-de Reviers et al., 1995). Unlike in females, male germ cells are prevented from entering meiosis, due at least in part to the secretion of CYP26B1, a cytochrome P450 enzyme which acts by catabolizing retinoic acid, a known meiosis inducer, into inactive substrates (Griswold et al., 2012) by Sertoli cells (Li et al., 2009; Childs et al., 2011). Prior to development of the HPG axis around mid-gestation, testicular growth occurs in the absence of gonadotropin regulation (O’Shaughnessy and Fowler, 2011). However, the testes appear to become both LH- and FSH-dependent shortly thereafter, as fetal ram lambs that were hypophysectomized (Hochereau-de Reviers et al., 1995) or treated with buserelin, a GnRH agonist that initially stimulates, but later desensitizes and inhibits gonadotropin secretion (Thomas et al., 1994), displayed reduced Leydig cell area (Hochereau-de Reviers et al., 1995) and a decrease in Sertoli cell number and nuclear area (Thomas et al., 1994; Hochereau-de Reviers et al., 1995), indicating that Leydig and Sertoli cells, as the main target cells for LH and FSH, respectively, were compromised. Gonadotropin and androgen concentrations reach peak
levels and subsequently decline from mid–late gestation (Attal, 1969; Sklar et al., 1981), likely due to maturation of the negative feedback system (Sklar et al., 1981).

1.2.2 Early Postnatal Testicular Development

At birth, the seminiferous epithelium is comprised of two cell types: Sertoli cells, sometimes called supporting or pre-Sertoli cells, and gonocytes, also referred to as pro- or pre-spermatogonia (Culty, 2013), which gradually differentiate into type A spermatogonia throughout early postnatal testicular development (Figure 1.5; Steger and Wrobel, 1996). While more proliferative before birth (Hochereau-de Reviers et al., 1995), Sertoli cells replicate substantially throughout early postnatal testicular development in rams, approximately doubling in number between 25 and 40 days of age (Monet-Kuntz et al., 1984). Additionally, an increase in Sertoli cell size becomes apparent (Monet-Kuntz et al., 1984) as they gradually start to elongate and form apical cytoplasmic extensions (Gondos and Berndston, 1993). A 4-fold increase in the total number of germ cells occurs as they differentiate from pre-spermatogonia I, a large, round cell, typically with multiple nucleoli and located centrally in the seminiferous cords, to pre-spermatogonia II, located near the periphery of the cord with one single, large nucleolus, and finally type A spermatogonia, a smaller, oval-shaped cell containing one or two nucleoli (Steger and Wrobel, 1996); however, the volume fraction of germ cells in the testis is only 10–20% (Steger and Wrobel, 1996). A wave of germ cell apoptosis occurs throughout the latter phase of early postnatal development which affects mostly pre-spermatogonia I that fail to make contact with the basement membrane (Steger and Wrobel, 1996). Overall, these changes in the seminiferous epithelium induce a steep increase in length and a more constant increase in width of the seminiferous cords (Steger and Wrobel, 1996) and a rise in volume fraction of the
cords from 42% during fetal life (Hochereau-de Reviers et al., 1995) to 60% when type A spermatogonia first start to appear (Steger and Wrobel, 1996).

Figure 1.5 Histological cross-section through an early postnatal ram lamb testis. Pre-Sg, pre-spermatogonia I; Pre-Sg II, pre-spermatogonia II; SC, Sertoli cell. A degenerating cell (arrow) is present in the centre of a seminiferous tubule.

The expansion of the Sertoli cell population during early postnatal testicular development is critically dependent on FSH secretion, as ram lambs immunized against FSH from birth had a 57–82% reduction in Sertoli cell numbers per testis at 100 or 160 days of age compared to controls (Kilgour et al., 1998). A rise in FSH concentration is generally seen around 1–2 months of age in ram lambs (Lee et al., 1976; Yarney and Sanford, 1990; Chandolia et al., 1997a;
Tilbrook et al., 1999) and is typically followed by a rise in LH levels between 1.5–3 months of age (Foster et al., 1978; Wilson and Lapwood, 1979; Wańkowska et al., 2010). The rise in LH coincides with a rapid increase in the number of adult Leydig cells (Rawlings et al., 2008), a population separate from fetal Leydig cells which differentiate from fibroblast-like or mesenchymal cells in the interstitium (Mendis-Handagama and Ariyaratne, 2001), and a transient slow rise in testosterone secretion (Rawlings et al., 2008). Testosterone is known to induce morphological and functional differentiation of the Sertoli cells (Rey et al., 2009), while estradiol, which is also elevated during early pre-meiotic postnatal development in rats (Walczak-Jędrzejowska et al., 2009), stimulates proliferation of gonocytes (Li et al., 1997) and spermatogonia (Kula, 1988). Interestingly, the timing and magnitude of the rise in gonadotropin levels, particularly LH, is associated with the rate of pubertal maturation (Evans et al., 1995; Aravindakshan et al., 2000) and possibly even future adult reproductive performance (Yarney and Sanford, 1990). In comparison with rodents, in which activation of the HPG axis and Sertoli cell proliferation during fetal/neonatal life are continuous with and indistinguishable from subsequent pubertal development (Sharpe et al., 2003; Ebling, 2005), ram lambs display distinct developmental periods as seen in humans, and are thus more suitable experimental models for studying early postnatal development.

1.2.3 Prepubertal Testicular Development

Prepubertal testicular development (Figure 1.6) encompasses the onset of spermatogenesis, which is initiated when SSCs divide into spermatogonia committed to the differentiation pathway (Kolasa et al., 2012; Oatley and Brinster, 2012), and is complete upon achievement of the first spermatogenic wave, at a testicular weight of 70–80 g (Courot, 1978), or
when an ejaculate contains at least 50 million sperm with at least 10% progressive motility (Wolf et al., 1965), endpoints that are suitable for both bulls and rams (Courot, 1978; Amann and Schanbacher, 1983). At this point, the rams are said to have reached puberty, although the entire phase of prepubertal development is sometimes referred to as “puberty” (Hafez, 2000). Most rams reach puberty at the age of 4–6 months or 40–60% of mature body weight (Jainudeen et al., 2000). In general, body weight provides a better indication of testicular maturation than chronological age (Courot, 1978; Jainudeen et al., 2000; Salhab et al., 2001); however, the precise timing of puberty is influenced by a complex interplay of genetic (e.g., breed), endogenous (e.g., metabolic state) and environmental or exteroceptive (e.g., photoperiod, temperature, nutrition, socio-sexual cues) factors (Valasi et al., 2012). Typically, the first differentiated spermatogonia become increasingly prevalent between approximately 6–10 weeks of age (Monet-Kuntz et al., 1984), with early primary spermatocytes first appearing around 9–10 weeks of age (Skinner et al., 1968; Monet-Kuntz et al., 1984; Herrera-Alarcón et al., 2007), followed by late primary spermatocytes and the first round spermatids at roughly 12 weeks of age (Skinner et al., 1968; Monet-Kuntz et al., 1984; Herrera-Alarcón et al., 2007) and finally elongated spermatids at 15 weeks of age (Herrera-Alarcón et al., 2007). Spermatozoa are first seen in the seminiferous tubule lumen and ejaculate around 16 and 18 weeks of age, respectively (Hafez, 2000). The stages of the seminiferous epithelial cycle become apparent shortly after the first appearance of primary spermatocytes (Courot et al., 1970; Drumond et al., 2011); however, during spermatogenic onset the stage of spermatogenesis is commonly described by the presence of the most mature germ cell type, likely due to the considerable variation in germ cell composition among the seminiferous tubules (Snyder et al., 2010).
Figure 1.6 Histological cross-section through a prepubertal ram lamb testis. Sg, spermatogonia; Sc, spermatocytes; SC, Sertoli cell.

The establishment of the seminiferous epithelial cycle has recently been suggested to be mediated by retinoic acid (RA; Snyder et al., 2011; Davis et al., 2013), the primary active metabolite of vitamin A (Griswold et al., 2012). Retinoids (i.e., compounds chemically related to vitamin A) as a class are well-known for their ability to induce cell differentiation and/or reduce proliferation or promote cell apoptosis (Ross, 2010). During spermatogenic onset, retinoic acid acts on gonocytes and undifferentiated spermatogonia to transform them into differentiated spermatogonia (Hogarth and Griswold, 2010; Busada et al., 2014). This is consistent with previous findings that gonocytes do not have to pass through the undifferentiated spermatogonia
stage before becoming differentiated spermatogonia (Yoshida et al., 2006). A periodic appearance of RA signalling was seen in the testes of neonatal mice (Snyder et al., 2011), indicating that spermatogenic onset does not occur uniformly throughout the testis. However, in some species such as boars and stallions the pattern of RA signalling may not be periodic since the initiation of spermatogenesis occurs along a maturation gradient, in which spermatogenesis and seminiferous tubule maturation proceed from the interior region of the testes towards the outer tunica albuginea (Clemmons et al., 1995; Ford and Wise, 2009; Avelar et al., 2010). The precise spatiotemporal organization of spermatogenesis appears to be supported by a delicate balance between RA and CYP26 degrading enzymes, which may be overwhelmed when exogenous RA is administered prior to the first appearance of preleptotene spermatocytes in neonatal mice, resulting in highly synchronous spermatogenesis as adults (Snyder et al., 2011; Davis et al., 2013).

Spermatogenic onset can be divided into two key phases: the mitotic and post-mitotic phases, with spermatogonia or spermatocytes and/or spermatids present in the majority of seminiferous tubule cross-sections as the most mature germ cell types, respectively. In the mitotic phase, gonadotropin levels are decreasing and testosterone secretion is low (Bagu et al., 2006). The microscopic appearance of the testis during the mitotic phase is quite similar to that during early postnatal development, except for a reduction in the number of pre-spermatogonia and an expanded population of both undifferentiated and differentiated spermatogonia. By contrast, in the post-mitotic phase gonadotropin secretion is low, while testosterone levels rise rapidly, due likely to an increase in gonadotropin receptor concentration and affinity (Bagu et al., 2006). In the ram, testosterone concentration rises dramatically beginning at 12 weeks of age (Herrera-Alarcón et al., 2007; Wańkowska et al., 2010) and LH receptor concentration increases
7-fold between 90–120 days (~13–17 weeks of age; Yarney and Sanford, 1989). Several changes in testicular histomorphology occur during the post-mitotic phase of spermatogenic onset including: i) the completion of Sertoli cell proliferation which occurs in ram lambs at 13.5–14.5 weeks of age (Oluwole et al., 2013); ii) continued Sertoli cell elongation and formation of multiple surface projections (Gondos and Berndston, 1993) such that cellular and nuclear area increase by a factor of 3.5 and 1.5, respectively, from 25–100 days of age (~3.5–14 weeks of age; Monet-Kuntz et al., 1984); iii) development of inter-Sertoli cell tight junctions comprising the blood-testis barrier (Pelletier and Byers, 1992); iv) lumen formation, occurring predominantly after the establishment of the blood-testis barrier (Russell et al., 1989), concurrent with Sertoli cell fluid production (Griswold, 1998) as well as fluid transportation from the interstitial tissue to the seminiferous tubules by Sertoli cells (Alves et al., 2013); and v) a round of germ cell apoptosis which may result from establishment of the proper germ:Sertoli cell ratio or could be related to functional immaturity of the blood-testis barrier (Steger and Wrobel, 1996), which primarily affects mid-pachytene spermatocytes in rats (Jahnukainen et al., 2004). Final adult testis size and daily sperm production are determined by the number of Sertoli cells present in the testes (Sharpe et al., 2003), which have been shown to vary with breed as well as individual (de Reviers et al., 1980; Berndtson et al., 1987a; Berndtson et al., 1987b); therefore, prepubertal development is critical in the establishment of future fertility.

1.2.4 Postpubertal Testicular Development

Beyond puberty, a period of increasing testicular growth and spermatogenic efficiency ensues prior to reaching adult levels (Courot et al., 1970). The tubular volume fraction of the testis increases from approximately 63% during the prepubertal period to 84% as adults and
Seminiferous tubule diameter doubles to more than 200 µm (Steger and Wrobel, 1996). Testosterone levels and testicular size are comparable with adult levels by 27–28 weeks of age (Olster and Foster, 1986; Olster and Foster, 1988; Yarney and Sanford, 1990; Yarney et al., 1990) and ejaculates consistent with normal fertility (3–5 x 10⁹ spermatozoa) are produced shortly afterwards, by around 30–36 weeks of age according to breed (Courot, 1979). Depending on the definition of puberty used in a particular study, the postpubertal period may begin when the first round of spermatogenesis has been completed or when ejaculate parameters reach a specific level, as previously stated; therefore, early postpubertal development is often referred to as peripubertal development.

1.3 Scrotal Ultrasonography

Scrotal ultrasonography was first reported in men in 1974 (Miskin and Bain), and later in bulls (Pechman and Eilts, 1987) and other animals. As a cost-effective, easily repeatable, portable and non-invasive technique, it has since become integrated into numerous evaluation protocols for the detection of scrotal pathologies (e.g., scrotal masses, acute scrotal pain, microlithiasis) in addition to male infertility (Ragheb and Higgins, 2002). Furthermore, when combined with computer-assisted image analysis, scrotal ultrasonography may provide an indication of tissue microstructure and reproductive hormone secretion (Evans et al., 1996; Brito et al., 2012) which could greatly enhance visual interpretation.

1.3.1 Image Formation

Ultrasound is a form of sound that exceeds the range of audible sound on the acoustic spectrum. As a wave, sound vibrates at specific frequencies, measured in hertz (Hz), such that
one hertz is one cycle per second and a megahertz (MHz) is one million cycles per second.

Humans are capable of hearing sound that vibrates at a frequency of 20–20,000 Hz, far below the frequencies generally used in medical ultrasound, which range from 2–10-MHz (Ginther, 1995). The transducer is the component of a medical ultrasound device that emits ultrasound waves when an electric current is applied to its piezoelectric crystal elements. Upon entering the tissue being scanned, the ultrasound beam takes five possible trajectories: i) transmission through the tissue; ii) reflection back along the same direction; iii) scattering in many directions; iv) refraction in a different direction; and v) absorption into the tissue by heat conversion (Ginther, 1995). The progressive weakening of the ultrasound beam as it becomes reflected, scattered, refracted and absorbed is referred to as attenuation (Ginther, 1995). Sound waves that have been reflected or scattered back to the transducer (i.e., backscatter) are converted into electrical signals by the piezoelectric crystal elements in the transducer. These are displayed as minute picture elements (i.e., pixels) of varying shades of gray (i.e., pixel intensities), comprising the image or ultrasonogram seen on the monitor (Ginther, 1995). Reflection and scattering are also referred to as specular (derived from Latin “mirror-like”) and non-specular or diffuse reflection, respectively, whereby interfaces that are smooth, wider than the beam wavelength and perpendicular to the beam generate specular reflection and interfaces that are rough or narrower than the beam wavelength generate non-specular reflections (Ginther, 1995; Kremkau, 2006).

The majority of reflections within a tissue are non-specular, which are much less intense than specular reflections (~1/1000 in amplitude; Gayrard et al., 2010); however, they contribute greatly in the formation of the distinct gray-scale pattern, or echotexture of a given tissue (Ginther, 1995). It should be noted, however, that some of the scattered ultrasound waves reinforce or interfere with each other so tissue echotexture is representative but does not
necessarily correspond with specific physical structures (Ginther, 1995). Brightness or intensity of the interspersed shades of gray on an ultrasonogram depends on tissue echogenicity or the ability of the tissue to reflect ultrasound waves (Ginther, 1995). Tissue echogenicity is determined by the magnitude of the difference in acoustic impedance, or resistance to the propagation of ultrasound waves (Ginther, 1995), related to tissue density and compressibility, (Venables, 2011) at multiple interfaces within the tissue (e.g., different macromolecular and cellular constituents), as well as the size, shape, concentration and distribution of the individual scatterers (Silverman and Noetzel, 1990; Hunt et al., 1995; Vlad et al., 2005; Ghoshal et al., 2013). Hyper- or hypoechoic/-echogenic structures appear bright or dark within an ultrasonogram, respectively, while anechoic structures, which do not reflect any ultrasound waves (typically fluids), appear black. Depending on the uniformity of the tissue being scanned, tissue echotexture ranges from fine to course.

Table 1.1 Typical ultrasound properties at 5–10-MHz. Information obtained from Kremkau (2006).

<table>
<thead>
<tr>
<th></th>
<th>5-MHz</th>
<th>7.5-MHz</th>
<th>10-MHz</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Wavelength (mm)</strong></td>
<td>0.31</td>
<td>0.21</td>
<td>0.15</td>
</tr>
<tr>
<td><strong>Axial resolution (mm)</strong></td>
<td>0.31</td>
<td>0.20</td>
<td>0.15</td>
</tr>
<tr>
<td><strong>Imaging depth (cm)</strong></td>
<td>12</td>
<td>8</td>
<td>6</td>
</tr>
</tbody>
</table>

The testes are well-suited for ultrasound scanning as they are easy to access, lack surrounding organs, are symmetrical and may easily be compared with one another (Gouletso and Fthenakis, 2010). In rams, testes are typically scanned at a frequency of 5–10-MHz (Chandolia et al., 1997a; Sargison, 2008; Vencato et al., 2014). With increasing frequency, the beam wavelength decreases and image resolution improves (typically best along the beam axis, referred to as “axial resolution”), but the depth of penetration decreases (Table 1.1; Kremkau,
A linear-array transducer, in which the piezoelectric crystals are arranged in a row, is most often used when scanning ram testes (Chandolia et al., 1997a; Sargison, 2008; Gayrard et al., 2010; Vencato et al., 2014). This type of transducer allows for broad surface contact and produces a rectangular-shaped image of constant and sufficient width to cover the region being examined (Gayrard et al., 2010). Scrotal ultrasonography is most easily performed with rams restrained in dorsal recumbency on the floor or in a metallic cradle, or possibly in a standing position (Viñoles-Gil et al., 2010). As the acoustic impedance of air is much less than soft tissue, with more than 99% of the energy reflected at the air/soft tissue interface (Gayrard et al., 2010), scrotal hair should be clipped and a coupling gel applied to eliminate air (Gouletsou and Fthenakis, 2010). Testes may be scanned in longitudinal and transverse planes (Chandolia et al., 1997a) and their ultrasonographic appearance optimized using ultrasound machine settings.

### 1.3.2 Image Analysis

Gray-scale (B-mode), two-dimensional ultrasonography permits the rapid visualization of internal, macroscopic structures. In rams, the main discernible features of a scrotal ultrasonogram are i) the rete testis, observed as a hyperechogenic linear or circular region in the centre of the testis in longitudinal and transverse views, respectively, ii) the testicular parenchyma surrounding the rete testis, which is typically described as homogenous with a course medium echotexture (Gouletsou and Fthenakis, 2010; Vencato et al., 2014), and iii) the tunica albuginea and scrotum, seen as a hyperechogenic line surrounding the testicular parenchyma with an anechoic layer due to the presence of fluid separating the visceral and parietal layers of the tunica vaginalis (Figure 1.7; Wittenberg et al., 2006). The head and tail of the epididymis, spermatic cord and scrotal septum may also be viewed depending on the
placement of the transducer in the longitudinal plane (Gouletsou and Fthenakis, 2010). Based on the ultrasonographic appearance of the scrotal contents, testicular size and certain abnormal conditions may be readily identified. However, visual analysis alone does not allow for a detailed assessment of testicular echotexture; determination of the precise shades of gray comprising an ultrasonogram is a highly subjective process, is dependent on ultrasound machine settings and is limited by the capabilities of the human eye, which can distinguish only about 20 different shades of gray (Lieu, 2010).

![Figure 1.7 Scrotal ultrasonograms of ram lambs in longitudinal and transverse views. rt, rete testis; tp, testicular parenchyma; ta, tunica albuginea; s, scrotum.](image)

A quantitative, objective analysis tissue echotexture may be achieved using computer-assisted image analysis. In this procedure, real-time ultrasound scanning is recorded and a still image is later selected and saved in an un-compressed format (i.e., bitmap; Singh et al., 2003) for analysis using a specialized imaging software program that assigns each pixel a numerical pixel value (NPV) ranging from 0, representing black, to 255, representing white. The square pixels number several thousand within an ultrasound image, and their size may be determined by dividing the anatomic width or length spanning the image by the corresponding number of
pixels; however, it should be noted that image resolution is usually limited by the resolution capability of the transducer, not pixel density (Kremkau et al., 2006). In the testes, echotextural attributes of the testicular parenchyma are commonly obtained using multiple circular areas that are placed on the testicular parenchyma, known as the spot metering technique (Pierson and Adams, 1995; Singh et al., 2003). Measures of central tendency and variation, typically mean NPVs and pixel standard deviation (PSD; i.e., pixel heterogeneity) are determined for specific area(s) of interest (Pierson and Adams, 1995; Kastelic and Brito, 2012). Pixel heterogeneity of the NPVs reflects the existence of interspersed hyper- and hypoechoic areas in a tissue, indicating the level of tissue uniformity. During scanning, ultrasound settings must be kept constant for all animals throughout the entire period of collection (i.e., all days of scanning) so that the images may be comparable with one other. While this technique is currently used only for research purposes, it considerably enhances the amount of extractable information that may be obtained from an ultrasonogram and is currently being investigated in a number of different tissues in the body including the carotid arteries (Lal et al., 2002), lymph nodes (Nguyen et al., 2012), ovaries (Singh et al., 2003) and testes (Kastelic and Brito, 2012).

1.3.3 Testicular Echotextural Trends

Using computerized-image analysis, testicular echogenicity demonstrates a distinct temporal trend and is correlated with several histomorphological and endocrine attributes throughout sexual development. In general, an increase in testicular echogenicity has been observed with age from birth to puberty in several species including humans (Hamm and Fobbe, 1995), bulls (Evans et al., 1996; Chandolia et al., 1997b; Aravindakshan et al., 2000; Brito et al., 2012) and rams (Chandolia et al., 1997a). However, this increase does not occur linearly; rather,
numerous fluctuations occur (Evans et al., 1996; Chandolia et al., 1997a; Chandolia et al., 1997b; Aravindakshan et al., 2000; Brito et al., 2012), especially with more frequently-taken ultrasound evaluations (Evans et al., 1996). Beyond puberty, minimal changes in testicular echogenicity are seen (Hamm and Fobbe, 1995; Brito et al., 2002; Brito et al., 2012).

Currently, the specific reason(s) for the changes in testicular echogenicity throughout sexual maturation are unknown. However, in bull calves from birth to puberty, testicular echogenicity was found to be negatively correlated with the percentages of seminiferous tubule cross-sections with no germ cells present or with gonocytes and pre-spermatogonia, spermatogonia or early spermatocytes present as the most mature germ cell type, and positively correlated with the percentages of seminiferous tubule cross-sections with late spermatocytes, round and elongated spermatids and mature sperm cells as the most advanced germ cell type present, the inner (luminal) and outer (tubular) seminiferous tubule diameters and serum testosterone concentration (Evans et al., 1996). In postpubertal yearling bulls, testicular echogenicity was positively correlated with total daily sperm production (total and per gram of testis; Brito et al., 2012) and negatively correlated with seminiferous tubule diameter and/or area (Gábor et al., 1998; Brito et al., 2012) and seminiferous epithelium area (Brito et al., 2012). Additionally, testicular NPVs and PSD have been correlated with semen parameters, especially in the weeks following ultrasound evaluations (Arteaga et al., 2005; Ahmadi et al., 2012; Brito et al., 2012). These results indicate that quantitative echotextural analysis of scrotal ultrasonograms is capable of detecting changes in testicular microstructure and endocrine secretion; however, further verification of the echotextural attributes of different testicular components and developmental events is needed to determine the precise diagnostic and prognostic capabilities of scrotal ultrasonography.
Chapter 2

RATIONALE, OBJECTIVES AND HYPOTHESES

Scrotal ultrasonography is a commonly used imaging modality for the evaluation of internal, macroscopic features. However, using computer-assisted image analysis, the range of potential clinical applications is greatly enhanced, as testicular echotextural attributes are associated with testicular microstructure, reproductive hormone secretion and future semen quality. Additionally, as a cost-effective, portable and non-invasive technique with unlimited frequency of use, computer-assisted image analysis of scrotal ultrasonograms offers several unique benefits that conventional methods of reproductive assessment do not provide. However, a more thorough understanding of which histomorphological and endocrine attributes affect testicular echotexture and how their relationship may change over time, as well as the influence of non-parenchymal tissue elements on testicular echotexture is needed. Specific research objectives of the studies presented in this thesis are given below.

**Objective 1:** To evaluate the effect of the scrotal and testicular integumentary layers, blood flow and the presence of blood on testicular echotexture and its relationship with seminiferous tubule histomorphology in peripubescent ram lambs.

Together, the scrotal and testicular integuments surround the testis in 10 layers of skin, muscle and connective tissue. During ultrasound scanning, the ultrasound beam must first pass through these layers before reaching the testicular parenchyma. With increasing penetration depth, there
is loss of ultrasound beam strength, known as attenuation. Throughout sexual maturation or with aging, and under a number of different pathological conditions, the thickness of the scrotal and/or testicular integument increases; furthermore, differences in testicular capsule thickness across the surface of the testis have been demonstrated in several species. Additionally, blood flow and the presence of blood may affect the trajectory of the ultrasound beam and present an additional interface between the ultrasound beam and the testicular parenchyma. Therefore, it is important to understand how these non-parenchymal tissue elements affect testicular echotexture and its ability to predict testicular microstructure. Our hypothesis for this study was that the scrotal and testicular integument, blood flow and presence of blood reduce testicular pixel intensity and heterogeneity of ultrasound images and weaken their relationship to seminiferous tubule histomorphology in peripubescent ram lambs.

Objective 2: To determine correlations among testicular echotexture and an array of histomorphological attributes during specific developmental phases in prepubescent ram lambs.

Testicular echogenicity generally increases with age from birth to puberty; however, considerable fluctuations exist, especially with more frequently taken ultrasound evaluations. Beyond puberty, minimal changes in testicular echogenicity occur. Correlations among testicular pixel intensity and various histomorphological variables have been found in both pre- and postpubertal bull calves; however, the direction of some correlations including seminiferous tubule size does not remain consistent. During the onset of spermatogenesis, a number of changes occur in the seminiferous tubules, including lumen formation and increased germ cell mitosis, differentiation and apoptosis. Therefore, we hypothesized that the temporal trend in
testicular echogenicity and correlations among echotextural and histomorphological attributes differ according to the phase of spermatogenic onset in prepubescent ram lambs.

**Objective 3:** To examine the influence of retinoic acid exposure in early postnatal ram lambs on spermatogenic onset during prepubertal development and to identify associated changes in testicular histomorphology, endocrine status and ultrasonographic attributes of the testes.

The testis is a complex, heterogeneous organ with over 30 different cell types. Testicular echotextural attributes are closely associated with spermatogenic development; however, precise characterization of specific cell types is difficult due to germ cell heterogeneity. Recently, a protocol was developed in which highly synchronized spermatogenesis was achieved using a single administration of retinoic acid in neonatal mice. If this works in other mammals, such as rams, in which distinct phases of testicular maturation are observed, it could represent a valuable *in vivo* experimental model for determining the echotextural characteristics of specific germ cell types. Therefore, we hypothesized that exposure to retinoic acid in early postnatal ram lambs induces the synchronous onset of spermatogenesis during prepubertal development and alters testicular histomorphology, endocrine status and testicular echotextural attributes.
Chapter 3

A STUDY OF INTEGUMENTARY, HISTOMORPHOLOGICAL AND
HAEMODYNAMIC DETERMINANTS OF TESTICULAR ECHOTEXTURE IN RAM

LAMBS

Jennifer L Giffin, Sarah E Franks, Jose Rafael Rodriguez-Sosa§, Ann C Hahnel, Pawel M Bartleewski

Department of Biomedical Sciences
Ontario Veterinary College
University of Guelph
Guelph, ON, Canada N1G 2W1

§Department of Anatomy
College of Veterinary Medicine
Midwestern University
Glendale, AZ, USA 85308

N. B. A version of the contents presented in this chapter has previously been published (Giffin et al., 2009; doi: 10.3181/0812-RM-364).
3.1 Abstract

The ultrasonographic appearance of an organ is a product of scattering and reflection of high-frequency sound waves by discrete units of tissue. The number and types of acoustic tissue interfaces affect the quantitative characteristics of ultrasound images. This study was undertaken to examine the influences of the scrotal and testicular integumentary layers, blood flow and the presence of blood on testicular echotextural parameters in the ram. Serial ultrasonograms of the testes were obtained during surgical castration of seven ram lambs aged 20–22 weeks. The first two sets of images were taken through the scrotum prior to and after induction of anaesthesia, the third and fourth sets were taken through the tunica vaginalis and tunica albuginea, respectively, the fifth set was taken when the spermatic cord and internal blood vessels were clamped, and the final set was taken after allowing the blood to drain from dissected testicles. Ultrasonograms were then subjected to computerized image analysis and the testes were processed for histology. The removal of the scrotal skin and tunica vaginalis increased numerical pixel values (NPVs) and pixel standard deviation (PSD) of the testicular parenchyma by approximately 25 and 9%, respectively. However, no differences in testicular echotexture were found between images taken just before or after clamping the spermatic cord vessels, or after draining. NPVs predicted tubular and luminal areas of the seminiferous tubules (STs) at nearly all stages of castration and had a consistent moderately positive slope in the regression equation. PSD predicted tubular and luminal ST area at only a limited number of stages in the castration process and the slope of the regression equation fluctuated greatly. Therefore, it was concluded that ultrasound attenuation by the scrotal and testicular integumentary layers, but not vascular blood flow or presence of blood affects testicular echotexture and that NPVs are a reliable indicator of ST microstructure in situ and ex vivo.
3.2 Introduction

The ultrasonographic display of an organ or tissue is a product of reflection and scattering of high-frequency ultrasound beams by discrete units of tissue (i.e., acoustic tissue interfaces). Interfaces that are large and smooth produce strong reflections, known as specular reflections, depending on beam angle, whereas interfaces that are rough or smaller than the ultrasound wavelength (0.77–0.15 mm for most medical ultrasound frequencies of 2–10-MHz) produce less intense reflections referred to as non-specular reflections or scattering (Kremkau, 2006). Specular reflections are seen as bright or hyperechogenic regions on the ultrasonogram whereas non-specular reflections, which form the bulk of the ultrasound image in cellular tissues, result in the formation of a unique gray-scale pattern, or echotexture, depending on the echogenicity, or ability of the various tissue interfaces to reflect ultrasound waves (Ginther, 1995). In addition to reflection and scattering, the ultrasound waves also become refracted and absorbed into the tissue by heat conversion; this collective loss in energy of the ultrasound beam is referred to as attenuation, which progressively increases as the ultrasound beam passes through the tissue, and ultimately limits the depth of penetration (Ginther, 1995).

Scrotal ultrasonography is a frequently performed imaging procedure for the evaluation of male reproductive health in both humans as well as domestic species. This approach offers many benefits as it allows for the repeated and non-invasive visualization of scrotal contents using a portable and cost-efficient device. In the testis, the main specular reflectors are the rete testis, a series of anastomosing channels located centrally in rams, and the outer integumentary layers, consisting of the scrotum, a multilayered tissue composed largely of connective tissue fascia along with the dartos and cremaster muscles (Davis et al., 1970), and the testicular capsule, comprised of 3 layers: i) the tunica vaginalis visceralis, a thin serous membrane forming
the outermost layer of the capsule (Davis et al., 1970); ii) the *tunica albuginea*, a thick collagenous layer interspersed with smooth muscle cells (Davis et al., 1970); and iii) the *tunica vasculosa*, a loose connective tissue rich in blood vessels (Jequier, 2000) in direct contact with the seminiferous tubules (STs). These specular reflectors appear as hyperechogenic regions on the ultrasonogram and are easily differentiated from the testicular parenchyma, consisting of the STs and interstitial tissue. The testicular parenchyma is typically described as homogenous with a course medium echotexture (Gouletsou and Fthenakis, 2010; Vencato et al., 2014). However, a more detailed assessment of testicular echotexture may be obtained using computer-assisted image analysis. In this procedure, shades of gray on the ultrasonogram are converted into numerical pixel values (NPVs), ranging from 0, representing black, to 255, representing white, which are positively associated with tissue echogenicity. Regions of interest on the ultrasonogram are selected for analysis, and various echotextural attributes may be determined, including average NPV and pixel heterogeneity/standard deviation (PSD). Studies in bulls and rams have demonstrated that testicular echotextural parameters are closely related to the histomorphological characteristics of STs (Gábor et al., 1998; Evans et al., 1996; Brito et al., 2012). Therefore, scrotal ultrasonography in conjunction with computerized-image analysis may have potential as a non-invasive method for assessing testicular microstructure.

In order to be used for diagnostic purposes, the effect of non-parenchymal tissues on testicular echotexture needs to be investigated. Scrotal thickness may increase during pathological conditions (Ragheb and Higgins, 2002) and thickness of the *tunica albuginea* has been shown to increase throughout sexual maturation (Mitchell and Seeley, 1977) and with aging (Arenas et al., 1997) and to vary across the surface of the testis in several species (Davis et al., 1970; Aire and Ozegbe, 2007). Furthermore, blood has known echotextural properties (Wang
and Shung, 2001) and the flow of blood is constantly in a state of flux, due to nervous and hormonal factors, temperature, posture and other variables (Setchell, 1970). To what extent these changes affect testicular echotexture and its relationship with tissue microstructure is currently unknown. Therefore, the objective of the present experiment in ram lambs was to examine the influence of the scrotal/testicular tissue layers and the presence of blood and blood flow on testicular echotexture and to assess the relationship among testicular echotextural attributes and histomorphological characteristics of the STs.

3.3 Materials and Methods

3.3.1 Animals and Experimental Design

The Animal Care Committee at the University of Guelph approved all experimental procedures performed on live sheep described in this section according to the Canadian Council on Animal Care guidelines. Seven spring-born Rideau Arcott x Polled Dorset ram lambs aged 20–22 weeks (July) were housed in a field research station in Ponsonby, ON, Canada (latitude: 43° 33’N). Animals were kept in outdoor pens, under ambient light and temperature conditions, with easy access to indoor facilities. All lambs were weaned at 50 days of age. Starting at 14 days of age and until 50 days after weaning, the lambs were fed a 16%-protein lamb grower (Floradale Seeds, Floradale, ON, Canada) ad libitum. After 100 days of age, rams were fed a diet of 80% whole barley and 20% whole corn with a 36% crude protein sheep supplement (Shur-Gain Feedmills, St. Marys, ON, Canada) and hay, and daily consumption averaged 1 pound of grain and 0.5 pounds of sheep supplement per ram. Water was always accessible to the rams.

Serial scrotal ultrasonograms were taken throughout surgical castration of both testes performed under general anaesthesia induced with xylazine (Rompun®, Bayer, Toronto, ON,
Canada; 0.2 mg/kg i.m.) and maintained by i.v. administration of ketamine (Ketalean, Animal Health Inc., Cambridge, ON, Canada; 2 mg/kg) and diazepam (Diazepam, Sabex, Boucherville, PQ, Canada; 0.2 mg/kg). The first set of images was taken in animals examined in a standing position prior to Rompun® injections. From that point on until the removal of testicles, the images were taken of animals placed in dorsal recumbency. The second set of images was taken through the scrotum just prior to the surgical procedure, the third set was taken through the tunica vaginalis, the fourth set was obtained through the tunica albuginea, and the fifth set was taken after the spermatic cord and internal blood vessels had been clamped off with forceps. The final set of images was recorded after allowing the blood to drain from dissected testicles for 5 min. Testes were then processed for histology. 14 testes were studied in accordance with the sample size calculation using the formula below:

\[
n = 3 + \frac{31.4}{\left[ \ln \left( \frac{1 + r}{1 - r} \times \frac{1 - r_0}{1 + r_0} \right) \right]^2}
\]

with Type I error set to \(\alpha=0.05\) and Type II error set to \(\beta=0.20\) (Dell et al., 2002), where values for \(r\) and \(r_0\) represent a 0.69 correlation coefficient found in bull calves from birth to puberty between testicular pixel intensity and outer seminiferous tubule (ST) diameter (Evans et al., 1996) and a zero value correlation coefficient it is different from, respectively.

### 3.3.2 Ultrasonography

Ultrasonography utilized an Aloka SSD-900 echo camera equipped with a 7.5-MHz linear array probe (Aloka Inc., Tokyo, Japan) placed in a plastic bag filled with lubricant gel (Light Lube, Centaur VA Animal Health, Guelph, ON, Canada) for easier cleaning and disinfected with isopropyl ethanol between sequential examinations. Both left and right testes
were examined in longitudinal view at constant settings of the ultrasound scanner for overall gain, near and far gains and focal points, and were recorded on a Digital Versatile Disk (DVD) recorder (Pioneer® DVD Recorder DVR-510H, Pioneer Electronics of Canada Inc., Markham, ON, Canada). The DVD recording was downloaded to a computer workstation (Intel®-Xeon® Processor MP; 2.8 GHz; Intel Co., Santa Clara, CA, USA) and still images containing the largest cross-sectional area in a longitudinal-plane view of each testicle were selected using Adobe Premiere® Pro 2.0 (Adobe Systems Inc., San Jose, CA, USA). Computerized-image analysis was carried out with Image ProPlus® (Media Cybernetics Inc., San Diego, CA, USA) using the “spot meter” technique, as previously described by Pierson and Adams (1995). Six spots of 60 pixels in diameter (~6 mm) were placed on the image of the testicular parenchyma; three computer-generated spots were placed above and three below the central rete testis, excepting the rete testis itself and any imaging artifacts. The mean numerical pixel values (NPVs) and standard deviations of NPVs (pixel heterogeneity– PSD) in each spot were recorded. Figure 3.1a depicts the general appearance of the ultrasonograms that were analyzed. Three individuals to whom the identities of rams were not known performed all echotextural analyses.

3.3.3 Tissue Processing and Histology

Testicular tissue samples were taken from the medial aspect of a longitudinally dissected testis (Fig. 3.1b) and fixed overnight in modified Davidson’s fluid (Lattendresse et al., 2002). Samples were washed in 70% ethanol and embedded into paraffin wax blocks, sectioned at a thickness of 5 µm and then deparaffinized in xylene and rehydrated in a graded isopropanol-water series for staining with hematoxylin and eosin. Coverslips were applied using Cytoseal XYL mounting medium (Richard-Allen Scientific, Kalamazoo, MI, USA).
Digital images of the histological slides (one tissue section/testis, three images per tissue section) were taken under 200x image magnification using the computer program Q Capture® (Quorum Technologies Inc., Guelph, ON, Canada). All histomorphological analyses were done using the Image ProPlus® analytical software. The outer seminiferous tubule (ST) circumference was manually outlined and used to calculate the relative tubular ST area (i.e., proportion of the total area occupied by STs; Fig. 3.1c). Similarly, the relative luminal ST area was computed from a manual outline of the inner seminiferous tubule circumference (Fig. 3.1c). All ST cross-sections per image were analyzed. Three individuals to whom the identities of rams were not known performed all histomorphological analyses.

3.3.4 Statistical Analyses

All data sets were initially screened for outliers using Grubb’s test (the maximum normed residual test; http://www.graphpad.com/www/grubbs.htm). Differences in mean NPVs as well as pixel heterogeneity (PSD) were evaluated using a mixed linear model generated by the PROC MIXED procedure on SAS (SAS Institute Inc., Cary, NC, USA) that included the main effects of the individual performing the analyses (3 individuals), testicle examined (left versus right), spot location (3 spots above versus 3 spots below the rete testis), set of images obtained, and the interactions among those factors. The relationships between echotextural and histomorphological parameters were studied by simple linear regression (SigmaStat®3.0 for Windows®, 2003, Systat Software, Inc., Richmond, CA, USA); the input variables were the mean NPVs and PSD values determined using Image ProPlus® software in the upper 3 spot meters (i.e., spots encompassing the regions of testicles from which tissue samples were dissected for histological examinations).
and the output variables were the relative (%) tubular and luminal ST areas. In all analyses, \( P < 0.05 \) was considered statistically significant. All results were presented as mean ± SEM.

**Figure 3.1** Testicular echotextural and histomorphological analysis of selected tissue regions and the abnormal histological appearance of a testicle removed from statistical analyses. (a) A digital ultrasonographic image of the ram’s testis, in longitudinal section, used for computer-assisted echotextural analysis. Arrows delineate the skin of the scrotum (top) and the scrotal septum (bottom). The rete testis is the hyperechogenic elongated area in the center. Circles represent potential placement areas for computer-generated spot meters used for the assessment of numerical pixel values (NPVs) and pixel heterogeneity (standard deviation of pixel values–PSD). (b) One half of a longitudinally dissected testis. Arrows point to the middle section from which samples were taken for histological examinations. (c) Outlines of all outer seminiferous tubule (ST) circumferences (black) were used to calculate the relative tubular area of STs. Similarly, the relative luminal area of STs was computed from outlines of the inner ST circumferences (dark gray). (d) Histological appearance of testicular tissue samples from one ram with the lowest relative tubular and luminal ST areas identified by the Grubb’s test; the ultrasonographic and histomorphological data for this testis were consequently withdrawn from all statistical analyses.
3.4 Results

3.4.1 General Results

The Grubb’s test revealed the existence of a single consistent outlier in the data set, denoted “5L” in the following graphs. Inspection of histological slides revealed small size of STs in the three tissue samples obtained from this testicle (Fig. 3.1d). Therefore, the data for this testicle were removed from all statistical analyses.

The mixed linear model revealed no differences among echotextural parameters determined by the three individuals, in the spot meters placed above or below the rete testis, or between left and right testes ($P > 0.05$). Therefore, echotextural parameters for the entire testis were based on the average of all eighteen values (six spots per image, three individuals/measurement). Echotextural parameters for the upper region of the testis, from which tissue samples were taken, were based on the average of nine values/testis (three upper spots per image, three individuals/measurement).

3.4.2 Testicular Echotexture

The removal of the scrotal skin, both before and after the induction of anaesthesia, and the *tunica vaginalis* both resulted in significant increments in NPVs of the testicular parenchyma ($P < 0.05$; Fig. 3.2a). Removal of the scrotal skin also resulted in increased pixel heterogeneity (PSD) values ($P < 0.05$; Fig. 3.2b). There were no differences in either echotextural parameter between images taken just prior to or after clamping the spermatic cord vessels and draining the testes of blood ($P > 0.05$; Fig. 3.2a and b).
Figure 3.2 Mean (± SE) numerical pixel values (NPVs; a) and pixel heterogeneity (PSD; b) for the testicular parenchyma determined by computer-assisted image analyses of testicular ultrasonograms obtained during castration in seven Rideau-Arcott x Polled Dorset ram lambs. “Scrotum*” refers to testicles scanned before sedation and placing the transducer in a lubricant-filled plastic bag; “Scrotum” refers to testicular images obtained through the intact scrotal skin; “Tunica vaginalis” and “Tunica albuginea” refer to testes with scrotum removed and images taken through tunica vaginalis and tunica albuginea, respectively; “Clamped” refers to testes after the spermatic cord and internal blood vessels were clamped with forceps; and “Drained” denotes testes after allowing for the blood to drain from for 5 min. Means with different letters are significantly different (P < 0.05).
3.4.3 Linear Regression Models

At all stages, NPVs of the three upper spot meters (placed above the rete testis) predicted tubular and luminal ST areas (Fig. 3.3; $P < 0.05$), except for images obtained through the *tunica albuginea*, in which there was no association between NPVs and luminal ST area ($P=0.20$; Fig. 3.3h). The association between NPVs and tubular ST area approached significance for testicular images recorded through the *tunica albuginea* ($P=0.06$; Fig. 3.3g) and after draining ($P=0.10$; 3.3k). Tubular ST area accounted for 33 to 58% of the variability in NPVs in the model when the testis was scanned through the scrotum prior to induction with anesthesia or through the *tunica vaginalis*, respectively ($P < 0.01$; Fig. 3.3e), while luminal ST area accounted for 29 to 53% of the variability in NPVs in the model when the testis was scanned through the *tunica vaginalis* or through the *tunica albuginea* when the spermatic cord was clamped, respectively ($P < 0.01$; Fig. 3.3j). The slope of the regression equation predicting tubular and luminal ST areas from NPVs was consistently positive across all stages of the castration.

PSD of the upper three spot meters was not indicative of tubular or luminal ST areas in the model ($P > 0.05$; Fig. 3.4), except when the testes were scanned through the *tunica albuginea* with the spermatic cord clamped, during which PSD was associated with the tubular area ($P < 0.05$; Fig. 3.4i), and when the testis was scanned through the scrotum prior to induction with anesthesia and through the *tunica albuginea*, during which an association between PSD and luminal ST area approached significance ($P=0.07$; Fig. 3.4b), or was significant ($P < 0.01$; Fig. 3.4h), respectively. The slope of the regression equation predicting tubular and luminal ST areas from PSD was positive when testes were scanned through the scrotum prior to and after induction with anesthesia (Fig. 3.4a-d); however, it became quite variable thereafter. In testes
Figure 3.3 (above, page 47) Scatter plots, regression lines and equations, and squared correlation coefficients ($r^2$) of the relationship between numerical pixel values (NPVs; input variable) and tubular (left) or luminal (right) seminiferous tubule (ST) areas (output variables) of testicles scanned at various time points during surgical castration of seven Rideau-Arcott x Polled Dorset ram lambs. Thin and thick lines represent the regression lines determined before or after removal of the data for “5L” (ram #5, left testicle; values identified by the outlier test), respectively, and regression equations and $r^2$ values are for the thick lines. 1-7: animal numbers. L, R denote left and right testicle.

Figure 3.4 (below, page 49) Scatter plots, regression lines and equations, and squared correlation coefficients ($r^2$) of the relationship between pixel heterogeneity values (PSD or standard deviation of numerical pixel values; input variable) and tubular (left) or luminal (right) seminiferous tubule (ST) areas (output variables) of testicles scanned at various time points during surgical castration of seven Rideau-Arcott x Polled Dorset ram lambs. Thin and thick lines represent the regression lines determined before or after removal of the data for “5L” (ram #5, left testicle; values identified by the outlier test), respectively, and regression equations and $r^2$ values are for the thick lines. 1-7: animal numbers. L, R denote left and right testicle.
Relative (%) tubular ST area

Relative (%) luminal ST area

Pixel heterogeneity (PSD)

Scrotum*

Tunica vaginalis

Tunica albuginea

Clamped

Drained

(a)

(b)

(c)

(d)

(e)

(f)

(g)

(h)

(i)

(j)

(k)

(l)
scanned through the *tunica vaginalis*, the regression equation predicting tubular ST area from PSD demonstrated a strong positive slope (Fig. 3.4e), whereas a weak negative slope was present in the regression equation predicting luminal ST area from PSD (Fig. 3.4f). There was a strong negative slope in the regression equation predicting tubular and luminal ST areas from PSD when the testes were scanned through the *tunica albuginea* (Fig. 3.4g and h) and a strong positive slope when the spermatic cord had been clamped (Fig. 3.4i and j). A low negative or positive slope was demonstrated in the regression equation predicting tubular and luminal ST area, respectively, from PSD when the testes were scanned through the *tunica albuginea* after the blood had been drained from testes (Fig. 3.4k and l).

### 3.5 Discussion

The results of the present study demonstrate how non-parenchymal cells and tissues in the testis affect testicular echotexture and its relationship with microscopic attributes of the seminiferous tubules (STs). Specifically, the effect of the scrotum and *tunica vaginalis* was evaluated by removing these two layers, the vascular blood flow was assessed by clamping the spermatic cord and allowing blood to pool in the testis, and the presence of blood was investigated by draining the testis after castration. Interestingly, numerical pixel values (NPVs) and pixel heterogeneity (*i.e.*, pixel standard deviation–PSD), the two echotextural parameters studied, did not always demonstrate a similar response to each step of the castration process. The increase in NPVs when the scrotum and *tunica vaginalis* were removed indicates that these two layers are a significant source of attenuation for testicular echogenicity. However, PSD increased only with the removal of the scrotum, indicating that tissue uniformity remained at a similar level prior to and after the removal of the *tunica vaginalis*. Both NPVs and PSD were unaffected by changes to blood flow and presence, indicating that these two factors do not influence testicular
echotexture. While tubular and luminal ST areas were associated with NPVs at nearly all stages of castration, there were only a limited number of associations with PSD; furthermore, the slope of the regression equation predicting tubular and luminal ST areas was consistently moderately positive when NPVs was the explanatory variable, but fluctuated greatly when PSD was the explanatory variable, indicating that changes in ST histomorphology are reliably detected in NPVs, but only occasionally detected in PSD.

Besides the non-parenchymal structures of the testis examined in the present study, several others exist, which may provide a more detailed explanation of the current findings. For instance, the *tunica albuginea* is capable of undergoing spontaneous or induced autonomic contractions (Davis *et al*., 1970; Mitchell and Seeley, 1977; Setchell and Breed, 2006) due to the smooth muscle content, which has been demonstrated in rams (Chacon-Arellano and Woolley, 1980), and may have undergone changes in surface appearance throughout the castration process, resulting in the sustained increase in PSD upon removal of the scrotum. Similarly, contractions of the *tunica albuginea* may have also elicited the drastic fluctuations in the relationship between PSD and tubular and luminal ST areas due to shifts in tissue uniformity. The *tunica vasculosa* may have also affected testicular echotexture due to the extensive vascularisation of this layer, as demonstrated in bulls (Ekstedt *et al*., 1986). The arteries in particular, with their large diameters and winding course (Polguy *et al*., 2011) may have easily attenuated the ultrasound beam and could explain some of the weaker relationships between NPVs and tubular and luminal ST areas. The rete testis is another non-parenchymal structure that may affect testicular echotexture. However, no supporting evidence for disruption of testicular echotextural attributes by the rete testis was obtained in the present study as there were no differences in NPVs and PSD between the upper and lower spots analyzed on the ultrasonograms.
The results of the present study confirm and enhance previous findings that testicular echogenicity is related to ST histomorphology (Evans et al., 1996; Gábor et al., 1998; Brito et al., 2012). The positive association that was observed between NPVs and tubular and luminal ST areas is consistent with the positive correlation between pixel intensity and outer (tubular) and inner (luminal) ST diameters reported by Evans et al. (1996) in bull calves from birth to puberty. However, it differs from two other studies in which pixel intensity was negatively correlated with ST diameter and/or area (Gábor et al., 1998; Brito et al., 2012) and seminiferous epithelium area (Brito et al., 2012) in postpubertal yearling bulls. This discrepancy may be accounted for by differences in the period of sexual development examined since the contents of the seminiferous tubules differ markedly throughout pubertal maturation; however, further investigation into the direction of the relationship between testicular echogenicity and tubular and luminal ST size is needed. Even though for the purpose of obtaining a standardized set of testicular echotextural values, the rams were age- and weight matched and the images were obtained in a consistent manner (e.g., in one plane of view), the consistency of the association between NPVs and tubular and luminal ST areas under various conditions throughout the castration process provides strong evidence that ST histomorphology is a major determinant of testicular echogenicity. Based on these results, it would be reasonable to expect that this relationship would persist even with varying placement of the transducer on the testicular surface, and with thickening of the scrotum or testicular capsule due to pathological conditions or normal testicular maturation and/or aging. Therefore, determining testicular echogenicity may have relevant clinical applicability. The relationship between PSD and ST histomorphology has not previously been studied and the present results are not conclusive, but suggest that other structures or events, such as the castration process, may have more greatly influenced PSD.
Scanning of the testes, while easy to access, lacking surrounding organs, symmetrical and easily comparable with one another (Gouletsou and Fthenakis, 2010), presents a unique challenge in the interpretation of testicular echotexture, due to the surrounding scrotal and testicular integumentary layers. An increase in testicular echogenicity upon removal of the scrotum and *tunica vaginalis* indicates that these structures are a significant source of attenuation. However, testicular echogenicity is closely related to ST histomorphology, regardless of the presence of these two layers. Indeed, the size of the STs, which occupied 67% to 83% of the testicular parenchyma by area (Figs. 3.3 and 3.4) appears to be a major determinant of testicular echotexture. The contracting ability of the *tunica albuginea* and the extensive vascularisation of the *tunica vasculosa* may impinge on the testicular echotextural attributes and their ability to predict tubular and luminal ST areas, although future studies are needed to provide conclusive evidence. Echotextural analysis of scrotal ultrasonograms is a promising technique for the reliable, non-invasive assessment of testicular microstructure.

### 3.6 Acknowledgements

We would like to thank Ms. Sabrina Sangupta, Ms. Stephanie Wilson, Mr. Sean Karnani and Mr. Karan Dhir for their efforts and contributions in data collection, analysis and interpretation, and the Ponsonby Station staff (Ms. Pam Hasson and Mr. Jeff McFarlane) for the care and management of experimental animals. This study was funded by the Ontario Ministry of Agriculture, Food and Rural Affairs (PMB and ACH), and the Natural Sciences and Engineering Research Council of Canada (ACH).
Chapter 4

CORRELATIONS AMONG ULTRASONOGRAPHIC AND MICROSCOPIC CHARACTERISTICS OF PREPUBESCENT RAM LAMB TESTES

Jennifer L Giffin, Pawel M Bartlewski and Ann C Hahnel

Department of Biomedical Sciences
Ontario Veterinary College
University of Guelph
Guelph, ON, Canada N1G 2W1

N. B. A version of the contents presented in this chapter has previously been published (Giffin et al., 2014; doi: 10.1177/1535370214543063).
4.1 Abstract

The onset of spermatogenesis during prepubertal development is accompanied by dynamic changes in testicular microstructure. Computer-assisted analysis of scrotal ultrasonograms may allow us to track these changes in a non-invasive manner; however, the echotextural characteristics of different histomorphological variables remain unclear. Hence the objective of this study was to compare echotextural and microscopic attributes of the testis over the first wave of spermatogenesis in prepubescent ram lambs. Bi-weekly ultrasound examinations and weekly testicular biopsies were carried out in 22 ram lambs from 9.5–10 weeks of age or the attainment of 15 cm³ in testicular volume, respectively, to the first detection of elongated spermatids (ESt). Testicular echogenicity was highly variable with age; however, after the alignment of data to the first detection of ESt, there was an initial increase followed by a decline, corresponding to the mitotic and post-mitotic phases of spermatogenesis in prepubescent ram lambs. Testicular echotextural attributes (mean numerical pixel values and pixel heterogeneity) correlated with tubular seminiferous tubule (ST) diameter, the number of degenerating cells/ST cross-section (XS) and the number of ubiquitin C-terminal hydrolase L-1 (a marker for pre-spermatogonia and undifferentiated spermatogonia) staining cells/ST XS during the mitotic and post-mitotic phases. Additionally, in the post-mitotic phase, significant correlations were recorded among the quantitative echotextural characteristics and ST cell density, nuclear : ST area and percentages of ST XS with different spermatogenic cells as the most mature germ cell type present. These results indicate that ram testes exhibit distinctive echotextural characteristics during the mitotic and post-mitotic phases of germ cell differentiation. It is concluded that scrotal ultrasonography in conjunction with computerized
image analysis holds potential as a non-invasive alternative to testicular biopsy in monitoring the reproductive status throughout different stages of testicular development.

4.2 Introduction

During prepubertal testicular development, there is a remarkable increase in cellular number and diversity within the seminiferous tubules (STs) owing mainly to the onset of spermatogenesis. Spermatogenic onset is initiated when pre-spermatogonia are converted into spermatogonial stem cells (SSCs), which divide by mitosis into new SSCs and spermatogonia committed to the differentiation pathway (Kolasa et al., 2012). Following several further rounds of mitosis, spermatocytes are formed and undergo meiosis, involving the recombination and reduction of genetic material to form haploid spermatids. Initially round in shape, the spermatids undergo extensive cytoplasmic and nuclear remodeling, becoming elongated spermatids. Maturation of the Sertoli cells is an essential part of spermatogenic onset as it allows for the physical and metabolic support of the germ cells, as well as the formation of a fluid-filled central ST lumen (Sharpe et al., 2003), into which the elongated spermatids are released for passage into the rete testis. Additionally, as the Sertoli cells mature and reach the end of their mitotic lifespan (Sharpe et al., 2003), their supportive capacity for germ cells becomes established (Orth et al., 1988), and a wave of germ cell apoptosis occurs to achieve the optimal germ to Sertoli cell ratio (Aitken et al., 2011). How these events proceed throughout prepubertal development determines the future reproductive capability of the adult male. Therefore, it is important to closely monitor this phase of sexual development in order to be able to predict future fertility and to diagnose any reproductive complications. Testicular biopsies allow for a detailed microscopic evaluation of the testes; however, due to the expense and risk of tissue damage they are not suitable for routine
clinical assessments and simply not practical to carry out in farmed livestock operations. A less invasive and more cost-effective alternative to testicular biopsies in prepubertal individuals would therefore be beneficial.

Scrotal ultrasonography is a commonly used imaging modality for examining internal, macroscopic features of the testis, epididymis and proximal genital tract. Recently however, a novel method of analyzing ultrasonograms has made the detection of microscopic changes in testicular and epididymal histomorphology a realistic possibility (Evans et al., 1996; Chandolia et al., 1997a; Chandolia et al., 1997b; Aravindakshan et al., 2000; Arteaga et al., 2005; Giffin et al., 2009; Kauffold et al., 2011; Brito et al., 2012; Kastelic and Brito, 2012). In this procedure, brightness and uniformity of gray-scale values on the ultrasonogram are quantified using computer-assisted image analysis of minute picture elements, or pixels, comprising the image. Pixel intensity is described in terms of numerical pixel values (NPVs), which range from 0 (“absolute black”) to 255 (“absolute white”) and provide an indication of tissue echogenicity, or the ability of the tissue to reflect or scatter ultrasound waves (Ginther, 1995). Pixel standard deviation (PSD) or heterogeneity of the NPVs reflects the existence of interspersed hyper- and hypoechoic areas in a tissue. Together, mean NPVs and PSD typically serve as measures of central tendency and variation, respectively, in the ultrasonographic appearance of organs and tissues (Pierson and Adams, 1995; Singh et al., 2003; Kastelic and Brito, 2012). Quantitative echotextural analysis in bull calves and ram lambs has revealed an overall increase in testicular echogenicity with age throughout sexual maturation (Evans et al., 1996; Chandolia et al., 1997a; Chandolia et al., 1997b; Aravindakshan et al., 2000; Brito et al., 2012); however, there is considerable variability, especially with more frequently performed ultrasonographic evaluations (Evans et al., 1996).
In early postnatal and prepubertal bull calves and peripubertal ram lambs, several associations have been found among ultrasonographic and microscopic attributes of the testes including outer (tubular) and inner (luminal) diameters of the STs (Evans et al., 1996), tubular and luminal ST areas (Giffin et al., 2009), and the percentages of ST cross-sections with different germ cells as the most mature cell type present (Evans et al., 1996). However, some of these correlations did not remain consistent in postpubertal yearling bulls (Brito et al., 2012; Gábor et al., 1998). As the majority of postnatal microscopic changes in the testes occur during prepubertal development and there are no shifts in testicular echogenicity beyond this period (Brito et al., 2012), this strongly suggests that echotextural variables are related to an array of histomorphological attributes of the developing testes.

While previous studies used castrations of age-matched groups of animals to evaluate how tissue microstructure is related to testicular echotexture, a direct comparison between two or more developmental phases necessitates multiple evaluations of the testicular tissue from the same individuals over time. Therefore, the primary objective of this study in prepubertal ram lambs was to employ scrotal ultrasonography in conjunction with computer-assisted image analysis and testicular biopsies to determine correlations among echotextural and histomorphological attributes of the testis at distinct developmental stages throughout the first wave of spermatogenesis.

4.3 Materials and Methods

4.3.1 Animals and Experimental Design

Twenty-two spring-born Rideau Arcott x Polled Dorset ram lambs were housed in a field research station in Ponsonby, ON, Canada (latitude: 43° 33’N) and kept under ambient light and
temperature conditions in sheltered outdoor facilities. From 7 to 100 days of age, they were fed a 16% crude protein lamb grower diet (Shur-Gain Feedmills, St. Marys, ON, Canada) ad libitum. Hay was offered after weaning at 50 days of age, and it was estimated that from this point until 100 days of age, daily consumption of the grower averaged 2.5 pounds per lamb. After 100 days of age, rams were fed a diet of 80% whole barley and 20% whole corn with a 36% crude protein sheep supplement (Shur-Gain Feedmills, as above) and hay, and daily consumption averaged 1 pound of grain and 0.5 pounds of sheep supplement per ram. Water was always accessible to the rams. All experimental procedures performed on live animals were approved by the Animal Care Committee at the University of Guelph according to the Canadian Council on Animal Care guidelines.

Bi-weekly ultrasound evaluations and weekly testicular biopsies commenced at 9.5–10 weeks of age or at the time of attainment of 15 cm³ in testicular volume, respectively, and continued until elongated spermatids (ESt) were first detected by histological evaluation of testicular biopsies, with an additional 1-2 weeks of scanning to allow for tissue processing and histological detection of ESt. To detect a significant correlation between testicular pixel intensity and outer seminiferous tubule diameter, as was found in Evans et al. (1996) in bull calves from birth to puberty, it was determined that sample size needed to be at least 14 using the formula below:

\[
n = 3 + \frac{31.4}{\left[\ln \left(\frac{1 + r}{1 - r} \times \frac{1 - r_0}{1 + r_0}\right)\right]^2}
\]

with Type I error set to \(\alpha=0.05\) and Type II error set to \(\beta=0.20\) (Dell et al., 2002), where values for \(r\) and \(r_0\) represent the specified correlation of 0.69 found by Evans et al. (1996) and a zero correlation coefficient it is different from, respectively. Therefore, the sample size of 22 used in
the present study is sufficient for detecting correlations among testicular echogenicity and seminiferous morphology, and possibly other echotextural and histomorphological attributes.

4.3.2 Ultrasonographic Examinations

Scrotal ultrasonography utilized an Aloka SSD-900 portable ultrasound machine equipped with a 7.5-MHz linear-array transducer (Aloka Inc., Tokyo, Japan). Settings for main, near and far gains, and focal points were optimized and kept constant throughout the study. Lubricating gel (Light Lube; Centaur VA Animal Health, Guelph, ON, Canada) was applied as a coupling material to eliminate air between the scrotum and the transducer. Left and right testes were scanned in longitudinal and transverse planes, and real-time images were recorded on a Digital Versatile Disk (DVD) recorder (Pioneer® DVD Recorder DVR-510H; Pioneer Electronics of Canada Inc., Markham, ON, Canada) for later analysis. Testicular length and width were measured using the built-in electronic calipers or a caliper instrument if the entire area could not be visualized on the screen, and used to calculate testicular volume (TV) with the formula \( TV = \frac{1}{6\pi} \times \text{length} \times \text{width}^2 \times 0.945 \) developed by Wrobel (1990) for ruminant species.

Still images from the DVD recordings containing the largest cross-sectional surface area were captured using a computer station (Intel®-Xeon® Processor MP; 2.8 GHz; Intel Co., Santa Clara, CA, USA). Computer-assisted image analysis of the ultrasonograms was performed using the “spot meter” technique previously described (Pierson and Adams, 1995; Singh et al., 2003; Giffin et al., 2009) with Image ProPlus® 7.0 analytical software (Media Cybernetics Inc., San Diego, CA, USA). A total of six spots in longitudinal view and four spots in transverse view, each measuring 60 pixels (~6 mm in diameter), were placed randomly on the “surface” of the testicular parenchyma with an equal number of spots above and below the rete testis, excepting
any image artifacts or scarring from biopsies. Mean numerical pixel values (NPVs) and standard deviation (pixel heterogeneity–PSD) were determined for each animal based on the images of both left and right testes (20 spots in total), for each day of examination.

4.3.3 Histology and Immunohistochemistry

Core needle biopsies (12, 14 or 16 gauge E-Z Core Single Action Biopsy Device; Products Group International, Lyons, CO, USA) were taken from the left testis only. Prior to tissue collection, ram lambs were weighed and then sedated with xylazine (Rompun®, Bayer, Toronto, ON, Canada; 0.2 mg/kg i.m.). Tissue was fixed overnight in modified Davidson’s solution (Latendresse et al., 2002), washed in 70% ethanol and embedded in paraffin wax blocks. Tissue was sectioned at a thickness of 5 µm, and then deparaffinized in xylene and rehydrated in a graded isopropanol-water series for staining with hematoxylin and eosin, using standard procedures, or for ubiquitin C-terminal hydrolase L-1 (UCHL-1; also referred to as protein gene product 9.5, PGP 9.5), a marker for pre-spermatogonia and undifferentiated spermatogonia in sheep (Rodriguez-Sosa et al., 2006), using an immunohistochemical protocol.

For UCHL-1 immunohistochemistry, heat-induced antigen retrieval was performed by microwaving slides in 10-mM citrate buffer (pH 6.0) on high power for 8 min. The sections were then treated with 3% hydrogen peroxide in distilled water for 15 min to block endogenous peroxidase activity, rinsed and incubated with 5% normal goat serum (GS; Vector Laboratories, Burlington, ON, Canada) in phosphate buffered saline (PBS; PBS-GS) for 30 min at room temperature to block non-specific binding. Polyclonal rabbit anti-PGP 9.5 (Dako, Carpinteria, CA, USA) primary antibody (2.5 µg/ml) in PBS-GS or PBS-GS alone was applied to test and negative control sections, respectively, and allowed to incubate overnight in a moist chamber at
4°C. The following day, sections were rinsed 3 times in PBS for 5 min each and incubated with 2.5 μg/ml biotinylated goat anti-rabbit immunoglobulin G (IgG; Invitrogen, Burlington, ON, Canada) in PBS-GS for 45 min at room temperature. After rinsing in PBS as above, the sections were exposed to avidin horseradish peroxidase R.T.U. Vectastain Elite ABC Reagent and NovaRed substrate according to the manufacturer’s instructions (above from Vector Laboratories, as above). Subsequently, the sections were counterstained in Mayer’s hematoxylin : water (1 : 1; Fisher Scientific, Ottawa, ON, Canada) for 30 s and mounted with Cytoseal XYL (Richard-Allen Scientific, Kalamazoo, MI, USA).

Histomorphological analysis was performed using Image ProPlus® on biopsy micrographs covering the entire tissue area taken by Q Capture® (Quorum Technologies Inc., Guelph, ON, Canada) at 200x image magnification. The following parameters were determined for all round seminiferous tubule (ST) cross-sections (XS): i) the tubular and luminal ST diameter, computed from manual outlines of ST XS; ii) the percentages of ST XS with pre-spermatogonia (pre-Sg) or spermatogonia (Sg), spermatocytes (Sc), round or elongated spermatids (RSt and ESt, respectively) as the most mature germ cell type present (Fig. 4.1 a-d), assessed manually; and iii) the number of UCHL-1+ cells (classified as light, medium or dark, based on staining intensity; Fig. 4.1 e-f) per ST XS, assessed manually. Additionally, in 10 round ST XS/biopsy we determined: iv) ST cell density, or the number of cells based on automated nuclear counts using a manually selected colour intensity range per ST μm², computed from manually outlined ST XS; v) the ratio of average cell nuclear area to tubular ST area (nuclear : ST area), calculated from nuclear area determined from the automated nuclear counts divided by ST area; and vi) the number of degenerating cells displaying classical histomorphological
Figure 4.1 Prepubertal ram lamb testicular micrographs representing four different stages of germ cell development and the general appearance of ubiquitin C-terminal hydrolase L-1 (UCHL-1) immunohistochemical staining. Pre-spermatogonia and/or spermatogonia ((Pre-)Sg; a), spermatocytes (Sc; b), round spermatids (RSt; c) and elongated spermatids (ESt; d) are observed as the most advanced germ cell type present. UCHL-1 test sections treated with primary antibody revealed dark (D), medium (M) and light (L) staining in (pre)-Sg (e) compared to negative control sections not treated with primary antibody (f). Images taken at 200x image magnification.
attributes of apoptosis (\textit{i.e.}, chromatin condensation and fragmentation, cytoplasmic vacuolization and eosinophilia and cell shrinkage; Gobé and Harmon, 2008), assessed manually.

4.3.4 Statistical Analyses

All statistical procedures were carried out using SigmaPlot\textsuperscript{®} (version 11.0; Systat Software, Inc., Richmond, CA, USA). Serial echotextural and histomorphological data were assessed using one-way repeated measures analysis of variance (ANOVA; general linear model) after alignment to both chronological age and the age of first ESt detection. The first week of ESt occurrence could not be ascertained in two ram lambs that had missed biopsies due to temporary illnesses unrelated to the experimental procedures; therefore, the data from these animals were removed from analyses with respect to the age at first ESt detection. All other statistical analyses used the data obtained from twenty-two ram lambs. Differences between individual means were determined using the Holm-Sidak method. A paired \( t \)-test was used to evaluate differences in testicular volume between left and right testes. Correlations among echotextural and histomorphological parameters were assessed during the mitotic and post-mitotic phases, with (pre-)spermatogonia as the most mature germ cell type in \( > \) or \( < 50\% \) of ST XS, respectively, as well as for the entire study period using the Pearson Product Moment. In all analyses, \( P < 0.05 \) was considered statistically significant and \( P \leq 0.10 \) was considered approaching statistical significance. All results were presented as mean \( \pm \) SEM.

4.4 Results

4.4.1 General Results
Testicular volume reached 15 cm³ at an average age of 10.7 ± 0.2 weeks (range: 9.5 to 13 weeks). (Pre-)spermatogonia had the highest prevalence in seminiferous tubule (ST) cross-sections (XS) as the most mature germ cell type from 10 to 13 weeks of age or from −6 to −3 weeks relative to the first detection of elongated spermatids (ESt); therefore this period was denoted the mitotic phase and the subsequent period the post-mitotic phase. The first detection of ESt occurred on average at 15.3 ± 0.4 weeks of age (range: 12 to 18 weeks), with a testicular volume averaging 80.1 ± 6.5 cm³ (range: 42.1 to 122.4 cm³) and a body weight of 42.4 ± 1.2 kg (range: 37.5 to 51.5 kg).

4.4.2 Body Weight and Testicular Growth

Body weight increased linearly from 11 to 16 weeks of age (P < 0.05) and then stayed relatively constant until 19 weeks of age (P > 0.05; Fig. 4.2a). Average testicular volume increased steadily from 10 to 15 weeks of age (P < 0.05) and then reached a plateau (Fig. 4.2b). There were no differences in testicular volume between the left and right testes except at 16 and 17 weeks of age, when the right testis was significantly larger compared to the left testis (P < 0.05).

Body weight increased in a curvilinear manner in the 6 weeks before ESt detection (P < 0.05) and then stayed relatively constant until 2 weeks after ESt were first detected (P > 0.05; Fig. 4.2c). Average testicular volume increased gradually from 8 until 5 weeks before the first detection of ESt (P < 0.05) and then rapidly increased until ESt were first detected (P < 0.05) before reaching a plateau in the following 2 weeks (P > 0.05; Fig. 4.2d). No differences in testicular volume were found between the left and right testes after data alignment to the time of first detection of ESt (P > 0.05).
Figure 4.2 Mean (± SE) body weight (a, c) and testicular volume (b, d) in prepubescent ram lambs with respect to weeks of age (a, b) and weeks relative to the first detection of elongated spermatids (ESt; c, d). Different letters indicate significant differences between weeks ($P < 0.05$). Asterisks indicate that testicular volume was significantly different ($P < 0.05$) between left and right testes.

4.4.3 Testicular Echotexture

Temporal changes in mean numerical pixel values (NPVs) and pixel heterogeneity (PSD) with respect to age and the first detection of ESt are demonstrated in Figures 4.3. NPVs
Figure 4.3 Mean (± SE) numerical pixel values (NPVs) and pixel standard deviation (PSD) from testicular ultrasonograms taken at twice weekly intervals in prepubescent ram lambs, aligned to chronological age of animals (a) or weeks relative to the first detection of elongated spermatids (ESt; b). Different letters indicate significant differences between mean values ($P < 0.05$).
increased significantly from a minimum value at 10.5 weeks of age to a maximum at 13 weeks of age, decreased to a nadir by 15.5 weeks of age ($P < 0.05$), and then continued to fluctuate ($P > 0.05$) over the next 4 weeks. The greatest increase in PSD occurred from 11 to 14 weeks of age ($P < 0.05$), after which it remained relatively constant. In contrast, NPVs rose steadily from 8 to 3 weeks before the first detection of ESt and then decreased gradually until 2 weeks after ESt were first detected ($P < 0.05$). PSD displayed a similar trend, with a significant increase from 7 to 3.5 weeks before the first detection of ESt ($P < 0.05$) followed by a numerical decrease until 2 weeks after ESt were first detected ($P > 0.05$).

### 4.4.4 Testicular Histomorphology

Tubular and luminal ST diameters rose relatively linearly from 10 to 19 weeks of age ($P < 0.05$; Fig. 4.4a) as did tubular ST diameter from 6 weeks before to 2 weeks after the first detection of ESt ($P < 0.05$); however, luminal ST diameter increased only from 6 weeks to 1 week before the first detection of ESt ($P < 0.05$) before reaching a plateau until 2 weeks after ESt were first detected ($P < 0.05$; Fig. 5.4a). ST cell density increased exponentially from 10 to 18 weeks of age ($P < 0.05$; Fig. 4.4b) or from 6 weeks before to 2 weeks after the first detection of ESt ($P < 0.05$; Fig. 4.5b). The nuclear : ST area decreased rapidly from 10 to 15 weeks of age ($P < 0.05$) and then stayed relatively constant until 19 weeks of age ($P > 0.05$; Fig. 4.4c), while from 6 weeks before to 2 weeks after the first detection of elongated spermatids, it decreased gradually ($P < 0.05$; 4.5c). The number of degenerating cells per ST XS increased numerically from 10 to 14 weeks of age ($P > 0.05$) or significantly from 5 to 2 weeks prior to the first ESt detection ($P < 0.05$) before numerically declining until 19 weeks of age or 2 weeks after the first detection of ESt ($P > 0.05$; Figs. 4.4d and 4.5d). Following a decrease in the total number of
ubiquitin C-terminal hydrolase L-1 (UCHL-1) positive cells/ST XS from 10 to 11 weeks of age ($P < 0.05$), the total number of UCHL-1$^+$ cells peaked numerically at 14 weeks of age ($P > 0.05$; Fig. 4.4e), or at 3 to 2 weeks before ESt were first detected ($P > 0.05$; Fig. 4.5e), before reaching a nadir. There were no significant changes in the numbers of different UCHL-1$^+$ cell subpopulations with age; however, there was a notable decline in the number of light-staining cells from 10-11 weeks of age, and a peak in the number of medium-staining cells at 14 weeks of age ($P > 0.05$; Fig. 4.4f). The number of medium-staining UCHL-1$^+$ cells/ST XS increased from 5 to 2 weeks before ESt were first detected ($P < 0.05$) and numerically decreased thereafter ($P > 0.05$; Fig. 4.5f).

The percentage of ST XS with (pre-)spermatogonia as the most advanced germ cells decreased from 10 to 15 weeks of age ($P < 0.05$), or from 6 weeks to 1 week before ESt were first detected ($P < 0.05$), and stayed relatively constant thereafter ($P > 0.05$; Fig. 4.6a and e). The percentage of ST XS with spermatocytes as the most mature germ cell type was highest between 12 and 17 weeks of age; however, there were no significant differences with increasing age ($P > 0.05$; Fig. 4.6b). Conversely, there was a steady increase in the percentage of ST XS with spermatocytes as the most mature germ cell type from 6 weeks to the week before the first detection of ESt followed by a rapid decrease until 2 weeks after ESt were first detected ($P < 0.05$; Fig. 4.6f). The percentages of ST XS containing round or elongated spermatids as the most mature germ cell type increased gradually starting at 15 weeks of age and then reached a peak at 18 or 19 weeks of age, respectively ($P < 0.05$; Fig. 4.6c and d). In the weeks relative to first ESt detection, there was a sudden increase in the percentages of ST XS with round and elongated spermatids as the most mature germ cell type at the time the first ESt were detected followed by
Figure 4.4 Mean (± SE) tubular and luminal seminiferous tubule (ST) diameter (a), ST cell density (b), nuclear : ST area (c), the number of degenerating cells/ST cross-section (XS; d), the total number of ubiquitin C-terminal hydrolase L-1 (UCHL-1) staining cells/ST XS (e) and the number of dark-, medium- and light-staining UCHL-1 cells/ST XS (f) in ram lamb testes from 10 to 19 weeks of age. Different letters indicate significant differences between mean values (P < 0.05).
Figure 4.5 Mean (± SE) tubular and luminal seminiferous tubule (ST) diameter (a), ST cell density (b), nuclear : ST area (c), the number of degenerating cells/ST cross-section (XS; d), the total number of ubiquitin C-terminal hydrolase L-1 (UCHL-1)+ cells/ST XS (e) and the number of dark-, medium- and light-staining UCHL-1+ cells/ST XS (f) in ram lamb testes from −6 to 2 weeks relative to the first detection of elongated spermatids (ESt). Different letters indicate significant differences between mean values ($P < 0.05$).
Figure 4.6 Mean (± SE) percentages of seminiferous tubule (ST) cross-sections (XS) with pre-spermatogonia and/or spermatogonia ((Pre-)Sg; a, e), spermatocytes (Sc; b, f), round spermatids (RSt; c, g) or elongated spermatids (ES; d, h) as the most mature germ cell type present in ram lamb testes from 10 to 19 weeks of age (a-d) or from −6 to 2 weeks relative to the first detection of ES (e-h). Different letters indicate significant differences between mean values ($P < 0.05$).

*As the most mature germ cell type present.
a marked decrease or increase, respectively, for the following 2 weeks \((P < 0.05; \text{Fig. 4.6g and h})\).

### 4.4.5 Correlations

Correlations among testicular echotextural and histomorphological attributes are summarized in Table 4.1. In the mitotic phase, NPVs and PSD were positively correlated with tubular \((r=0.35, P=0.03\) and \(r=0.40, P=0.01\), respectively) and luminal \((r=0.48, P=0.004\) and \(r=0.56, P=0.0004\), respectively) diameters of the STs, the number of degenerating cells/ST XS \((r=0.32, P=0.06\) and \(r=0.37, P=0.02\), respectively), the number of dark- \((r=0.30, P=0.08\) and \(r=0.30, P=0.07\), respectively) and light- \((r=0.35, P=0.04)\) staining UCHL-1+ cells/ST XS and the total number of UCHL-1+ cells/ST XS \((r=0.45, P=0.005\) and \(r=0.52, P=0.0008\), respectively). Additionally, the number of medium-staining UCHL-1+ cells/ST XS was also correlated with PSD \((r=0.32, P=0.06)\).

In the post-mitotic phase, NPVs and PSD were negatively correlated with tubular diameter \((r=–0.51, P=0.000007\) and \(r=–0.36, P=0.0007\), respectively), ST cell density \((r=–0.20, P=0.08\) and \(r=–0.20, P=0.09\), respectively) and percentages of ST XS with round \((r=–0.23, P=0.04\) and \(r=–0.20, P=0.07\), respectively) and elongated \((r=–0.36, P<0.0007\) and \(r=–0.28, P=0.01\), respectively) spermatids as the most mature germ cell type. NPVs were positively correlated with the nuclear : ST area \((r=0.46, P=0.00002)\), the number of degenerating cells/ST XS \((r=0.20, P=0.07)\), the numbers of dark- \((r=0.24, P=0.03)\) and medium- \((r=0.36, P=0.001)\) staining UCHL-1+ cells/ST XS, the total number of UCHL-1+ cells/ST XS \((r=0.20, P=0.07)\) and the percentages of ST XS with (pre-)spermatogonia \((r=0.25, P=0.02)\) and spermatocytes \((r=0.34, P=0.001)\) as the most mature germ cell type. PSD was positively correlated with the nuclear : ST area.
Table 4.1 A summary of Pearson product moment correlations among echotextural and histomorphological attributes in the testes of twenty-two 10 to 19 week old Rideau-Arcott x Polled Dorset ram lambs at different phases of spermatogenic onset.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Mitotic Phase</th>
<th>Post-Mitotic Phase</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NPVs</td>
<td>PSD</td>
<td>NPVs</td>
</tr>
<tr>
<td>Tubular ST diameter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$r=0.35$</td>
<td>$P=0.03$</td>
<td>$r=-0.51$</td>
</tr>
<tr>
<td></td>
<td>$r=0.40$</td>
<td>$P=0.01$</td>
<td>$r=-0.36$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminal ST diameter</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$r=0.48$</td>
<td>$P=0.004$</td>
<td>$r=-0.20$</td>
</tr>
<tr>
<td></td>
<td>$r=0.56$</td>
<td></td>
<td>$r=0.09$</td>
</tr>
<tr>
<td>ST cell density</td>
<td>NS</td>
<td>NS</td>
<td>$r=0.46$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$r=0.24$</td>
</tr>
<tr>
<td>Nuclear : ST area</td>
<td>NS</td>
<td>NS</td>
<td>$r=0.46$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$r=0.24$</td>
</tr>
<tr>
<td># Degenerating cells/ST XS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$r=0.32$</td>
<td>$P=0.06$</td>
<td>$r=0.20$</td>
</tr>
<tr>
<td></td>
<td>$r=0.37$</td>
<td>$P=0.02$</td>
<td>$r=0.29$</td>
</tr>
<tr>
<td># Dark UCHL-1+ cells/ST XS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$r=0.30$</td>
<td>$P=0.08$</td>
<td>$r=0.24$</td>
</tr>
<tr>
<td></td>
<td>$r=0.30$</td>
<td>$P=0.07$</td>
<td>$r=0.36$</td>
</tr>
<tr>
<td># Medium UCHL-1+ cells/ST XS</td>
<td>NS</td>
<td>$r=0.32$</td>
<td>$r=0.36$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$P=0.06$</td>
<td>$r=0.36$</td>
</tr>
<tr>
<td># Light UCHL-1+ cells/ST XS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$r=0.35$</td>
<td>$P=0.04$</td>
<td>$r=0.20$</td>
</tr>
<tr>
<td></td>
<td>$r=0.35$</td>
<td>$P=0.04$</td>
<td>$r=0.20$</td>
</tr>
<tr>
<td>Total # UCHL-1+ cells/ST XS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>$r=0.45$</td>
<td>$P=0.005$</td>
<td>$r=0.28$</td>
</tr>
<tr>
<td></td>
<td>$r=0.52$</td>
<td>$P=0.0008$</td>
<td>$r=0.28$</td>
</tr>
<tr>
<td>% ST XS with (Pre-)Sg*</td>
<td>NS</td>
<td>NS</td>
<td>$r=0.25$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$r=0.25$</td>
</tr>
<tr>
<td>% ST XS with Sc*</td>
<td>NS</td>
<td>NS</td>
<td>$r=0.34$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$r=0.31$</td>
</tr>
<tr>
<td>% ST XS with RSt*</td>
<td>NA</td>
<td>NA</td>
<td>$r=0.23$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$r=0.23$</td>
</tr>
<tr>
<td>% ST XS with ESt*</td>
<td>NA</td>
<td>NA</td>
<td>$r=0.36$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$r=0.28$</td>
</tr>
</tbody>
</table>

NPVs, numerical pixel values (pixel intensity); PSD, pixel standard deviation (pixel heterogeneity); ST, seminiferous tubule; XS, cross-section; UCHL-1+, ubiquitin C-terminal hydrolase L-1 positive; (Pre-)Sg, (pre-)spermatogonia; Sc, spermatocytes; RSt, round spermatids; ESt, elongated spermatids; * as the most mature germ cell type present; NS, non-significant; NA, non-applicable.
area ($r=0.24, P=0.03$), number of degenerating cells/ST XS ($r=0.29, P=0.008$), the number of medium-staining UCHL-1$^+$ cells/ST XS ($r=0.36, P=0.001$) and the percentage of ST XS with spermatocytes as the most advanced germ cell type ($r=0.31, P=0.004$).

During the entire period of study, NPVs and PSD were positively correlated with luminal ST diameter ($r=0.19, P=0.04$ and $r=0.15, P=0.10$, respectively), the number of degenerating cells/ST XS ($r=0.25, P=0.005$ and $r=0.31, P=0.0006$, respectively), the number of dark- ($r=0.25, P=0.007$ and $r=0.18, P=0.06$, respectively), medium- ($r=0.28, P=0.003$ and $r=0.34, P=0.0002$, respectively) and light- ($r=0.23, P=0.02$ and $r=0.21, P=0.02$, respectively) staining UCHL-1$^+$ cells/ST XS, the total number of UCHL-1$^+$ cells/ST XS ($r=0.28, P=0.002$ and $r=0.26, P=0.003$) and the percentage of ST XS with spermatocytes ($r=0.29, P=0.001$ and $r=0.28, P=0.002$, respectively) as the most mature germ cell type and negatively correlated with the percentage of ST XS with elongated spermatids ($r=-0.19, P=0.04$ and $r=-0.17, P=0.06$, respectively) as the most mature germ cell type.

4.5 Discussion

Prepubertal testicular development is accompanied by complex changes in testicular echotexture. In agreement with previous studies (Evans et al., 1996; Chandolia et al., 1997a; Chandolia et al., 1997b; Aravindakshan et al., 2000; Brito et al., 2012), testicular echogenicity in the present ram lambs was highly variable with age. However, when the echotextural parameters were aligned to the first detection of elongated spermatids, they exhibited a much more distinct pattern of changes, suggesting that ultrasonographic attributes are more directly associated with spermatogenic development than with chronological age of animals. Similarly, Brito et al. (2012) showed that testicular echogenicity in bull calves was much less variable when aligned to
the attainment of puberty as defined by semen parameters compared with an alignment to the animals’ age. In the present study, when testicular echotextural parameters were aligned with respect to weeks relative to the first detection of elongated spermatids, there was an initial increase followed by a decrease in mean numerical pixel values (NPVs) of the testicular parenchyma, which corresponded to two distinct spermatogenic periods, namely the mitotic and post-mitotic phases. Clearly, testicular echotexture is affected by microstructural changes associated with spermatogenic development during the first wave of spermatogenesis in ram lambs.

Histomorphological attributes were evaluated from unilateral testicular biopsies taken on a weekly basis. Studies of repetitive testicular biopsies performed in sexually mature llamas and prepubertal sheep have demonstrated that it is a safe procedure with maintenance of normal testicular size and function when appropriate care is taken to avoid hematoma, lesions and infections (Lunstra and Echternkamp, 1988; Heath et al., 2002). Indeed, the average testicular volume and body weight of ram lambs in the present study displayed normal growth trajectories for pubertal development. A period of rapid testicular growth was demonstrated in the final five weeks prior to elongated spermatid detection, at approximately 10–15 weeks of age, which is similar to the findings reported in Blackbelly sheep by Herrera-Alarcón et al. (2007). Body weight displayed a similar increase throughout pubertal development, in accordance with its positive correlation with testicular size (Salhab et al., 2001). Furthermore, serial histological evaluations revealed no pathological consequences of multiple biopsies taken from the left testis in this study.

In rams, the relative seminiferous tubule (ST) volume increases from about 50 to 84% of the testicular parenchyma from the early prepubertal to the adult developmental periods (Steger
and Wrobel, 1996). There are two major contributing factors to ST volumetric expansion during prepubertal development: i) the growing cell population of the seminiferous epithelium; and ii) lumen formation and enlargement. Initially, there is a lengthening of the STs due to Sertoli cell proliferation, followed by an increase in ST diameter as the germ cell number rises with spermatogenic onset (Irfan et al., 2011). Therefore, both the tubular and luminal ST diameter and ST cell density increased as expected throughout the present study.

The relationship between testicular NPVs and ST size in ruminants has previously been evaluated; however, the findings have been inconsistent. A positive correlation with ST diameter was reported in bull calves from birth to puberty (Evans et al., 1996), while a negative correlation with ST area was reported in postpubertal yearling bulls (Gábor et al., 1998; Brito et al., 2012). Notably, no other ST histomorphological attributes that were evaluated demonstrated a similar switch in the direction of the correlation with testicular echotexture; therefore, future studies will need to identify which ST components could be contributing to this. Recently, the protein content of testicular parenchyma was shown to be negatively correlated with echotextural parameters in rams (Ahmadi et al., 2013). The proteome profile of a mouse testis during spermatogenic onset reveals a high level of protein expression during spermatogonial proliferation as well as during round and elongated spermatid formation, however there appears to be a reduction in the total amount of protein synthesized during the initiation of meiosis in spermatocytes (Huang et al., 2008). Therefore, shifts in testicular protein content may have contributed to the switch in the direction of the correlations between ST diameter and testicular parenchymal echogenicity in the present study. At present, it is uncertain how the level of protein expression in the testes may affect the resulting testicular echogenicity. Not unlikely, specific organelles involved in protein synthesis may have distinct echotextural properties that impinge
on the overall tissue echogenicity. Alternatively, protein molecules may interact with the ultrasound waves in a specific way. Tubular and luminal diameter of the STs had some of the strongest correlations with testicular echotexture; therefore, it is important to thoroughly investigate how cellular and biochemical changes in both the tubular and luminal compartments affect the resulting ultrasound image.

Throughout spermatogenesis, germ cell nuclei undergo dynamic changes in size and chromatin arrangement. In the present study, nuclear : ST area demonstrated a relatively constant decrease throughout spermatogenic onset. While there is an overall decrease in nuclear volume between spermatogonia and spermatids (Wrobel et al., 1995) the present findings were somewhat unexpected since there is also a dramatic 3-fold increase in nuclear volume between early and late primary spermatocytes in sheep (Wrobel et al., 1995). However, this discrepancy can be explained by the fact that a peak in the number of degenerating cells occurred when spermatocytes had the highest prevalence in ST cross-sections (XS) as the most mature germ cell, in agreement with a study that reported increased apoptosis predominantly in primary (mid-pachytene) spermatocytes in rats during the first wave of spermatogenesis (Jahnukainen et al., 2004). Chromatin condensation or decreased nuclear size is a hallmark morphological feature of apoptosis (Gobé and Harmon, 2008) which may have maintained the decreasing trend in nuclear : ST area. Additionally, there is a nearly 2-fold increase in Sertoli cell cytoplasmic volume from 70–100 days of age in ram lambs (Monet-Kuntz et al., 1984). As Sertoli cells occupy approximately one third of the adult ovine seminiferous epithelium (Wrobel et al., 1995), this increase in cytoplasmic volume could greatly affect the nuclear : ST area.

Recent evidence has indicated that the nucleus is likely the dominant source of subcellular ultrasound scattering and changes in nuclear configuration (e.g., mitosis, apoptosis)
greatly affect tissue echogenicity (Czarnota and Kolios, 2010). A reduction in the nuclear : ST area was therefore hypothesized to result in a corresponding decrease in testicular echogenicity, as the ultrasound waves are less likely to be scattered by STs with cells having a smaller nuclear size. Indeed, a positive correlation was found among nuclear : ST area and testicular echotextural parameters in the post-mitotic phase; however, no correlation was found in the mitotic phase. This may be explained by the increase in the number of degenerating cells observed towards the end of the mitotic phase, which had a positive correlation with NPVs and PSD in both the mitotic and post-mitotic phases. Cells undergoing apoptosis exhibit changes in nuclear morphology (e.g., condensation and fragmentation) that greatly enhance ultrasound scattering strength (Czarnota and Kolios, 2010). Nuclear morphology holds promise as a strong determinant of testicular echotexture; future studies on other changes in nuclear configuration during spermatogenesis such as meiosis and spermiogenesis may provide further insight into interpreting testicular echotextural fluctuations during the first wave of spermatogenesis.

Spermatogonial stem cells (SSCs) are a rare germ cell population in the testis, constituting only about 10.6% of undifferentiated spermatogonia, 1.25% of all spermatogonia or 0.03% of all spermatogenic cells in the adult mouse (Tegelenbosch and de Rooij, 1993). At present, there are no morphological or molecular markers that can specifically identify SSCs (Oatley and Brinster, 2008). Ubiquitin C-terminal hydrolase L-1 (UCHL-1) has been shown to be exclusively expressed in undifferentiated spermatogonia in boars, with a high level of UCHL-1 expression associated with maintenance of spermatogonia in the undifferentiated state, and a reduction of UCHL-1 expression associated with their differentiation towards meiosis (Luo et al., 2009). These findings are consistent with the heterogeneity in UCHL-1 expression found in the present study and suggest that the lower numbers of dark-staining UCHL-1⁺ cells may
represent a SSC-enriched population and the higher numbers of medium- and light-staining UCHL-1+ cells may represent undifferentiated spermatogonia that have become committed to the differentiation pathway. The number of UCHL-1+ cells at different levels of intensity remained relatively stable throughout the study, consistent with the generally low rate of self-renewal described for all stem cells under steady-state conditions (Oatley and Brinster, 2012).

The total number of UCHL-1+ cells and the numbers of dark-, medium- and light-staining UCHL-1+ cells/ST XS were all positively correlated with testicular echotextural parameters throughout spermatogenic onset. However, the numbers of dark- and medium-staining UCHL-1+ cells/ST XS were more strongly correlated with echotextural parameters during the post-mitotic phase, whereas the number of light-staining UCHL-1+ cells/ST XS was correlated with NPVs and PSD only in the mitotic phase, indicating that even subtle differences in cellular histomorphology and/or function of spermatogonial populations may affect testicular echotexture. A role for UCHL-1 in promoting a wave of apoptosis during spermatogenic onset has previously been described (Kwon et al., 2005); therefore, it is feasible that the numbers of dark- and medium-staining UCHL-1+ cells/ST XS are more strongly correlated with testicular echotextural parameters during the post-mitotic phase due to their association with germ cell apoptosis, which was highest at the start of this period. The existence of correlations among testicular echotextural attributes and UCHL-1+ cells opens up several intriguing possibilities for using ultrasound as a non-invasive tool to evaluate the success of spermatogonial stem cell transfer procedures and/or monitor the SSC population during gonadotoxic cancer treatment.

Spermatogenesis is not simply initiated at a particular age; rather, its timing is precisely determined by a complex interaction of genetic, endogenous and environmental or exteroceptive cues (Valasi et al., 2012). Indeed, even among rams born in the same season, raised in the same
climate under the same feeding regimens and having a similar cross-bred genetic background, the first appearance of elongated spermatids was found to vary by 6 weeks in the current study. Furthermore, the rate of spermatogenic onset appeared to vary within ST XS of the same individual, with peaks in the percentages of ST XS with different germ cells as the most mature cell type present at specific times throughout prepubertal development. Spermatocytes were present in ST XS as the most mature germ cell type at the outset of the study at 10 weeks of age, in agreement with previous studies in ram lambs demonstrating the first appearance of primary spermatocytes at approximately 9–10 weeks of age (Skinner et al., 1968; Monet-Kuntz et al., 1984; Herrera-Alarcón et al., 2007). The percentage of ST XS with spermatocytes as the most mature germ cell type continued to increase until one week before the first detection of round and elongated spermatids, which occurred at 15 weeks of age, also in agreement with earlier reports (Skinner et al., 1968; Herrera-Alarcón et al., 2007). Therefore, the first wave of spermatogenesis proceeded gradually in the ram lambs of the present study, with differences among individuals as well as within the testes of each individual.

During spermatogenic onset, the developing germ cells undergo a number of changes in their morphological (e.g., size, geometry) and mechanical (e.g., density, compressibility) properties, which are both known to affect tissue echogenicity (Vlad et al., 2005; Ghoshal et al., 2013). Nearly all germ cell types have been correlated with testicular echotexture (Evans et al., 1996). The percentages of ST XS with (pre-)spermatogonia and spermatocytes as the most advanced cell type were both positively correlated with testicular echogenicity; however, differing physical properties of these germ cell types may explain this occurrence. In a study that used density centrifugation to physically separate different germ cell types, spermatogonia were found to have a relatively high density, and subsequently the density level decreased until the
formation of spermatocytes undergoing the first meiotic division before gradually increasing and reaching the highest level in elongated spermatids (Meistrich and Trostl, 1975). Therefore, the positive correlation among the percentage of ST XS with (pre-)spermatogonia as the most mature germ cell type and testicular echotextural parameters in the post-mitotic phase may be explained by the high density of this germ cell population. Spermatocytes, however, have a relatively low density (Meistrich and Trostl, 1975), but undergo a 5-fold increase in cellular volume and a 3-fold increase in nuclear volume during the first meiotic prophase to become the largest members of the germ cell line (Wrobel et al., 1995), which could explain the positive correlation observed among the percentage of ST XS with spermatocytes as the most advanced germ cell type and NPVs and PSD in the post-mitotic phase. The percentages of ST XS with round and elongated spermatids as the most mature germ cell type were both negatively correlated with testicular echotextural parameters, which may be a consequence of the comparatively small cellular volume of both cell types, especially for elongated spermatids (Wrobel et al., 1995). Additionally, the transition in shape from a round to an elongated cell would likely reduce cellular echogenicity further due to a dependence of ultrasound beam scattering on a perpendicular cell orientation for optimal scattering, which may have resulted in the slightly stronger negative correlation among the percentage of ST XS with elongated spermatids as the most mature germ cell type and testicular NPVs and PSD. Correlations among the percentages of ST XS with different spermatogenic cell types as the most advanced cell present and echotextural attributes have previously been evaluated by Evans et al. (1996) in bull calves studied from birth to puberty. While the strength of these correlations was similar to the current findings, the directions of correlations were opposite for all but one germ cell type, namely the (late) spermatocytes. The reason(s) for these differences is currently unknown;
however, future experimental protocols targeting different developmental stages or in vitro approaches may provide further insight into the echotextural characteristics of specific germ cell types. Acquiring this knowledge would be useful for potentially monitoring the stage of spermatogenesis using echotextural analysis in prepubertal individuals as well as in adults whose spermatogenesis has been interrupted by testicular recrudescence, contraceptive use or various pathological conditions.

In summary, the results of this study provide considerable insight into how tissue microstructure affects testicular echotexture during the first wave of spermatogenesis in ram lambs. STs are the largest morphological constituent of the testis, and accordingly their dimensions (tubular and luminal diameter) had the strongest correlations with testicular echotexture; however, these were comparable to the correlations with ST cell density, the numbers of degenerating and UCHL-1+ cells per ST XS and the percentages of ST XS with various germ cells as the most mature type at the cellular level, as well as with the nuclear : ST area at the sub-cellular level. There are several avenues of research that need to be explored before computerized image analysis of ultrasonograms may be used in a clinical setting, including a more detailed evaluation of the cellular and biochemical changes in the seminiferous epithelium and lumen accompanying ST maturation and the effect of this on testicular echotexture, further investigation of how nuclear configurations associated with different spermatogenic phases and germ cell apoptosis affect ultrasonographic attributes, and a precise determination of the echotextural characteristics of SSCs and different types of germ cells. Additionally, the echotextural attributes of Sertoli cells, which are intricately involved in spermatogenic onset as well as Leydig cells, which are responsible for secreting testosterone, a hormone that has previously been correlated with testicular echogenicity (Evans et al., 1996).
should be investigated more thoroughly. To recapitulate, our present findings could have important implications for the diagnostic and prognostic capabilities of scrotal ultrasonography including the non-invasive assessment of the rate and progression of spermatogenesis as well as microstructural testicular anomalies in prepubertal and sexually mature individuals.

4.6 Acknowledgements

The authors gratefully acknowledge Vicki Watts, Jose Rafael Rodriguez-Sosa, Andrew Bertolini, Kanwal Minhas, Stephanie Wilson and Bret McLeod for their assistance with data collection and histomorphological analysis and Pam Hasson and Jeff McFarlane at the Ponsonby Sheep Research Station for the care and management of experimental animals. J L Giffin was supported by the Ontario Ministry of Food, Agriculture, and Rural Affairs Highly Qualified Personnel Graduate Scholarship. Preliminary results were presented at the Institute of Animal Reproduction and Food Research/Society for Biology of Reproduction joint meeting (27 February - 1 March, 2013 in Gdańsk, Poland). This study was funded by the Ontario Ministry of Agriculture, Food and Rural Affairs (PMB), and the Natural Sciences and Engineering Research Council of Canada (PMB and ACH).
Chapter 5

HISTOMORPHOLOGICAL, ENDOCRINE AND ECHOTEXTURAL CHANGES ASSOCIATED WITH RETINOIC ACID-INDUCED SYNCHRONOUS SPERMATOGENESIS IN PREPUBESCENT RAM LAMBS

Jennifer L Giffin, Pawel M Bartlewski and Ann C Hahnel

Department of Biomedical Sciences
Ontario Veterinary College
University of Guelph
Guelph, ON, Canada N1G 2W1
5.1 Abstract

Spermatogenesis is initiated in a temporally and spatially asynchronous manner during prepubertal development. Retinoic acid (RA) appears to be a key regulator of this event and may be exogenously administered to induce synchronous spermatogenesis. Testicular echotexture is closely associated with spermatogenic development; therefore, exposure to RA may allow for precise determination of the echotextural characteristics of specific germ cells. The objective of this study was to determine whether RA exposure is capable of reducing germ cell heterogeneity during spermatogenic onset in ram lambs and to evaluate associated changes in echotextural, histomorphological and endocrine variables. Blood samples and scrotal ultrasound recordings were obtained from 17 ram lambs, treated with or without RA at 8 weeks of age, until castration 24 h or 2.5 weeks later. At 8.2 weeks of age, the nuclear : seminiferous tubule (ST) area was higher in the treated compared to the control group. Numerical pixel values (NPVs) and testosterone level reached a peak at 9 weeks of age in the treated group with a similar tendency in the control group. At 10.5 weeks of age, the percentage of ST cross-sections (XS) with pre-spermatogonia I as the most mature germ cell type was higher in the treated group and the percentage of ST XS with spermatogonia and spermatocytes as the most mature germ cell type and tubular and luminal ST area were higher in the control group. Testicular echotextural attributes were correlated with the percentages of ST XS with pre-spermatogonia I and II, spermatogonia and spermatocytes as the most mature germ cell type, tubular and luminal ST areas, the total number of cells/ST XS, the nuclear : ST area and the number of degenerating cells/ST XS as well as mean serum follicle-stimulating hormone, testosterone and estradiol concentrations, and these correlations differed between treatment groups. These results indicate that testicular echotexture is significantly altered by RA-induced synchronous spermatogenesis.
RA manipulation of spermatogenesis in large domestic animals may provide a suitable model for further investigation into the echotextural characteristics of specific germ cells.

5.2 Introduction

Following the initiation of spermatogenesis during prepubertal testicular development in rams, the production of sperm occurs year-round (Rosa and Bryant, 2003), ranging from 2–4 billion sperm/testis/day, depending on the season and breed (Dacheux et al., 1981). However, the process of spermatogenesis is quite lengthy, lasting in rams approximately 50 days (Wrobel, 1998). Therefore, to ensure the continuous production of sperm, different rounds of spermatogenesis must occur asynchronously within the seminiferous epithelium. This is achieved through the seminiferous epithelial cycle, in which different germ cell types (typically 4-5; Wrobel et al., 1995; Zeng et al., 2006; Griswold et al., 2010) associate together and differentiate into successive stages (8 stages described in rams; Wrobel et al., 1995; Zeng et al., 2006) per each cycle (approximately 10.4–10.6 days in rams; Amann and Schanbacher, 1983; França et al., 2005). These stages are phased in an orderly sequence along the length of the seminiferous tubule (ST), referred to as the spermatogenic wave (Hogarth and Griswold, 2010). The stages of the seminiferous epithelial cycle become apparent during the first wave of spermatogenesis, even before all germ cell types are formed (Courot et al., 1970; Drumond et al., 2011); however, spermatogenesis is commonly described on the basis of the most mature germ cell types present during this developmental stage, due likely to the considerable variation among different STs (Snyder et al., 2010).

A role for retinoic acid (RA), the primary active metabolite of vitamin A, in instigating germ cell entry into meiosis in both male and female mammals has recently emerged (Griswold
et al., 2012). In the testes, it acts by inducing gonocytes (also called pro- or pre-spermatogonia; Culty, 2013) and/or undifferentiated spermatogonia to transform into differentiated spermatogonia (Hogarth and Griswold, 2010; Busada et al., 2014), the first germ cells to become committed to undergo meiosis (Olive and Cuzin, 2005). A periodic distribution of RA signalling was found in neonatal mice testes (Snyder et al., 2011) in agreement with descriptions of a random initiation of spermatogenesis throughout the testes of most species (Courot et al., 1970). However, in stallions and boars, spermatogenic onset and testicular developmental changes occur along a maturation gradient, with more advanced stages in the central region of the testis compared to the outer region (Clemmons et al., 1995; Ford and Wise, 2009; Avelar et al., 2010), suggesting that RA signalling does not occur periodically in all species. Interestingly, when exogenous RA was administered to neonatal mice prior to the first appearance of preleptotene spermatocytes, the seminiferous epithelial cycle became highly synchronized while the spermatogenic wave was eliminated in adults (Snyder et al., 2011; Davis et al., 2013). In addition to providing valuable insight into the spatiotemporal regulation of the initiation of spermatogenesis, this experimental model may also be useful for studying specific stages of the seminiferous epithelial cycle and/or major developmental stages during spermatogenic onset. With over 30 different cells (mostly constituting the seminiferous epithelium) present in the testes of most adult mammals, cellular heterogeneity presents a major challenge in characterizing specific germ cell types (Rodríguez-Casuriaga et al., 2013). Furthermore, this model would likely eliminate any regional differences in rate of testicular maturation that may exist.

Scrotal ultrasonography is a commonly performed imaging technique for the non-invasive assessment of internal, macroscopic structures. However, quantitative analysis of testicular ultrasonograms using computerized image analysis may provide additional information
on the structures at the microscopic, both cellular and sub-cellular levels (Evans et al., 1996; Giffin et al., 2009; Brito et al., 2012; Giffin et al., 2014). While direct visualization of tissue microstructure is not possible at conventional diagnostic ultrasound frequencies, a unique pattern of gray-scale dots or pixels, termed echotexture (Ginther, 1995), creates an anatomical representation of the scrotal contents based on differences in acoustic impedance, related to tissue density and compressibility (Venables, 2011), at multiple tissue interfaces and the morphological properties of specific tissue constituents (e.g., shape, size, number; Ghoshal et al., 2013). The brightness or intensity of the image varies according to the ability of the tissue to reflect ultrasound waves, referred to as echogenicity (Ginther, 1995). Typically, computer-generated numerical pixel values (NPVs) and pixel standard deviation (PSD)/heterogeneity serve as measures of central tendency and variation, respectively (Pierson and Adams, 1995; Singh et al., 2003; Kastelic and Brito, 2012). Testicular echotextural parameters have been correlated with an array of histomorphological variables and hormonal levels (Evans et al., 1996; Brito et al., 2012; Giffin et al., 2014); however, clarification is needed to determine how specific spermatogenic features and events affect testicular echotexture, as the presence and direction of some correlations, such as the percentages of STs with various germ cells as the most mature, appear to fluctuate depending on the specific developmental phase observed (Evans et al., 1996; Giffin et al., 2014). Therefore, the objective of this study was to determine whether RA exposure in ram lambs induces a more uniform onset of spermatogenesis and to evaluate associated changes in reproductive hormone secretion, testicular histomorphology and echotextural attributes of the testes.

5.3 Materials and Methods
5.3.1 Animals and Experimental Design

Seventeen spring-born Dorset x Rideau Arcott x Canadian Arcott ram lambs were housed at a field research station in Ponsonby, ON, Canada (latitude: 43° 33’N) in a sheltered outdoor facility, under ambient light and temperature conditions. From one week of age, lambs were fed a 16% crude protein lamb grower diet (Shur-Gain Feedmills, St. Marys, ON, Canada) *ad libitum*. Lambs were weaned after 50 days of age, and from this point hay was offered and daily consumption of the grower averaged 2.5 pounds per lamb. Water was always available to the animals. All experimental procedures performed on live animals were approved by the Animal Care Committee at the University of Guelph in accordance with the guidelines of the Canadian Council on Animal Care.

At 8 weeks of age, ram lambs were divided into two groups with equal breed percentage backgrounds. Treated animals \((n = 9)\) received 55 mg of all-trans retinoic acid (RA) in 1 ml of dimethyl sulfoxide (DMSO) and control animals \((n = 8)\) received 1 ml of DMSO alone (above from Sigma-Aldrich, St. Louis, MO, USA) injected s.c. in the neck region. The dose of RA was calculated using allometric scaling based on a previous study in neonatal mice which showed that a 75-µg dose of RA significantly increased the expression of stimulated by retinoic acid gene 8 (STRA8; Snyder *et al.*, 2011), a protein that is induced by RA (Zhou *et al.*, 2008). Castration was performed by Ruminant Field Services (University of Guelph, Guelph, ON, Canada) on a subset of animals 24 hours \((n = 4\) treated and control) or 2.5 weeks \((n = 5\) treated, \(n = 4\) control) after the injections were given. A minimum of four animals were assigned to each treatment group in accordance with the sample size calculation based on the formula below:

\[
n \approx \frac{16}{\left(\frac{\mu_1-\mu_2}{\sigma}\right)^2}
\]
with Type I error set to $\alpha=0.05$ and Type II error set to $\beta=0.20$ (van Belle and Kerr, 2012), where values for $\mu_1$, $\mu_2$, and $\sigma$ were derived from a previous study in neonatal mice which showed that 24 hours after receiving RA injections, the number of STRA8$^+$ cells/ST XS was significantly higher in treated animals than in vehicle-treated controls (~2 compared to 0.5, with a standard deviation of ~0.75; Snyder et al., 2011). Figure 5.1 depicts the sample sizes and groups of animals used in the present study.

**Figure 5.1** Experimental design and sample sizes.

Prior to castration surgery, forage and grain were withheld for 12 hours and the lambs were weighed and administered ketoprofen (Merial Canada Inc, Baie d’Urfé, QC, Canada; 3 mg/kg i.m.), xylazine (Rompun®, Toronto, ON, Canada; 0.1 mg/kg i.m.), and 1% lidocaine hydrochloride (Zoetis, Kirkland, QC, Canada; 0.5-1.0 ml into the spermatic cord proximal to the testes, 1.0-2.0 ml s.c. into the scrotal tissue at base of scrotum). Penicillin (Penpro, Vétoquinol,
Lavaltrie, QC, Canada; 0.7 ml/10 kg) was also administered on the day of surgery and for the following 2 days of recovery.

5.3.2 Tissue Processing and Histology

Following castrations, testes were transported to the laboratory on ice. Scrotal tissue and epididymides were removed, and the testes were cut longitudinally and then sectioned into four tissue samples of approximately 1 cm³ each collected from the medial and lateral regions of the intermediate and ventral aspects of the anterior plane of the testicular parenchyma. The tissue was fixed in modified Davidson’s solution (Lattendresse et al., 2002) overnight at 4°C and then kept in 70% ethanol at 4°C until paraffin wax embedment. Subsequently, the tissue was sectioned at a thickness of 5 µm and allowed to dry on glass slides (Fisherbrand Superfrost Plus, Fisher Scientific, Ottawa, ON, Canada) overnight and then deparaffinized in xylene and rehydrated in a standard graded isopropanol-water series for staining with hematoxylin and eosin. Coverslips were applied using Cytoseal XYL mounting medium (Richard-Allen Scientific, Kalamazoo, MI, USA).

Tissue micrographs were taken at 200x image magnification using Q Capture® (Quorum Technologies Inc., Guelph, ON, Canada). For each of the four tissue sections per animal, ten round seminiferous tubule (ST) cross-sections (XS) were analyzed using Image ProPlus® 7.0 (Media Cybernetics Inc., San Diego, CA, USA) to determine: i) the percentages of ST XS containing no germ cells or pre-spermatogonia I and II, spermatogonia and spermatocytes as the most mature cell type present, assessed manually based on their morphological description by Wrobel et al. (1995) and Steger and Wrobel (1996); ii) tubular and iii) luminal ST area (µm²), computed from manual outlines of the ST XS; iv) the total number of cells/ST XS, based on
automated counts of cell nuclei using a manually selected range of colour intensity values for manually outlined ST XS; v) the ratio of average cell nuclear area to tubular ST area (nuclear : ST area), calculated from nuclear area determined from the automated nuclear counts divided by ST area; and vi) the number of degenerating cells displaying classical histomorphological attributes of apoptosis (i.e., chromatin condensation and fragmentation, cytoplasmic vacuolization and eosinophilia and cell shrinkage; Gobé and Harmon, 2008) per ST XS, assessed manually.

5.3.3 Ultrasonography

Scrotal ultrasonography was performed before administering RA and/or DMSO and performing castrations as well as on a twice weekly for animals castrated 2.5 weeks after receiving injections (Fig. 5.1). Left and right testes were scanned in longitudinal and transverse planes using an Aloka SSD-900 portable ultrasound scanner equipped with a 7.5-MHz linear array transducer and recorded on a Digital Versatile Disk (DVD) recorder (Pioneer® DVD Recorder DVR-510H, Pioneer Electronics of Canada Inc., Markham, ON, Canada). Settings for main, near and far gains and focal points were optimized and kept constant throughout the study. A lubricant gel (Light Lube, Centaur VA Animal Health, Guelph, ON, Canada) was applied to eliminate air between the scrotum and the transducer, and minimal pressure was applied to the testis while scanning. All examinations were carried out by the same two operators.

Real-time scrotal ultrasound recordings initially saved on the hard drive of the DVD recorder were downloaded to a computer workstation (Intel®-Xeon® Processor MP; 2.8 GHz; Intel Co., Santa Clara, CA, USA) and saved as *.avi files. Representative still-images containing maximal cross-sectional surface area and of adequate image quality (i.e., devoid of artifacts)
were selected using Adobe Premiere® Pro 2.0 (Adobe Systems Inc., San Jose, CA, USA) and saved as *.bmp images. Computer-assisted image analysis of the digitalized ultrasonograms was performed using Image ProPlus® analytical software according to the “spot meter” technique, as described previously (Pierson and Adams, 1995; Singh et al., 2003; Giffin et al., 2009). A total of six spots in longitudinal view and four spots in transverse view, each measuring 40 pixels in diameter, were placed randomly on the surface of the testicular parenchyma with an equal number above and below the rete testis. Mean numerical pixel values (NPVs) and pixel heterogeneity (i.e., standard deviation of NPVs; PSD) were determined for each spot and averaged for left and right testes (20 spots in total/day/animal).

5.3.4 Hormone Analysis

Blood samples were taken before administering RA and/or DMSO and performing castrations as well as on a twice weekly basis for animals castrated 2.5 weeks after receiving injections (Fig. 5.1). Samples were collected via jugular venipuncture into 10 ml vacutainer tubes (Becton Dickinson, Rutherford, NJ, USA) and allowed to coagulate for 12–24 h at room temperature before removing the clots and centrifuging at 1500 x g for 10 min. Blood serum was decanted and stored at –20 °C until assayed (Prairie Diagnostic Services, University of Saskatchewan, Saskatoon, SK, Canada). Follicle-stimulating hormone (FSH), testosterone and estradiol concentrations were determined in single assays using validated radioimmunoassay procedures. The sensitivity of the assays was 0.1 ng/ml for FSH and testosterone and 1.0 pg/ml for estradiol. Reference FSH sera had mean concentrations of 0.37 ng/ml and 1.28 ng/ml with intra-assay coefficients of variation (CVs) of 16.4% and 7.3%, respectively. Reference testosterone sera had mean concentrations of 1.28 ng/ml and 4.75 ng/ml with an intra-assay CVs
of 8.7% and 11.1%, respectively. Reference estradiol sera had mean concentrations of 4.5 pg/ml and 13.0 pg/ml with an intra-assay CVs of 13.3% and 13.0%, respectively.

5.3.5 Statistical Analyses

All statistical procedures were carried out using SigmaPlot® (version 11.0; Systat Software, Inc., Richmond, CA, USA). Histomorphological data were analyzed using two-way analysis of variance (ANOVA) with group (treated or control) and anatomical section as the two input variables, and the differences between individual means were analyzed using the Holm-Sidak method. Endocrine and echotextural data for treated and control groups were analyzed using separate one-way repeated measures ANOVA models, and the differences between individual means taken at different ages were analyzed by the Holm-Sidak method; comparisons between means in treated and control groups was performed using t-tests. Correlations among echotextural, endocrine and histomorphological variables were analyzed using the Pearson Product Moment. In all analyses, $P < 0.05$ was considered statistically significant and $P \leq 0.10$ was considered approaching statistical significance. All results were presented as mean ± SEM.

5.4 Results

5.4.1 General Results

Histomorphological, endocrine and echotextural variables varied between treated and control groups ($P < 0.05$), but not among different anatomical sections of the testis ($P > 0.05$); therefore, data from all anatomical sections were pooled for the treatment groups prior to subsequent analyses.
5.4.2 Testicular Histomorphology

Testicular micrographs depicting the typical appearance of treated and control ram lamb testes at 8.2 and 10.5 weeks of age are shown in Figure 5.2. Several differences in testicular histomorphology were detected between the two treatment groups. At 8.2 weeks of age, the percentage of seminiferous tubule (ST) cross-sections (XS) with no germ cells present or with spermatogonia as the most mature germ cell type was numerically greater in the treated compared to the control group ($P > 0.05$; Fig. 5.3a and d) and the nuclear : ST area was significantly greater in the treated compared to the control group ($P < 0.05$; Fig. 5.3i). There was also a numerically higher number of degenerating cells/ST XS at 8.2 weeks of age in the treated compared to the control groups ($P > 0.05$; Fig. 5.3j). At 10.5 weeks of age, the percentage of ST XS with pre-spermatogonia I was greater in the treated group compared to the control group ($P < 0.05$; Fig. 5.3b), while the percentage of ST XS with spermatogonia or spermatocytes as the most mature germ cell type was significantly greater in the control compared to the treated group ($P < 0.05$; Fig. 5.3d and e). Tubular and luminal ST area were also significantly greater in the control compared to the treated group at 10.5 weeks of age ($P < 0.05$; Fig. 5.3f and g) and the number of degenerating cells/ST XS was numerically higher in the control compared to the treated group ($P > 0.05$; Fig. 5.3j).
Figure 5.2 Testicular micrographs of retinoic acid-treated and control ram lambs at 8.2 (a, b) and 10.5 (c, d) weeks of age. Pre-spermatogonia I (Pre-Sg I) were most prevalent in 8.2 week old ram lambs in both treated (a) and control (b) groups. Pre-spermatogonia II (Pre-Sg II) were more apparent at 10.5 weeks of age as seen in the treated (d) group, while spermatogonia (Sg) were seen more frequently and spermatocytes (Sc) were seen only in control ram lambs (c) at this age. Degenerating cells (arrows) indicate cells displaying classical histomorphological features of apoptosis. All other cells constituting the seminiferous epithelium are Sertoli cells. Occasionally, seminiferous tubule cross-sections without germ cells (stars) were observed. Note the presence of a lumen in the seminiferous tubules of the control group at 10.5 weeks of age. Images were taken at 200x image magnification.
**Figure 5.3** Mean (± SE) percentages of seminiferous tubule (ST) cross-sections (XS) with no germ cells present (a) or with pre-spermatogonia I (Pre-Sg I; b), pre-spermatogonia II (Pre-Sg II; c), spermatogonia (Sg; d) or spermatocytes (Sc; e) as the most mature germ cell type present, tubular (f) and luminal (g) ST area, total number of cells/ST XS (h), nuclear : ST area (i) and the number of degenerating cells/ST XS (j) in treated and control ram lambs at 8.2 and 10.5 weeks of age. Bars connected with dashed lines indicate significant differences ($P < 0.05$).
5.4.3 Hormone Concentrations

Mean serum concentrations of follicle-stimulating hormone (FSH), testosterone and estradiol from 8 to 10.5 weeks of age in control and treated ram lambs are shown in Figure 5.4. There were no significant differences in hormone concentrations between control and treated groups at any age studied ($P > 0.05$). A 2.3- or 1.8-fold numerical increase in peripheral FSH concentration occurred in control and treated ram lambs at 8.2 and 8.5 weeks of age, respectively ($P > 0.05$), followed by a gradual numerical decline to nadir values at 10.5 weeks of age ($P > 0.05$; Fig. 5.4a). Serum testosterone concentration rose 16-fold from 8.2 to 9 weeks age ($P < 0.05$), followed by a return to the nadir at 10.5 weeks of age in treated ram lambs ($P < 0.05$) and there was a similar numerical trend for the control group ($P > 0.05$; Fig. 5.4b). A 3.4- to 11.1-fold numerical increase in circulating estradiol concentration occurred in control and treated ram lambs, respectively, from 8.2 to 9 weeks of age ($P > 0.05$), before returning to a nadir at 10.5 weeks of age ($P > 0.05$; Fig. 5.4c).
Figure 5.4 Mean (± SE) serum concentrations of follicle-stimulating hormone (FSH; a), testosterone (b) and estradiol (c) in retinoic acid-treated and control ram lambs from 8 to 10.5 weeks of age. Values with different letters represent statistically significant differences among ages within treatment groups ($P < 0.05$).
5.4.4 Testicular Echotexture

Numerical pixel values (NPVs) and pixel standard deviation (PSD) from 8 to 10.5 weeks of age in the control and treated ram lambs are shown in Figure 5.5. NPVs increased numerically from 8.2 to 9 weeks of age in both the control and treated ram lambs; thereafter, NPVs decreased in the treated group to 10.5 weeks of age ($P < 0.05$), while the control group decreased numerically to 10 weeks of age ($P > 0.05$), then increased to 10.5 weeks of age ($P > 0.05$; Fig. 5.5a). PSD decreased numerically from 8 to 8.2 or 8.5 weeks of age in the treated and control groups, respectively ($P > 0.05$), before rising to a peak at 9 weeks of age and then decreasing to 10.5 weeks of age in the treated group ($P > 0.05$), or staying relatively constant until rising at 10.5 weeks of age in the control group ($P > 0.05$; Fig. 5.5b). Both NPVs and PSD were greater in the control compared to the treated groups of ram lambs at 10.5 weeks of age ($P < 0.05$).
Figure 5.5 Mean (± SE) numerical pixel values (NPVs; a) and pixel standard deviation (PSD)/heterogeneity (b) in retinoic acid-treated and control ram lambs from 8 to 10.5 weeks of age. Values with different letters represent statistically significant differences between ages within treatment groups (P < 0.05). Values with an asterisk represent statistically significant differences among ages within treatment groups (P < 0.05).
5.4.5 Correlations

Correlations among testicular echotextural parameters and histomorphological attributes at 8.2 and 10.5 weeks of age and hormone concentrations from 8-8.2 or 8-10.5 weeks of age in treated and control ram lambs are summarized in Table 5.1. At 8.2 weeks of age, there was a negative correlation among NPVs and tubular ST area ($r=-0.72; P=0.05$), the total number of cells/ST XS ($r=-0.71; P=0.05$) and the number of degenerating cells/ST XS ($r=-0.64, P=0.09$) and a positive correlation between NPVs and the nuclear : ST area ($r=0.84; P=0.04$) in the treated group. There was a positive correlation among NPVs and the percentage of ST XS with spermatogonia as the most mature germ cell type ($r=0.66; P=0.07$) and circulating estradiol level ($r=0.81; P=0.01$) in the control group at 8.2 weeks of age or from 8–8.2 weeks of age, respectively.

At 10.5 weeks of age, NPVs were positively correlated with the percentage of ST XS with pre-spermatogonia I as the most advanced germ cell type ($r=0.70; P=0.02$) and the nuclear : ST area ($r=0.74; P=0.01$) and negatively correlated with the percentage of ST XS with pre-spermatogonia II as the most mature germ cell type ($r=-0.78; P=0.007$) in the treated group. Serum testosterone and estradiol concentrations were positively correlated with NPVs ($r=0.45; P=0.01$) and PSD ($r=0.40; P=0.06$), respectively, from 8–10.5 weeks of age in the treated group. In the control group at 10.5 weeks of age, NPVs were negatively correlated with the percentage of ST XS with pre-spermatogonia I as the most mature germ cell type ($r=-0.73; P=0.04$) and the nuclear : ST area ($r=-0.91; P=0.002$), and positively correlated with the percentage of ST XS with spermatogonia and spermatocytes as the most advanced germ cell type ($r=0.78; P=0.02$ and $r=0.90; P=0.003$, respectively), tubular and luminal ST area ($r=0.98; P=0.00003$ and $r=0.82; P=0.01$), the total number of cells/ST XS ($r=0.93; P=0.0008$) and the number of degenerating
Table 5.1 Pearson product moment correlation coefficients among echotextural, histomorphological and endocrine variables in control (n = 8) and retinoic acid-treated (n = 9) Dorset x Rideau-Arcott x Canadian Arcott ram lambs from 8 to 10.5 weeks of age.

<table>
<thead>
<tr>
<th></th>
<th>8.2 / 8-8.2 weeks of age</th>
<th>10.5 / 8-10.5 weeks of age</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T&lt;sub&gt;1&lt;/sub&gt;</td>
<td>C&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>NPVs, PSD</td>
<td>NPVs, PSD</td>
<td>NPVs, PSD</td>
</tr>
<tr>
<td>% STs with Pre-Sg I*</td>
<td>NS, NS</td>
<td>NS, NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% STs with Pre-Sg II*</td>
<td>NS, NS</td>
<td>NS, NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% STs with Sg*</td>
<td>NS, r=0.66, p=0.07</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% STs with Sc*</td>
<td>NA, NA</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubular ST area</td>
<td>r=-0.72, p=0.05</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminal ST area</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total # cells/ST XS</td>
<td>r=-0.71, p=0.05</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nuclear : ST area</td>
<td>r=0.84, p=0.04</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td># Degenerating cells/ST XS</td>
<td>r=-0.64, p=0.09</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum FSH concentration</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum testosterone concentration</td>
<td>NS, r=0.45, p=0.01</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum estradiol concentration</td>
<td>NS, r=0.81, p=0.01</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NPVs, numerical pixel values (pixel intensity); PSD, pixel standard deviation (pixel heterogeneity); ST, seminiferous tubule; XS, cross-section; Pre-Sg, pre-spermatogonia; Sg, spermatogonia; Sc, spermatocytes; RSt, round spermatids; ESt, elongated spermatids; FSH, follicle-stimulating hormone; *as the most mature germ cell type present; NS, non-significant; NA, non-applicable.
cells/ST XS \( (r=0.73; P=0.04) \). Peripheral FSH concentrations were positively correlated with NPVs and PSD \( (r=0.36; P=0.36) \) from 8-10.5 weeks of age in control rams.

### 5.5 Discussion

This study demonstrated that, similar to mice, exogenous retinoic acid (RA) administration during pre-meiotic spermatogenic development in ram lambs resulted in delayed but synchronized progression of early spermatogenesis. This was evidenced by the fact that 2.5 weeks after RA injections, when the ram lambs were 10.5 weeks of age, there were 5 different spermatogenic stages in the control group, while the treated group was comprised of only 3 spermatogenic stages. There are two proposed mechanisms for this delay in spermatogenic onset: i) a round of apoptosis that affects differentiated spermatogonia following RA-induced expression of differentiation markers (Snyder et al., 2011; Davis et al., 2013); and ii) increased expression of RA degrading enzymes which suppress RA availability to the germ cells (Busada et al., 2014), thereby inhibiting the initiation of meiosis. As the number of degenerating cells/seminiferous tubule (ST) cross-section (XS) did not differ between control and treated groups, it suggests that the second mechanism was implicated in the present study, although apoptosis may have occurred in the absence of typical morphological features, as previously suggested (Busada et al., 2014).

In addition to spermatogenic onset, the rate of ST growth was also delayed in treated ram lambs 2.5 weeks after RA administration. While the total number of cells/ST XS did not differ between treatment groups at this age, smaller tubular ST area in treated ram lambs may be attributed to the reduced size and/or lack of a lumen. The presence and size of a lumen are generally indicative of Sertoli cell maturation (Russell et al., 1989; van Haaster et al., 1993). RA
has previously been shown to induce differentiation of cultured Sertoli cells (Buzzard et al., 2003; Nicholls et al., 2013); however, a decreased rate of Sertoli cell maturation may have occurred in the present study due to an increase in RA-degrading enzymes, as mentioned above, or the interaction with germ cells, as the presence of zygotene-pachytene spermatocytes have been temporally associated with the formation of inter-Sertoli cell tight junctions (Cavicchia and Sacerdote, 1991), a key element of Sertoli cell maturation. Therefore, the exposure of ram lambs to RA appears to affect the differentiation of not only germ cells, but also Sertoli cells and the development of the ST as a whole.

Unlike boars and stallions, in which the onset of spermatogenesis and differentiation of somatic cell types proceeds along a maturation gradient from the innermost regions of the testis to the periphery (Clemmons et al., 1995; Ford and Wise, 2009; Avelar et al., 2010), the rate of testicular development appeared to occur evenly throughout early prepubertal ram lamb testes, as there were no differences in the histomorphological data among the four regions evaluated. However it remains to be determined whether a different regional arrangement exists, or if the maturation gradient becomes more apparent closer to the attainment of puberty, as occurs in porcine testicular development (Avelar et al., 2010).

Histomorphological analysis revealed a number of differences between the treated and control groups at 8.2 and 10.5 weeks of age. Short-term exposure to RA resulted in a considerably higher nuclear : ST area, which could be explained if the germ cell nuclei were increasing in size due to cell death. While apoptosis generally results in nuclear condensation (Gobé and Harmon, 2008), other forms of cell death, such as accidental cell death, also referred to as primary cell necrosis or oncosis, are accompanied by nuclear swelling (Darzynkiewicz et al., 2004). An alternate pathway of cell death induced by RA is consistent with the observation
made by Busada et al. (2014), that germ cells in RA-treated mice staining positive for caspase-3 activity did not display classical apoptotic features. At 10.5 weeks of age, the greater percentages of ST XS with pre-spermatogonia I in the treated ram lambs or with spermatogonia and spermatocytes in the control ram lambs support previous findings that RA exposure results in delayed spermatogenic onset (Snyder et al., 2011; Busada et al., 2014). Furthermore, the reduced tubular and luminal ST area provides direct evidence that ST growth is also affected. The perceived increase in the number of degenerating cells/ST XS in the control group at 10.5 weeks of age would reasonably be explained by the wave of germ cell apoptosis affecting post-spermatogonial cells during spermatogenic onset in ram lambs (Steger and Wrobel, 1996).

An early postnatal rise in gonadotropin secretion, accompanied by decreased hypothalamic sensitivity to gonadal steroids, is generally considered the trigger for the initiation of reproductive function during pubertal onset (Olster and Foster, 1986; Olster and Foster, 1988). In ram lambs, the level of follicle-stimulating hormone (FSH) typically reaches a peak around 1–2 months of age (Yarney and Sanford, 1990; Chandolia et al., 1997a; Tilbrook et al., 1999), whereas peak LH concentration normally occurs somewhat later, between 1.5–3 months of age (Foster et al., 1978; Wilson and Lapwood, 1979; Wańkowska et al., 2010), depending on breed prolificacy (Pelletier et al., 1981) and individual rate of maturation (Aravindakshan et al., 2000). The early postnatal rise in LH is followed by a rapid increase in the number of adult Leydig cells and a transient rise in testosterone level (Rawlings et al., 2008). The present results suggest that FSH levels reached their peak no later than 8.5 weeks of age, and that the rise in testosterone and therefore LH concentrations occurred at approximately 9 weeks of age. The rise in estradiol concentration closely resembled that of testosterone, and likely reflects aromatization of testosterone into estradiol which may occur in either Leydig or Sertoli cells in immature ram
lambs (Bilińska et al., 1997). As there were no differences in reproductive hormone levels between control and treated ram lambs, this suggests that RA acts in a local manner and does not impinge on the neuroendocrine maturation of the hypothalamo-pituitary-gonadal axis.

Throughout early postnatal and peripubertal development, there is an overall increase in testicular echogenicity (Evans et al., 1996; Chandolia et al., 1997a; Chandolia et al., 1997b; Aravindakshan et al., 2000; Brito et al., 2012). However, the trend is not linear but rather variable with increasing age, especially with more frequently taken ultrasound evaluations (Evans et al., 1996), reflecting the dynamic histomorphological changes occurring within the testis. Therefore, the fluctuating pattern in NPVs observed in the present study is consistent with previous findings (Evans et al., 1996; Chandolia et al., 1997a; Chandolia et al., 1997b; Aravindakshan et al., 2000; Brito et al., 2012). The first apparent increase in testicular echogenicity that occurred at 9 weeks of age in both control and treated ram lambs corresponded with the increase in testosterone and estradiol secretion, and therefore may be attributed to a rise in adult Leydig cell numbers, although direct histomorphological evaluation of the interstitial tissue was not performed. The second apparent increase in testicular echogenicity that occurred in control but not in RA-treated ram lambs at 10.5 weeks of age may be attributed to the larger percentages of ST XS with spermatogonia and spermatocytes as the most mature germ cell types present, the greater tubular and luminal ST areas and the perceived larger number of degenerating cells/ST XS in the control compared to the treated ram lambs, which were all positively correlated with NPVs of the testicular parenchyma. By contrast, the decrease in testicular echogenicity that occurred in the treated group from 9 to 10.5 weeks of age may have been due to the lack of these changes. Considering the synchronization of spermatogenesis that occurred with RA treatment, it is surprising that lower PSD was not observed in the treated
group, except at the final evaluation point at 10.5 weeks of age. As spermatogenesis progresses, however, it is likely that PSD would become increasingly lower in the treated compared to control ram lambs, since there are much fewer stages of the seminiferous epithelial cycle present in the testes of adult mice exposed to RA compared to the even distribution in non-exposed animals (Snyder et al., 2011; Davis et al., 2013). Overall, the quantitative echotextural attributes of the testes were variable with age; however, RA treatment resulted in lower NPVs and PSD at 10.5 weeks of age than normally occurs.

The correlations among histomorphological, endocrine and echotextural parameters presented in this study are the first to be obtained from an intervention study. Some of the correlations agree with those previously found, such as the negative correlation between testicular echogenicity and the percentages of ST XS with pre-spermatogonia I (group C₂) and II (group T₂) as the most mature germ cell type (Evans et al., 1996), and the positive correlations among NPVs and the percentage of ST XS with spermatogonia (Giffin et al., 2014) or spermatocytes as the most mature germ cell type (Giffin et al., 2014), tubular and luminal ST dimensions (Evans et al., 1996; Giffin et al., 2014), the nuclear: ST area (group T₁ and T₂; Giffin et al., 2014), the number of degenerating cells/ST XS (group C₂; Giffin et al., 2014) and serum testosterone concentration (Evans et al., 1996). However, some of the correlations have previously not been found, such as the correlations among NPVs and/or PSD and the total number of cells/ST XS and serum FSH and estradiol concentrations, or are contrary to those previously reported, such as the negative correlation among NPVs and tubular ST dimensions (group T₁; Evans et al., 1996; Giffin et al., 2014), the nuclear: ST area (group C₂) and the number of degenerating cells/ST XS (group T₁; Giffin et al., 2014) and the positive correlations among NPVs and the percentages of ST XS with pre-spermatogonia I (group T₂; Evans et al.,
1996) and spermatogonia (Evans et al., 1996) as the most mature germ cell type and tubular and luminal ST dimensions (group C2; Gábor et al., 1998; Brito et al., 2012; Giffin et al., 2014). It is important to note that variability in the correlations among echotextural, histomorphological and endocrine attributes do not imply a deficit in the image analysis procedure; rather, it demonstrates the complexity of the testis itself. Using different experimental procedures and protocols to compare and contrast correlations will allow previously unknown associations to emerge and will strengthen our understanding of the determinants of testicular echotexture.

Distinct correlations among echotextural, histomorphological and endocrine attributes were found for each experimental group. In treated ram lambs after 24 hours, there was a strong correlation between NPVs and nuclear : ST area, which suggests that increased nuclear size due likely to accidental cell death from RA exposure presents a highly reflective surface to ultrasound waves, in agreement with studies indicating that the size of the nucleus is a dominant ultrasound scattering structure (Taggart et al., 2007; Czarnota and Kolios, 2010). Germ cells exhibiting classical apoptotic death with condensed nuclei may present a comparatively less reflective surface to the ultrasound beam, resulting in the negative correlation between NPVs and the number of degenerating cells/ST XS. Prior to the onset of spermatogenesis, ST size is still largely a factor of the Sertoli cell population, which comprise about 80-90% of the testis by volume (Steger and Wrobel, 1996). Therefore, since ST cell number and tubular ST area are predominantly determined by Sertoli cells, which do not undergo cell death in response to RA (Davis et al., 2013) and are thus less echogenic, the negative correlation with NPVs makes sense. By comparison, in control rams, a strong positive correlation between NPVs and estradiol was seen, which was due likely to the cellular effects of estradiol such as increased numbers of undifferentiated and differentiated type A spermatogonia (Kula, 1988). The positive correlation
between NPVs and the percentage of ST XS with spermatogonia as the most mature germ cell type could be due to the increased amount of heterochromatin as a result of differentiation in this cell type (de Rooij and Russell, 2000), which would increase the density of this cell and thus improve tissue echogenicity.

2.5 weeks after treatment with RA or at 10.5 weeks of age, testicular echotextural attributes were correlated with a new set of histomorphological and endocrine variables. In the treated group, NPVs were positively or negatively correlated with the percentage of ST XS with pre-spermatogonia I or II, respectively, which could be explained by the distinct nuclear appearance of these two cell types. Pre-spermatogonia I have multiple nucleoli within the nucleus while pre-spermatogonia II have one, prominent nucleolus (Steger and Wrobel, 1996). The increased concentration of scatterers within pre-spermatogonia I nuclei may therefore be more echogenic in comparison with the nuclear arrangement of pre-spermatogonia II. The positive correlation between NPVs and nuclear : ST area is relatively easy to interpret; a greater average nuclear area per ST XS will provide a larger area for the ultrasound beam to reflect than a lower average nuclear area per ST XS. Finally, the positive correlation between echotextural parameters and serum testosterone and estradiol concentrations in the treated group could be explained by the effects of testicular steroid biosynthesis on the numbers of adult Leydig cells or spermatogonial differentiation, as previously mentioned, or on the size of the Leydig and/or Sertoli cell protein processing organelles such as the endoplasmic reticulum, which have an abundance of membranes that may be quite echogenic. Some of these histomorphological variables were also correlated with NPVs in the control group; however, the direction of these correlations was opposite. Specifically, there was a negative correlation among NPVs and the percentage of pre-spermatogonia I as the most mature germ cell type and the nuclear : ST area.
This may be explained by considering the advanced stage of spermatogenesis and seminiferous tubule maturation that the control group was in compared to the treated group, with a greater percentage of ST XS with spermatogonia and spermatocytes as the most mature germ cell type and a larger tubular and luminal ST area, and the strong positive correlation with each of these variables (described further in Chapter 4), which could have obscured the correlations of less echogenic factors. By controlling for some of the developmental events in the treated group, a more accurate estimation of the relationship between histomorphological and echotextural attributes was obtained.

This study is the first to demonstrate that RA has the ability to reduce germ cell heterogeneity during spermatogenic onset in a non-rodent, domestic livestock species. This finding opens up numerous possibilities to study events surrounding the initiation of spermatogenesis during prepubertal development that were previously obscured due to the establishment of the spermatogenic wave. Precise echotextural characterization of specific germ cell types or other spermatogenic features and events may be obtained using RA manipulation of spermatogenesis in future studies that extend throughout the first wave of spermatogenesis or into postpubertal development or adulthood. The results from these studies could greatly enhance the diagnostic and/or prognostic capability of scrotal ultrasonography as a potential alternative to more invasive methods of assessing reproductive function, such as testicular biopsy and semen evaluation (Ahmadi et al., 2012; Giffin et al., 2014). The present study indicates that when RA is administered during pre-meiotic spermatogenic development in ram lambs, differences in testicular histomorphology and echotexture are apparent by the time that spermatocytes normally first start to appear. Further investigation into these differences is merited.
5.6 Acknowledgements

The authors would like to thank Ms. Pam Hasson and Mr. Jeff McFarlane for the care and management of experimental animals. Ms. Taylor VanDuzer, Ms. Kamila Skalski and Mr. Longfei Gao are gratefully acknowledged for their assistance performing ultrasound scanning, taking blood samples and processing tissue samples. JL Giffin was supported by the Ontario Ministry of Food, Agriculture, and Rural Affairs Highly Qualified Personnel Graduate Scholarship. This study was funded by the Natural Sciences and Engineering Research Council of Canada (PMB).
Chapter 6

SUMMARY DISCUSSION AND FUTURE RESEARCH APPLICATIONS

6.1 Summary Discussion

Over the past 20 years, our understanding of the temporal trends as well as the microstructural and hormonal basis of testicular echotexture in a variety of mammals has considerably expanded. Specifically, we know that testicular echogenicity generally increases throughout sexual maturation (Hamm and Fobbe, 1995; Evans et al., 1996; Chandolia et al., 1997a; Chandolia et al., 1997b; Aravindakshan et al., 2000; Brito et al., 2012) and that it is related to seminiferous tubule (ST) size (Evans et al., 1996; Brito et al., 2012; Gábor et al., 1998), the percentages of ST cross-sections (XS) with different germ cells as the most mature (Evans et al., 1996), and testosterone biosynthesis (Evans et al., 1996). However, a number of gaps in the literature have emerged. For instance: why does testicular echogenicity fluctuate with age?; why does the correlation between testicular echogenicity and ST size switch from positive during early postnatal and prepubertal development (Evans et al., 1996) to negative during postpubertal development (Brito et al., 2012; Gábor et al., 1998)?; do the correlations among testicular echogenicity and the percentages of ST XS with different germ cells as the most mature and serum testosterone level remain consistent throughout different phases of sexual maturation?; what other correlations among testicular echogenicity and histomorphology or hormone secretion exist? Furthermore, some questions simply have not been asked or studied. For example: how do non-parenchymal tissue elements affect testicular echotexture?; what information do other quantitative measures of testicular echotexture besides pixel intensity (e.g.,
pixel heterogeneity) reveal?: how do the known correlations change in response to altered
development or pathological conditions? The studies presented in this thesis directly answer or
provide pertinent information for further investigations into these questions. The major findings
of the studies can be summarized as follows:

- Testicular echotextural attributes (numerical pixel values and pixel standard
deviation/heterogeneity; NPVs and PSD, respectively) are significantly lowered by the
presence of the scrotum in peripubescent ram lambs; NPVs are further reduced by the
tunica vaginalis.
- Blood flow and the presence of blood do not impinge on testicular echotextural attributes
during peripubertal development in ram lambs.
- NPVs, but not PSD, are a reliable predictor of tubular and luminal ST area in
peripubescent ram lamb testes, with or without the presence of the scrotum or tunica
vaginalis, when the testicular blood flow is restricted or the blood is drained from the
testis.
- Testicular echotextural attributes fluctuate greatly with age during the first wave of
spermatogenesis; however, a distinct trend is observed when aligned with respect to the
first histological detection of elongated spermatids.
- During the mitotic phase of spermatogenic onset, NPVs and/or PSD are positively
correlated with tubular and luminal ST diameter, the number of degenerating cells/ST
XS, the number of dark-, medium- and light-staining ubiquitin C-terminal hydrolase L-1
(UCHL-1)+ cells/ST XS as well as the total number of UCHL-1+ cells/ST XS.
During the post-mitotic phase of spermatogenic onset, NPVs and/or PSD are negatively correlated with tubular ST diameter, ST cell density and the percentage of ST XS with round and elongated spermatids, and positively correlated with nuclear: ST area, the number of degenerating cells/ST XS, the number of dark- and medium-staining UCHL-1\(^+\) cells/ST XS, the total number of UCHL-1\(^+\) cells/ST XS, and the percentage of ST XS with (pre-)spermatogonia or spermatocytes as the most mature germ cell type.

Spermatogenic onset and ST histomorphology do not differ among medial, lateral, intermediate or ventral regions of the ovine testis during early postnatal and early prepubertal development.

Exposure to retinoic acid (RA) in early postnatal ram lambs increases the nuclear : ST area after 24 h and increases the percentage of ST XS with pre-spermatogonia I as the most mature germ cell type, lowers the percentage of ST XS with spermatogonia or spermatocytes as the most mature germ cell type and lowers tubular and luminal ST area after 2.5 weeks; inducing the synchronous, delayed onset of spermatogenesis during prepubertal development.

Exposure to RA in early postnatal ram lambs does not affect reproductive hormone secretion throughout early prepubertal development.

Differences in NPVs and PSD in ram lambs exposed to RA during early postnatal development are apparent by 10.5 weeks of age when spermatocytes normally first start to appear.

After 24 hours, NPVs are negatively correlated with tubular ST area, the total number of cells/ST XS and the number of degenerating cells/ST XS and positively correlated with the nuclear : ST area in RA-treated ram lambs, while NPVs are positively correlated with
the percentage of ST XS with spermatogonia as the most mature germ cell type and with circulating estradiol level in control ram lambs.

- After 2.5 weeks, testicular echotextural attributes are positively correlated with the percentage of ST XS with pre-spermatogonia I as the most mature germ cell type, the nuclear: ST area and serum testosterone and estradiol concentration, and negatively correlated with the percentage of ST XS with pre-spermatogonia II as the most mature germ cell type in RA-treated ram lambs, while testicular echotextural attributes are negatively correlated with the percentage of ST XS with pre-spermatogonia I as the most mature germ cell type and with the nuclear : ST area, and positively correlated with the percentage of ST XS with spermatogonia and spermatocytes as the most mature germ cell type, tubular and luminal ST area, the total number of cells/ST XS, the number of degenerating cells/ST XS and peripheral follicle-stimulating hormone concentrations in control ram lambs.

Most of the proposed research hypotheses turned out to be correct, with a few exceptions. In Chapter 3, the scrotal and testicular integument was shown to affect testicular echotexture; however, blood flow and the presence of blood did not. This could be explained by the relatively high density of the scrotum and *tunica vaginalis* compared to blood, which is only slightly more dense than water (Kremkau, 2006), as well as the abundant collagen content of the different layers of fascia in the scrotum, which have great elasticity, a property that has previously been postulated to largely account for tissue echotexture (Fields and Dunn, 1973). Additionally, the strength of the relationship that was observed between NPVs and tubular and luminal ST area in Chapter 3 did not differ between testes with or without the scrotum or *tunica vaginalis*, or when
the blood flow was restricted or blood was drained from the testes, indicating its level of
stability. In Chapter 5, RA exposure appeared to affect only testicular histomorphology and
echotexture but not endocrine status, in accordance with its role as a local hormonal signal
(Ghyselinck et al., 2006), and suggests that RA and the hypothalamo-pituitary-gonadal axis act
via separate signalling systems that typically operate in concert to bring about the initiation of
spermatogenesis, but may become disengaged when RA signalling is modified, at least
temporarily.

All of the studies performed may be followed up with future studies to confirm or expand
the present research findings in various mammalian species. In Chapter 3, a number of potential
confounding variables were identified. Specifically, contractions of the *tunica albuginea*
throughout the castration process and vascularisation of the *tunica vasculosa* may have precluded
the relationship between NPVs and especially PSD with the tubular and luminal ST area. The
first assumption could be tested by repeating the experiment and measuring action potentials
through electrodes placed directly on the *tunica albuginea* (Shafik, 1998), or could be performed
by administering adrenergic (*e.g.*, noradrenaline, NA) or purinergic (*e.g.*, adenosine 5’-
triphosphate, ATP) stimuli to induce contractions of the *tunica albuginea* (Banks et al., 2006),
and measuring the action potentials by electrodes placed on the scrotal skin (Shafik et al., 2005),
while scanning the testis and then obtaining a tissue sample through testicular biopsy or
castration. The second assumption could be tested by scanning the testis using Doppler
ultrasound, which is capable of detecting the presence, direction and speed of blood flow
(Kremkau, 2006), to compare testicular echotextural attributes of selected vascularised and non-
vascularised areas of interest on the scrotal ultrasonogram, as is possible with Colour Doppler or
colour flow imaging, which overlay hemodynamic information on top of the gray-scale image (Hoskins, 2010) by a manually controlled process.

By design, the findings reported in Chapter 4 as a correlational study are well-suited to further hypothesis testing. The correlations among testicular echotextural attributes and tubular ST diameter, and their switch in direction from positive during the mitotic phase of spermatogenesis to negative during the post-mitotic phase are particularly intriguing. The histomorphological analysis of cellular and sub-cellular components of the germ cell population and the seminiferous epithelial population as a whole did not provide an adequate explanation for this occurrence. However, direct microscopic analysis of Sertoli cell histomorphology may reveal an association with testicular echotexture that more closely resembles that of tubular ST diameter during the transition from the mitotic to post-mitotic phases of spermatogenesis, considering the remarkable plasticity of this cell type during the seminiferous epithelial cycle (Wrobel et al., 1995). For example, during stage 3, when primary spermatocytes reach the final diplotene stage, Sertoli cells reach their highest percentage and absolute mitochondrial volume (Wrobel et al., 1995), an organelle which has been postulated to cause a considerable amount of ultrasound scattering due to the high density of membranes and proteins (Baddour et al., 2005). Furthermore, at stage 4 of the seminiferous epithelial cycle, meiosis I and II are completed and Sertoli cells reach their highest absolute nuclear volume (Wrobel et al., 1995), which would be expected to result in a positive association with testicular echotexture. Additionally, increasing Sertoli cell differentiation throughout prepubertal development and during rising testosterone secretion induces a number of morphological changes (Sharpe et al., 2003; Rey et al., 2009), including increased cytoplasmic volume (Gondos and Berndston, 1993; Steger and Wrobel, 1996), which would be expected to reduce testicular echogenicity due to the anticipated increase
in the relative density of cytoplasmic matrix (typically comprising ~75% of Sertoli cell volume in adults; Wrobel et al., 1995), with its low organelle content (Wrobel et al., 1995). If testicular echotextural attributes are lower during other stages of the seminiferous epithelial cycle (i.e., stages 1, 2, 5-8) and become increasingly lower with Sertoli cell maturation during pubertal onset, this would imply that Sertoli cell histomorphology is a major determinant of testicular echotexture. Considering the high volume percentage of Sertoli cells in the adult ovine seminiferous epithelium (27–36%; Wrobel et al., 1995), more than most primary spermatocytes (5–29%; Wrobel et al., 1995) and comparable with round spermatids (34–40%; Wrobel et al., 1995), this theory has plausibility. It would also explain why the correlation between testicular echogenicity and ST size is negatively correlated in postpubertal yearling bulls (Gábor et al., 1998; Brito et al., 2012). No histomorphological analysis of Sertoli cells was carried out in the experiments conducted due to the lack of immunohistochemical markers for Sertoli cells in prepubescent ram lambs (Oluwole et al., 2013); however, identification based on morphological features would likely be effective in determining the overall echotextural characteristics of this cell population, as they have distinct cellular characteristics, which become increasing apparent throughout pubertal maturation (Sharpe et al., 2003).

The consistency of the correlation reported in Chapter 4 among testicular echotextural attributes and the number of degenerating cells/ST XS throughout both the mitotic and post-mitotic phases of spermatogenesis is also remarkable. This suggests that apoptosis is a key determinant of testicular echotexture, in agreement with previous reports that the nucleus and nuclear configuration dominate the scattering of ultrasound in cell ensembles (Taggart et al., 2010; Czarnota and Kolios, 2010). Further investigation of how different chromatin arrangements throughout spermatogenesis (e.g., mitosis, meiosis, spermiogenesis) influence
testicular echotexture may reveal other strong histomorphological associations. The correlations among testicular echotextural attributes and the total number of UCHL-1+ cells, including dark- and medium- staining subpopulations, were also fairly consistent between the mitotic and post-mitotic stages. This was surprising as the number of UCHL-1+ cells is so low in comparison with other germ cell types; spermatogonial stem cells (SSCs), which formed at least a portion of the UCHL-1+ cells, constitute only 0.03% of all spermatogenic cells in the adult mouse (Tegelenbosch and de Rooij, 1993). However, since SSCs are always present in the STs, regardless of the stage of the seminiferous epithelial cycle, this may explain their sizable contribution to testicular echotexture. Similarly, Leydig cells, while only constituting 1% of the testis (Setchell, 1991) may also be an important source of ultrasound scattering since they are always present throughout the entire testis. Furthermore, serum concentration of testosterone, the major hormone produced by Leydig cells, has been correlated with testicular echotextural attributes (Evans et al., 1996; Chapter 5). Therefore, future studies should address how changes in cell nuclear conformation during various homeostatic events (i.e., proliferation, differentiation, and apoptosis) affect testicular echotexture as well as the underlying echotextural properties of relatively stable, evenly distributed cell populations.

A third notable finding from Chapter 4 was the variability in correlations among testicular echotextural attributes and the percentages of ST XS with different germ cell types as the most mature, compared to the earlier study by Evans et al. (1996). While testicular echogenicity was negatively correlated with the percentages of ST XS with different germ cell types up to and including early spermatocytes, and positively correlated with the percentages of ST XS with late spermatocytes to spermatozoa in bull calves from birth to puberty (Evans et al., 1996), the direction of these correlations were opposite for ram lambs during the post-mitotic
phase of the first wave of spermatogenesis (Chapter 4), except for the positive correlation among testicular echotextural parameters and the percentage of ST XS with (late) spermatocytes. This indicates that while specific features of different germ cell types may affect testicular echotexture, it is necessary to take the developmental status of the testis into consideration. For example, testicular echotextural attributes and tubular ST diameter were positively correlated in the mitotic phase and negatively correlated in the post-mitotic phase, but had no correlation overall for the entire period of study. Without defining the phase of spermatogenic onset that a particular animal was in, no correlation would have been detected. The fact that the percentage of ST XS with spermatocytes, particularly late primary or secondary spermatocytes as the most mature germ cell type, retained a consistent positive correlation with testicular echotextural attributes throughout the first wave of spermatogenesis (Chapter 4) and until puberty (Evans et al., 1996), suggests that this cell type or developmental events associated with the first appearance of this cell type have a major influence on testicular echotexture. Due to the heterogeneity in the most advanced germ cell type present among ST XS within the same individual and the establishment of the seminiferous epithelial cycle during the first wave of spermatogenesis, the echotextural characteristics of specific seminiferous epithelial cycles or germ cells cannot be evaluated through a correlational study. Rather, an experimental approach is necessary, as was carried out in Chapter 5.

The experimental design of Chapter 5 was inspired by recent studies performed in mice in which RA exposure during pre-meiotic spermatogenic onset induced synchronization of the seminiferous epithelial cycle and elimination of the spermatogenic wave in adults (Snyder et al., 2011; Davis et al., 2013). While only encompassing spermatogenic onset in Chapter 5, a similar reduction in germ cell heterogeneity was observed when the experimental protocol was tested in
prepubescent ram lambs. Echotextural differences between RA-treated and control ram lambs were not observed until 2.5 weeks after the injections were given. It would be interesting to see how testicular echotexture would compare between control and treated groups throughout the remainder of the first wave of spermatogenesis and into postpubertal development or adulthood. The distribution of seminiferous epithelial stages throughout the ST XS of adult mice was relatively even, whereas in mice treated with RA as neonates, the distribution was markedly skewed, with small number of stages (Snyder et al., 2011; Davis et al., 2013). Therefore, the testicular echotextural properties of specific seminiferous epithelial stages could be acquired in future studies. Furthermore, if spermatogenesis could be manipulated using RA such that only one germ cell type (with the exception of SSCs) was present, or if different germ cell types were isolated using an in vitro procedure, the echotextural characteristics of specific germ cell types could be determined.

6.2 Future Research Applications

Computer-assisted image analysis of scrotal ultrasonograms is currently performed only for research purposes, with the goal of making this technique clinically applicable. Specifically, testicular echotextural analysis may be useful as an alternative to:

1. Testicular biopsy. Diagnostic testicular biopsies are generally only carried out in humans and companion animals to confirm or exclude the presence of a suspected pathology (Bergmann, 2006; McLachlan et al., 2007). A wealth of information may be obtained from testicular biopsies on tissue histomorphology and the expression of specific protein molecules. However, collecting testicular biopsies is an invasive procedure and could be
detrimental to testicular function especially for patients with severe spermatogenic impairment (Bergmann, 2006). Scrotal ultrasonography offers a non-invasive alternative to testicular biopsy as testicular echotextural attributes are associated with testicular histomorphology (Evans et al., 1996; Chapters 3–5). Furthermore, any regional differences in spermatogenesis throughout the testis are accommodated for by taking spot analyses across the entire surface area of the testicular parenchyma, whereas multiple testicular biopsy samples are recommended in clinical practice (Bergmann, 2006).

2. Semen evaluation. Semen evaluations are carried out in humans and animals, including farmed livestock species, to provide an indication of reproductive potential and identify sub- or infertile individuals. Collected samples are evaluated for various semen parameters, including viability, motility, concentration and morphology of the sperm (Tsakmakidis, 2010) and compared to standard values. However, semen evaluation is only representative of fertility at the time of assessment. It has no ability to predict future fertility due to the lengthy spermatogenic process (approximately 50 days in rams; Wrobel, 1998) and may vary according to ejaculation frequency in humans as there is minimal sperm storage in the epididymis (Sharpe, 2010). Furthermore, the electroejaculation procedure commonly used to collect semen in sheep raises ethical issues due to its stressful and painful nature (Damián and Ungerfeld, 2011). By contrast, testicular echotextural attributes are representative of semen quality several weeks in advance (Arteaga et al., 2005; Ahmadi et al., 2012; Brito et al., 2012), are not affected by sexual activity and are obtained non-invasively through scrotal ultrasonography.
3. Frequent blood sampling. Gonadotropin-releasing hormone (GnRH), the gonadotropins and testosterone are all secreted in an episodic manner (Wilson and Lapwood, 1979; Yarney and Sanford, 1989; Chandolia et al., 1997a); luteinizing hormone (LH) is particularly pulsatile likely due to its short half-life (Bliss et al., 2010). Therefore, frequent blood sampling needs to be performed to obtain an accurate hormonal profile. However, total blood volume loss needs to be carefully taken into account, in accordance with established guidelines (Landskroner et al., 2011). Significant correlations among testicular echotextural attributes and serum FSH (Chapter 5), testosterone (Evans et al., 1996; Chapter 5) and estradiol (Chapter 5) concentrations have been demonstrated, likely due to hormone-induced protein secretion or changes in cellular organelle dimensions. Therefore, scrotal ultrasonography may have the potential to non-invasively assess reproductive hormone levels.

Additionally, testicular echotextural analysis of scrotal ultrasonograms may hold potential as a:

4. Novel monitoring device. Unlike the invasive and/or stressful and painful nature of the above approaches to assessing testicular function, scrotal ultrasonography may be routinely performed with minimal bioeffects or safety concerns (Merritt et al., 1992; Lieu, 2010). Therefore, it is suitable to be used in cases where regular monitoring would be beneficial, but not typically performed using conventional methods due to the associated risks. For example, scrotal ultrasonography could be used to monitor testes in patients receiving gonadotoxic cancer therapy to assess and possibly alter the treatment course or treat any emerging conditions before the development of major reproductive health problems. Similarly, it could also be used to monitor spermatogonial stem cell
transplantation to intervene where necessary or fine tune the procedure for the best possible outcome. Specifically in farmed livestock species, it could be used as a screening tool for selection of optimal breeding stock prior to the breeding season or even as early as during prepubertal development, since earlier-maturing individuals with higher circulating LH and/or testosterone levels tend to have higher fertility at and beyond puberty (Yarney and Sanford, 1990; Yarney et al., 1990; Evans et al., 1995; Aravindakshan et al., 2000).

In time, with future studies, these potential applications for scrotal ultrasonography may well become realities. An automated system to perform echotextural analysis would greatly enhance the speed and ease of obtaining measurements, which increasing digitalization of the technology may allow (Schlegel, 2007). Computerized image analysis of scrotal ultrasonograms holds promise as a non-invasive diagnostic and prognostic tool and novel monitoring device of testicular microstructure and endocrine status, and has the potential to become integrated into a number of reproductive health evaluation protocols.
LIST OF PUBLICATIONS


de Rooij DG, Russell LD. (2000). All you wanted to know about spermatogonia but were afraid to ask. *Journal of Andrology, 21*(6), 776-798.


Lal BK, Hobson II RW, Pappas PJ, Kubicka R, Hameed M, Chakhtura EY, Jamil Z, Padberg


