CHANGES IN GENE EXPRESSION OF GOAT DEVELOPING TESTES AND SPERM DURING BREEDING AND NON-BREEDING SEASON

A Dissertation

by

AZURE NICOLE FAUCETTE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2012

Major Subject: Physiology of Reproduction

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Approved by:

Chair of Advisory Committee,	David W. Forrest
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ABSTRACT

Changes in Gene Expression of Goat Developing Testes and Sperm During Breeding and Non-breeding Season. (May 2012)

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Chair of Advisory Committee: Dr. David W. Forrest

Testicular function is fundamental to male fertility, since testicular cells act in collaboration with each other to signal sex differentiation, the initiation of puberty and spermatogenesis. Complications that can be influenced by many factors will affect sperm number, morphology, motility, chromatin quality and acrosomal integrity. The purpose of these studies was to analyze the changes in gene expression in the developing testes and analyze the seasonal changes in gene products in sperm of mature bucks. In the first experiment, testes were harvested from five Alpine bucks at 0, 2, 4, 6, and 8 months of age. Northern and in situ hybridization indicated that the largest change in gene expression occurred during the first 4 months of goat testes development. Sex determining region Y-box 9 (SOX9) and Heat Shock protein A8 (HSPA8) peaked at 2 months of age, and were expressed in Sertoli cells and spermatogonium, respectively. At 4 months, expression of Stimulated by Retinoic Acid gene 8 (STRA8), Protamine1 (PRM1) and Outer Dense Fiber protein 2 (ODF2) was strongly up-regulated in early and maturing germ cells, respectively. In the second experiment, RNA from ejaculated spermatozoa collected from mature Alpine bucks in peak (October) and non-peak (April) breeding season were analyzed on a 4 x 44K Agilent bovine microarray. One thousand

three hundred and eighteen gene products were differentially expressed 2-fold or more ($p \leq 0.05$) in mature goat sperm collected October and April. To eliminate the likelihood of false positives, the cut off was set to fold change of 3 or more at $p \leq 0.01$ which narrowed the list of genes to 50 transcripts. Real time PCR results confirmed the expression of Sperm Adhesion Molecule 1 (SPAM1) in April, and the expression of Glycerol kinase 2(GK2) and Myc Binding Protein 2 (MYCBP2) in October. Based on the results from both experiments, it can be concluded that: SOX9 and HSPA8 expression play an important role in tubular formation and germ cell maintenance; two months after SOX9 and HSPA8 expression, genes that are associated with spermatogenesis initiation and completion are upregulated; and validation of the seasonal changes in sperm mRNA levels may provide additional insight to testicular events as they relate to breeding and non-breeding season.

DEDICATION

To my mother, Belva A. Spann.

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I would like to thank my committee chair, Dr. David W. Forrest, for his guidance, patience and support throughout the course of my research. I would like to thank my committee members, Drs. Clay A. Cavinder, Nancy H. Ing, Gary R. Newton and Louis C. Nuti, for their assistance, advice and patience throughout my doctoral program.

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NOMENCLATURE

BSE	Breeding Soundness Exam
cDNA	Complementary Deoxyribonucleic Acid
cRNA	Complementary Ribonucleic Acid
ER-Sense	Estrogen Receptor Sense
EtOH	Ethanol
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GK2	Glycerol Kinase 2
HSPA8	Heat Shock Protein A8
IACUC	Institutional Animal Care and Use Committee
mRNA	Messenger RNA
MYCBP2	Myc-Binding Protein 2
NaCit	Sodium Citrate
NRR	Non-return rates
ODF2	Outer Dense Fiber 2
PAF	Paraformaldehyde
PRM1	Protamine 1
qPCR	Quantitative Polymerase Chain Reaction
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SOX9	SRY-like HMG box gene 9
SPAM1	Sperm Adhesion Molecule 1

STRA8	Stimulated by Retinoic Acid gene 8
SRY	Sex Determining Region on the Y-chromosome
WTA2	Whole Transcriptome Amplification Kit 2

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CHAPTER I

INTRODUCTION

Semen evaluation is an important component when estimating the potential capacity of a male for breeding. In the livestock industry, fertile males are an investment that impact the revenue generated by a herd. In order to access a male's breeding potential, producers use the Breeding Soundness Exam (BSE), which is used to make an assessment of testicular/epididymal function, help to identify clear cut cases of sub- and infertility (Rodriguez-Martinez, 2006). Results from the BSE will group males in 3 categories: satisfactory, unsatisfactory or questionable/undetermined. One of the main components of the evaluation is the quality of the semen, which includes normal morphology, progressive motility and absence of foreign cells such as immune or epithelial cells (Ott and Memon, 1979; Baker, 1980; Hafez and Hafez, 2000; Rodriguez-Martinez, 2006). A mature Alpine buck would need the scrotal circumference to be 34-35 cm, an ejaculate that has 1.5 ml semen volume with a concentration of 2-5 billion/sperm ml, greater than 50% progressive motility, and less than 10 % abnormal sperm in order to pass a goat BSE (Ott and Memon, 1979; Baker, 1980; Hafez and Hafez, 2000). Although the BSE has proven to be a useful tool for predicting the breeding potential of a male with unknown fertility, results are too general because sperm can undergo other changes both pre-and post-spermatogenesis that may impact fertility.

This dissertation follows the style of Journal of Animal Science.

In order to achieve a more accurate estimate of male fertility, more sensitive assays could evaluate mitochondrial, and membrane and DNA integrity, which will provide information about sperm motility and chromatin compaction, respectively. Assay that access mitochondrial integrity are variable (Rodriguez-Martinez, 2006), and assisted reproductive technologies techniques have been used to overcome problems with sperm motility. In regards to plasma membrane and DNA integrity, it is harder to overcome. There are several protocols that are used to evaluate the sperm plasma membrane which include utilizing dyes and fluorescence to determine the percentage of live/dead cells or whether or not the sperm have undergone capacitation, and proportion of cells outside the general population (COMPαt) or DNA fragment index (DFI) quantifies the percentage of spermatozoa with denatured DNA (Rodriguez-Martinez, 2003; Rodriguez-Martinez, 2006). Even with all these advances with the assessment of fertility, there are still unanswered questions.

In the livestock industry, the ability to produce offspring is essential for the continuous production of milk, fiber and meat (Shelton, 1979). The number of goat operations in the U.S. as of 2010 remains unchanged, but the total number of goats has decreased by 1% (NASS, 2010 a, b). In order to increase herd production, more detailed information concerning the basic biology of goat reproduction is needed. So, the working hypothesis of the current study is that genes involved in spermatogenesis may be a useful predictor for male fertility. Experiments were designed to achieve the following two objectives: 1) describe the histomorphology of the developing buck testis and time the expression of genes associated with tubule formation; and 2) characterize

specific mRNAs in the buck ejaculate that can be linked to sperm quality, and may be useful as potential markers for fertility.

CHAPTER II

LITERATURE REVIEW

Goat Reproduction: Overview

Reproduction plays a vital role in livestock production. Shelton (1978) noted that in livestock production, milk, fiber and meat rely on the ability of an animal to reproduce. This influences the current and future number of replacement animals for the herd. Between 1985 and 2006, U.S. goat population has doubled, which has been attributed to the change in demographic (Solaiman, 2007). However in the past 5 years, U.S. goat population has dropped 1% (NASS, 2011b); therefore, in order to increase herd production, one would have to understand the basic biology behind goat reproduction.

Overall, goats are among the most fertile and prolific domesticated animals with conception rates ranging from 90-98% (Bliss et al., 1992; Holtz, 2005). The average size of a litter is 1.5 kids, and may vary between breeds, season and environmental conditions. Comparing the domesticated ruminants, goats and sheep are more reproductively comparable than cattle due to their size, reproductive anatomy and physiology (Hafez and Hafez, 2000; Ensminger and Parker, 2002). Does reach puberty around 7 to 10 months of age (breed specific) or at an optimal weight of 80% of mature wt, however, bucks reach puberty at around 4 to 6 months of age (Bliss, 1992; Hafez and Hafez, 2000). Goats reared in temperate areas are more seasonal than those closer to the equator; however, this statement applies more to the doe than the buck because sperm

production and testicular size as well as libido decline rather than cease during the non-breeding season.

Spermatogenesis

Spermatogenesis is the process in which an immature germ cell undergoes mitosis, meiosis and differentiation to ultimately end up as an elongated spermatid. This process occurs within the seminiferous tubule of the testes (O'Donnell et al. 2001, Senger, 2003). In the seminiferous tubules, spermatogonia are in close contact with Sertoli cells, which separates them from the lumen of the testis via tight junctions forming the blood-testis barrier. Spermatogonia are classified as either A, A_2, A_3, A_4, I or B. Spermatogonia A must undergo numerous mitotic divisions that enable them to replenish germ cell population and produce spermatogonia B, which are recruited and transported adluminally to become a 1º spermatocyte (Senger, 2003). The primary spermatocytes begin the stages of meiosis. During prophase of the first meiotic division, primary spermatocytes experience nuclear changes. Secondary spermatocytes are yielded after the first meiotic division and are difficult to find histologically due to the rapid recruitment that results in a haploid round spermatid (O'Donnell et al. 2001; Senger, 2003). Round spermatids undergo a cascade of steps to eventually mature into elongated spermatids. This differentiation process is known as spermiogenesis. It involves the formation and development of the acrosome and flagellum, replacement of histones by protamines, reshaping and elongation of the nucleus and incomplete removal of the cytoplasm. At this point, the elongated spermatid is no longer transcriptionally functional.

The elongated spermatid with a cytoplasmic droplet is released from the Sertoli cell into the tubulus contortus. Ultimately, spermatozoa end up in the epididymus. The epididymus has multiple functions: 1) absorption of retes fluid, 2) sperm transport, 3) elimination of defective gametes, and 4) sperm maturation and storage (Senger, 2003, Sullivan et al., 2005). The epididymus is divided into 3 segments: caput, corpus and caudal. Transport through these sections is critical for maturation of spermatozoa. Retes fluid is absorbed in the caput segment where non-motile, non-fertile spermatozoa with low disulfide links are found. In the corpus segment, spermatozoa begin to show signs of motility and fertility (oocyte binding capability) and moderate to high degrees of disulfide links. The caudal segment is very important for storage and final maturation of sperm; in addition, spermatozoa gain fertilizing capability, showing high degrees of disulfide linkage. During transit, the cytoplasmic droplets begin to move from the proximal region to mid-tail and are ultimately absorbed by the caudal epididymus.

Testicular Development

In mammals, the initial steps of testis development have been linked to the activity of a gene located on the Y-chromosome (Jacobe and Strong, 1959; Ford et al., 1959; Welsons and Russell, 1959). This gene was initially known as the testes determining factor/gene (TDY), but was later renamed the sex determining region on the Y-chromosome (SRY). SRY is expressed in supporting cell precursors in the genital ridge and triggers differentiation along the Sertoli cell pathway (Lovell-Badge and Robertson, 1990; Cupp and Skinner, 2005). Lovell-Badge and Robertson (1990) used chimeric mice to look for mutations that would alter the sex determining pathway. They

reported that mice lacking *Sry* gene followed the female pathway of sex differentiation; conversely, XX mice that carried the *Sry* gene developed male genitalia. It was thought that in the mouse the *Sry* gene was expressed throughout development, from somatic cell differentiation to adult germ cells in the testes. This theory was hard to prove since after the initial SRY expression there is a sharp decline until SRY is no longer expressed (Hacker et al., 1995), which lead to the hypothesis that the Sry gene triggers other genes in the Sertoli cell but is not required for maintenance of testes differentiation.

One of the genes found downstream of SRY is SRY-like HMG (High Mobility Group) box-containing gene-9 (SOX9). The SOX9 gene is responsible for campomelic dysplasia (CD) in humans. Patients with CD are characterized by having both skeletal malformations and gonadal sex reversal, because of which the latter suggests that the gene plays a critical role in testes formation (Foster et al., 1994; Wagner et al.; 1994). The expression of SOX9 is upregulated shortly after SRY expression and it coincides with Sertoli cell development (Marshal and Harley, 2000). The role of SRY in sex differentiation is only linked to mammalian testes development; however, SOX9 is a major sex determining factor in reptilian, amphibian and avian species (Takase et al., 2000; Pask et al., 2002; Swain and Lovell-Badger, 1999).

Along with Steroidogenic factor-1 (SF-1) and Wilms tumor gene-1(WT-1), SOX9 activates Anti-Müllerian hormone (AMH) gene in the Sertoli cell (Marshal and Harley, 2000). The AMH is a transforming growth factor-β like glycoprotein hormone that causes regression of the Müllerian ducts. DeSanta Barbara et al. (1998) demonstrated that SOX9, through the Wnt canonical pathway, binds to the AMH

promoter and SF-1 and WT-1 synergizes AMH activation. However, SOX9 activates genes other than AMH for male sex differentiation because *Amh* knockouts (KO) have a psuedohermaphrodite phenotype (Marshall and Harley, 2000). In addition to Müllerian duct regression, *Sox9* signals extracellular matrix protein formation. Barrionuevo and Scherer (2010) reported that the ablation of *Sox9* at embryonic day 14 (E14) leads to late onset of sterility at 5 months in mice that is characterized by the down regulation of desert hedge-hog (*Dhh*) and *Amh*. The incomplete *Sox9* KO, with *Sox8* $^{-/-}$, caused degeneration during seminiferous tubule formation (Chaboisser et al., 2004; Barrionueveo et al., 2009). These studies show that SOX9, along with other genes, is necessary for the initiation of testes development and its continuous expression is needed for proper seminiferous tubule formation.

Genes Important in Testicular Development and Spermatogenesis

Heat Shock Protein A8 (HSPA8)

Heat shock proteins (HSP) are a specific set of highly conserved proteins that are produced by both prokaryotic and eukaryotic cells in response to environmental and physiological stressors. These proteins are classified into families according to their molecular weight that include HSP100, HSP90, HSP70, HSP60, and HSP27. Past research on the protective ability of the HSP, have focused on their chaperoning of the facilitation of protein folding and assembly, and stabilizer of damaged proteins (Matwee et al., 2001). However, Beere (2004) focused on the anti-apoptotic role HSP and discovered that their mechanism of action is not consistent with their chaperoning ability.

The HSP70 family of proteins consists of at least 8 different members that are ATP-dependent molecular chaperones (Eddy, 1999; Powers et al., 2008; Tzankov et al., 2008). Two members of the HSP70 proteins are products of genes expressed specifically during spermatogenesis: HSP70-2 (meiosis specific) and HSPA8 (post meiotic; Dix et al., 1996; Eddy, 1999). The HSPA8 gene has been linked to cancer (humans; Powers et al., 2008; Nirdé et al., 2010), coronary heart disease (human; He at al., 2010) and fertility (rodents; Krawczyl et al., 1988; Maekawa et al., 1989). Despite what the name suggest, HSPA8 is not a heat inducible HSP which suggests that HSPA8 may have a role is preventing apoptosis during spermatogenesis. Krawczyl et al. (1988) observed high levels of HspA8 during stages associated with the late pachytene spermatocytes, and decreased in stages associated with spermatid elongation.

Stimulated by Retinoic Acid Gene 8 (STRA8)

Vitamins, as cofactors, are a critical component of metabolic pathways. Vitamin A is a fat soluble vitamin that is not readily synthesized by goats, and deficiencies result in respiratory, digestive and reproductive abnormalities (Hart, 2008). Although there are no studies which look at deficiency of vitamin A and retinoid (inactive metabolite) intake on the development of the goat testis, there have been multiple studies using mice and rats as a model for deficiency and excess of vitamin A effect on reproductive function.

The active metabolite of vitamin A, retinoic acid (RA), plays a vital role in normal fetal development, pattern formation, cell proliferation and differentiation, and apoptosis (Zhou et al.; 2008). In the testis, RA operates through the nuclear retinoic acid

receptors (RARs) that can be found in the 3 cell types of the testis (Sertoli, pre-elongated spermatid and Leydig cells; Livera et al., 2002). Deficiency in RA has been characterized by degenerated germ cells because of the inability of RA to signal mitosis and apoptosis via RARα, which can be seen in RARα KO mice (Akmal et al, 1997; Boulogne et al., 1999; Dufour et al., 1999; Vernet et al., 2006). Conversely, this can be recovered by giving vitamin A or retinol injections (Thompson et al, 1964) and high doses of RA (Van Pelt and de Rooij, 1991).

The sex specific timing of meiotic initiation through STRA8 (stimulated by retinoic acid gene 8) is regulated by RA. Oulad-Abdelghani et al. (1996) isolated *Stra8* in P19 embryonal carcinoma cells which is induced by all *trans* and *9-cis* retinoic acid. They also observed that during mouse embryogenesis *Stra8* is restricted in the male gamete but later it is restricted to the pre-meiotic germ cells in the adults. Zhou et al. (2008) noted that peak expression of *Stra8* is at the onset of meiosis with the highest levels of both mRNA and protein in preleptotene/early leptotene spermatocytes. The increase in RA accelerates *Stra8* protein expression in preleptotene spermatocytes with 5-bromo-2-deoxyuridine incorporation, thus indicating synchrony in premeiotic DNA replication. Mice with a *Stra8*-null mutation undergo premeiotic DNA replication, display cytological features for the initiation of recombination and express numerous meiotic genes; however, there is a prolonged asynapsis and heterosynapsis that are important for chromosomal pairing (Mark et al., 2008). Therefore, the presence of STRA8 is important for the cellular preparation and DNA stability to commit spermatocytes to commence meiosis.

Protamine-1 (PRM1)

Round spermatids undergo a cascade of steps to eventually mature into elongated spermatids. One of the major changes is the exchange of histones with protamines. Protamines are small, positively charged proteins that condense sperm chromatin by forming disulfide bonds between the protamine's cysteine residues (Carrell and Liu, 2001). Histones are easily hyperacetylated and ubiquinated, which both aid in histone replacement with protamine (Dadoune, 2003; Carrell et al., 2007). Protamines are the last to appear in the mature spermatid nuclei. They provide a torus shape structure, which allows for a more dynamic nucleus and protection against nucleases (Brewer et al., 1999). The affect of protamines on fertility have been widely studied in human on both the protein and mRNA level. The most common problem occurs when there is insufficient protamine 1 (PRM1) and protamine 2 (PRM2) levels. Aoki et al. (2006b) suggested that abnormal protein synthesis is associated with defects in the translational regulation of protamine caused by mRNA retention; thus, causing an abnormal ratio of PRM1/PRM2. This ratio varies among species; however in most mammals, the ideal ratio equals 1 (Carrell and Liu, 2001). Infertility in some men has been attributed to a diminished level of PRM2. Abnormal PRM2 production significantly affects oocyte penetration rates, morphology and progressive motility (Carrell and Liu, 2001; Aoki et al., 2006a). A review by Oliva (2006) indicated that mouse models designed to express the *Prm1* gene prematurely or in excess resulted in early condensation of chromatin, abnormal morphology and the incomplete processing of *Prm2* resulting in histone retention. Zhang et al. (2006) reported that infertile men have a significantly higher

proportion of histone H2B to protamine than fertile men suggesting that histone retention is common among infertile men. In the bull, PRM2 is transcribed and translated in low levels; moreover, there are numerous point mutations which reduce the affinity of PRM2 to DNA (Maier et al., 1990). Therefore one can infer that in bovine, PRM2 is functionally deficient resulting in bovine PRM1 being more prevalent due to the amount of arginine residues allowing for a higher affinity to DNA (Brewer et al., 2003).

Outer Dense Fiber Protein 2 (ODF2)

After the DNA condensation occurs, the round spermatid continues to differentiate even more by a process known as spermiogenesis (previously described). Formation of the flagellum involves migration of the centrioles. The centrioles serve as a nucleation site for development of the axonemal microtubules which comprise the central core of the sperm flagellum (Xu et al., 2008). There is a recruitment of flagella/cilliary assembly proteins by the centrioles. The prominent component of the sperm tail is the outer dense fibers (ODF), which is unique to spermatozoa (Hoyer-Fender et al., 1998; Salmon et al. 2006; Hüber et al., 2008). Highly conserved among species of vertebrate and invertebrates, ODF2 protein is composed of ~560 amino acids with 2 leucine zipper motifs that interact heterozygously with ODF-1 or homozygously with another ODF2 protein (Donker et al., 2004). ODF2 was first described as the major protein in the mammalian sperm tail (Hoyer-Fender et al., 1998). Though it is the major protein, it is not directly associated with motility, but is involved in the elasticity and stability of the sperm tail (Salmon et al., 2006). Haidl et al. (1991) implicated that tail

abnormalities in sperm of teratozoospermic men are associated with abnormal development of ODF2. In the developing germ cell, the ODF2 transcripts have been isolated in the postmeiotic spermatids (Hoyer-Fender et al., 1998; Horowitz et al., 2005). Ing et al. (2004) found ODF2 transcripts were highly expressed in the "light" or spermatogenic testicular tissue colt. When there is a depletion of ODF2, mother centrioles lack their appendages and are unable to generate primary cilia (Ishikawa et al., 2005). Low expression of the ODF2 gene in adult tissue suggests that ODF2 is a widespread component of the centrosome (Nakagawa et al., 2001). Salmon et al. (2006) attempted a homozygous knockouts (KO) murine model for *Odf2*, but they were unable to recover any pups (homozygous lethal). These results suggest that ODF2 is essential for early embryo development.

Tests for Fertility

Semen evaluation is an important component when estimating the potential capacity of a male for breeding. In livestock production, diagnostic tools, such as the BSE, are used to make an assessment of testicular/epididymal function, help to identify clear cut cases of sub- and infertility, and in the case of AI or IVF, the degree of normalcy before processing (Rodriguez-Martinez, 2006). The main component of the evaluation is the quality of the semen, which includes normal morphology, progressive motility and absence of foreign cells such as immune or epithelial cells (Ott and Memon, 1979; Baker, 1980; Hafez and Hafez, 2000; Rodriguez- Martinez, 2006). Based on the BSE results, the males will be categorized as satisfactory, unsatisfactory or questionable/undetermined. In the case of the mature Alpine buck, it is ideal for the

scrotal circumference to be 34-35 cm, 1.5 ml volume with a concentration of 2-5 billion/ml, >50% progressive motility, and less than 10 % abnormal sperm (Ott and Memon, 1979; Baker, 1980; Hafez and Hafez, 2000). By setting the threshold for 'fertile' males, the BSE has proven to be a useful tool for predicting the breeding potential of a male with unknown fertility. Despite its usefulness, the BSE evaluation is too general because sperm can undergo other changes both pre-and post spermatogenesis that may impact fertility.

In order to get a more accurate estimate of male fertility, more sensitive assays would evaluate mitochondrial, membrane and DNA integrity. Mitochondria function and integrity of spermatozoa is essential for sperm motility; however, there is variability in establishing the relationship between the function and integrity of mitochondria (Rodriguez-Martinez, 2006). Artificial Reproductive Technologies (ART) techniques can be employed to overcome problems with sperm motility (i.e. Intracytoplasmic Sperm Injection or ICSI). Though motility is important, the stability of both plasma membrane and DNA integrity are harder to overcome. There are several protocols that are used to evaluate the sperm plasma membrane. The protocols utilize dyes and fluorescence to determine the percentage of live/dead cells, or whether or not the sperm has undergone capacitation. Normal sperm have highly condensed chromatin that is due to the association of DNA with protamine. The condensation of chromatin is essential to early embryonic development. The proportion of cells outside the general population (COMPαt) or DNA fragment index (DFI) quantifies the percentage of spermatozoa with denatured DNA (Rodriguez-Martinez, 2003; Rodriguez-Martinez, 2006).

Sperm mRNA

Amann and Hammerstedt (1993) proposed that there were multiple factors that influence male fertility. As stated before, the process of predicting male fertility in the field only covered obvious problems. Throughout the years, laboratory tests have been developed which are more sensitive in identifying sub- and infertile males. Even these tests/assays have not answered all the questions involving male fertility.

Pessot et al. (1989) stained rat testis section with RNAse-colloidal gold and revealed a high density of gold particles in the nucleus of elongated spermatid and fully differentiated testicular and epididymal spermatozoa. In addition, electrophoresis identified RNA molecules that ranged from tRNA to 5.8S and 5S rRNA. Since there was no labeling of the mitochondrial sheath, this ruled out that the RNA was not mitochondrial derived. Their findings initiated the ongoing hypothesis that the sperm contribute more than paternal DNA to embryo development.

Over the past decade, it has been hypothesized that male- derived transcripts are essential to characterizing male infertility. Since spermatozoa are transcriptionally inactive, the current working hypothesis is that the transcripts present are remnants of stored mRNAs from post meiotically active genes that can potentially give rise to proteins that are likely associated with sperm quality and important to the first steps of embryogenesis (Ostermeier et al., 2002; Ostermeier et al., 2004; Ostermeier et al., 2005; Martin and Krawetz, 2005; Carreau et al., 2007; Nanassy et al.; 2008). Specific mRNAs have been identified in human ejaculated sperm which have been linked with sperm quality and are important to embryonic development. Ostermeier et al. (2002), using

microarrays, was able to identify 2780 and 3281 cDNAs from single and pooled ejaculate. They concluded that the transcripts are left-over from spermatogenesis, and are important in classifying sperm health and important in embryogenesis (Ostermeier et al., 2002). Zhao et al. (2006) used serial analysis of gene expression (SAGE), and found 2459 and 2712 unique tags from individual and pooled sperm samples where 54 were novel to sperm health. Transcripts such as PRM1, Aromatase, nitric oxide synthase (eNOS and nNOS), PLC-zeta have been linked to infertile men with low sperm motility and non-obstructive azospermia (Lambard et al., 2004; Carreau et al., 2007; Lalancette et al. 2008a).

Because of the works done using mRNA as a potential non-invasive diagnostic tool, there has been interest in using this method to identify livestock sires with low non-return rates. Gilbert et al. (2007) looked at the difference between spermatid and spermatozoa transcript and found that they share over 900 mRNA transcripts. The same group noticed that fractionated sperm showed differences in genes associated with sperm motility (Bissonnette et al. 2009). Lalancette et al. (2008b) looked at the difference in transcripts found in bovine sperm that may be associated with non-return rate. They found the high fertile group had 29% transcripts associated with function (metabolism, and cell signaling) while the low fertile group only had 10% transcripts. In porcine, Yang et al. (2009) identified a broad spectrum of mRNA that correlated with transcript found in humans and bovine. To analyze the importance of select transcripts, Kempisty et al. (2008) found that sperm (porcine) introduced 3transcripts (clusterin, PRM1 and PRM2) to the oocytes which, upon fertilization, were present in the zygote and 2-cell

stage embryo. The use of mRNAs as potential markers for fertility appears feasible because studies suggest that sperm transport mRNAs that are necessary for successful fertilization (Kempisty et al.; 2008).

CHAPTER III

DIFFERENTIAL EXPRESSION OF GENES EXPRESSED IN THE CAPRINE TESTES DURING POSTNATAL DEVELOPMENT

Introduction

Testicular development is a complex process that requires the appropriate specification, proliferation and maturation of both testicular somatic and germ cells. Postnatal development marks the onset of seminiferous tubule formation and is characterized by mitotic division of the Sertoli and germ cells (Carmon and Green, 1952; Ren et al. 2009). In the ram (Carmon and Green, 1952) and buck (Nishimura et al., 2000), spermatocytes and other mature sperm cells become apparent around the ages of 3 and 4 months of age which corresponds with the onset of puberty.

Due to changes in the demographics of the U.S., the goat has become an animal of economical importance. Male reproductive literature is scarce, and the few articles available mainly relate to seminiferous tubule cycle (Bilaspuri and Guraya, 1984; França et al., 1999); changes in sperm nuclear proteins (Courten and Loir,1981); testis development and sexual behavior (Nishimura et al., 2000), and Sertoli cell efficiency (Leal at al., 2004). However, little is known about the genes involved in buck testicular development. Therefore, the objective of this study was to describe the histomorphology of the developing buck testis and time the expression of genes associated with tubule formation.

Materials and Methods

Animal and sample preparations

Twenty-five Alpine buck kids from the International Goat Research Center (Prairie View A&M University, Prairie View, TX) were castrated by a standard IACUC approved method. Castration occurred at 0, 2, 4, 6, and 8 months of age (n=5 each stage). Testes were separated from the epididymis and cut midsagittally. Sections from the right testis were minced, snap-frozen in liquid nitrogen and stored at -80˚C, and a 1 cm cube was placed in a 50-ml volume of 4% paraformaldehyde (PAF) fixative for 24 hr, washed in 70% ethanol (EtOH) and stored at 4˚C in 70% EtOH until paraffin embedding. Samples were used for northern blot analysis and in situ hybridization. All animal procedures were approved by the Prairie View A&M University Animal Care and Use Committee.

RNA isolation

Total RNA was isolated from testis samples collected from bucks, ages 0, 2, 4, 6 and 8 months, using Tripure Isolation Reagent (Roche, Mannheim, Germany) according to manufacturer's recommendations. To determine the concentration, 2 µl of total RNA was subjected, in duplicate, to a NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE).

Cloning of PRM1, STRA8, and SOX9 from goat testes

To make probes for protamine 1(PRM1), Stimulated by Retinoic Acid Gene 8 (STRA8) and SRY box containing 9 (SOX9) mRNA, 2 µg of mRNA from two bucks (ages 0 and 8 months) were reverse transcribed with Superscript II (Invitrogen, San

Diego, California) and random octomer primers. Two sets of primers were designed from *Bos taurus* cDNA sequences for PRM1, STRA8 and SOX9 (Genbank accession numbers BC108207, NM_182489, and AF278703, respectively, see table 1 for primers). In addition, the T7 promoter site was added to the second reverse primer. The second set of primers used for amplification was nested inside the first set. The generated cDNAs were cloned using the TA Cloning® Kit protocol (Invitrogen). Plasmids were purified using Qiagen Plasmid Purification kit (Qiagen technologies, Valencia, California). The goat PRM1, STRA8 and SOX9 cloned cDNAs were sequenced, and sequences were submitted to GenBank (accession numbers HM773246, HM773245 and HM773244, respectively).

Northern blot analysis

Total testes RNA (8µg) was denatured, subjected to electrophoresis on a 1.2% agarose gels and transferred to nitrocellulose membranes. RNA Millennium Markers (Ambion, Austin, Texas) were run alongside the samples. The blots were hybridized with radiolabeled anti-sense cRNA probes produced by in vitro transcription with [^{32}P]-UTP (3000 Ci/mmol; New England Nuclear, Boston, Massachusetts). The cDNA templates for PRM1, STRA8 and SOX9 probes were linearized with *Eco*R1 and amplified using the second set of PCR primers (table 1).The Outer Dense Fiber of Sperm Tail (ODF2), and Heat Shock Protein A8 (HSPA8) constructs were linearized as follows. Rat ODF2 plasmid (pBluescriptII vector; GenBank accession number U62821) was linearized with *Nco1* and transcribed with T7 RNA polymerase; and bovine HSPA8 (pBluescriptII vector; GenBank accession number NM_174345) was linearized *Sty1* and

transcribed with T3 RNA polymerase. After washing, hybridization signals were detected by exposing the blots to a PhosphoImager screen and visualized using a Typhoon 8600 variable mode imager (Molecular Dynamics, Piscataway, New Jersey).

In situ hybridization analysis

The mRNAs for PRM1, STRA8, SOX9, HSPA8, and ODF2 were localized on serial cross sections from the right testis of goat buck kids (ages 0, 2, 4, 6 and 8 months) by in situ hybridization. Tissue sections were hybridized with radiolabeled anti-sense or sense cRNA probes generated using in vitro transcription with [α-^{35}S] UTP (1250 Ci/mmol, New England Nuclear). After hybridization, washing and RNAse A digestion, slides were coated with photographic emulsion (Eastman Kodak, Rochester, New York) and developed after 1.5-6 weeks at 4ºCwith Kodak D-19 developer. Sections were counterstained with Harris hematoxylin (Sigma-Aldrich, St. Louis, Missouri), dehydrated and protected with coverslips. Digital photomicrographs of in situ hybridization were evaluated using a Nikon Eclipse 80i microscope (Nikon Instruments, Inc., Melville, New York), which was interfaced with a Nikon digital camera and Nikon Software.

Statistics

Data were analyzed as a complete random design, using the ANOVA procedure in SAS (SAS Institute, Inc., Cary NC.). Quantitative data were reported in case of significant differences ($p < 0.05$) in gene expression between the age groups, Tukey's was applied.

Table1. Primers designed from *Bos taurus* cDNA.

Gene	Forward Primer	Reverse Primer	Accession #†
PRM1	[a]5'- GCTGGCTCAACCAAGGCGG	5'- GGCATTGTTCGTTAGCAGGCTCC	BC108207
	[b]5'- CCTGCTCTGAGCATCCAGGC	5'-**TAATACGACTCACTATAGGG**TGGCATGTTCAAGATGTGGCA	
STRA8	[a]5'- AGGAGCTGGAGCAGCGGGT	5'- TCCTCAGGGGTGGAGATCAG	NM_182489
	[b]5'- GCTCTTCGGCAACCTCAGGAA	5'-**TAATACGACTCACTATAGGG**TGTCAACCCCGCTGTCCTTCA	
SOX9	[a]5'-GACCGACGAGCAGGAGAAGGG	5'- TCTCGCTCTCGTTCAGCAGTCTCCA	AF278703
	[b]5'- CGGCTCCGACACCGAGAACAC	5'-**TAATACGACTCACTATAGGG**AGCTTGCCCAGAGTCTTGCTGAGCTC	

†Bos taurus accession numbers, T7 promoter sequence in bold, [a, b] Corresponds to the outer and inner primer sequence, respectively.

Results

Development of the goat testis

The mean paired testis wt by buck age is graphically shown in Figure 1. Though there was a change in testes weight over time, mean paired testis weight did not differ between 0 and 2 months of age ($p > 0.05$). After 2 months, there was rapid increase ($p < 0.05$) in paired testicular weight. There was no difference between paired testis weight of 4 and 8 month old buck kids; however, 6 month old buck kids had significantly ($p < 0.05$) heavier paired testis weights.

In relation to age, the histological structures of the testis were noticeably different (Figure 2). At 0 month of age, seminiferous tubules were small in diameter, contained no lumen and contained a single layer of Sertoli and germ cell nuclei. Another cell type observed in the seminiferous tubules was a testicular macrophage. Some macrophages appear larger than others suggesting that they are actively removing damaged cells from the seminiferous tubules. The 2 month old seminiferous tubules are larger because of the increase in Sertoli and germ cell number. During the ages of 4, 6 and 8 months, fully formed seminiferous tubules are present. This was characterized by the presence of spermatocytes, round and elongated spermatids and spermatozoa in the lumen.

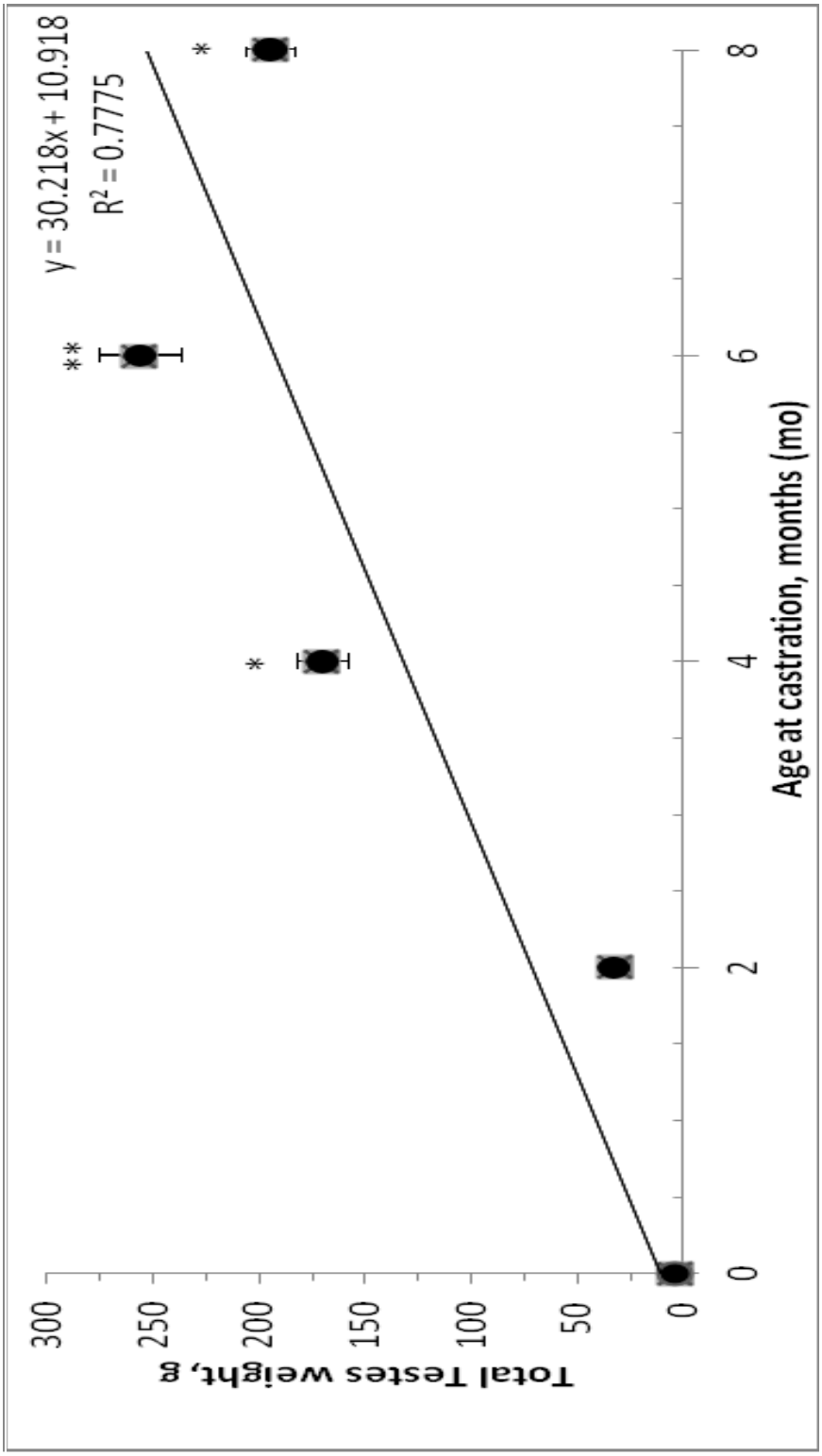

Figure 1. Mean total testes weight (g) of goat kids castrated at 0, 2, 4, 6, and 8 months of age. * or ** $P<0.05$.

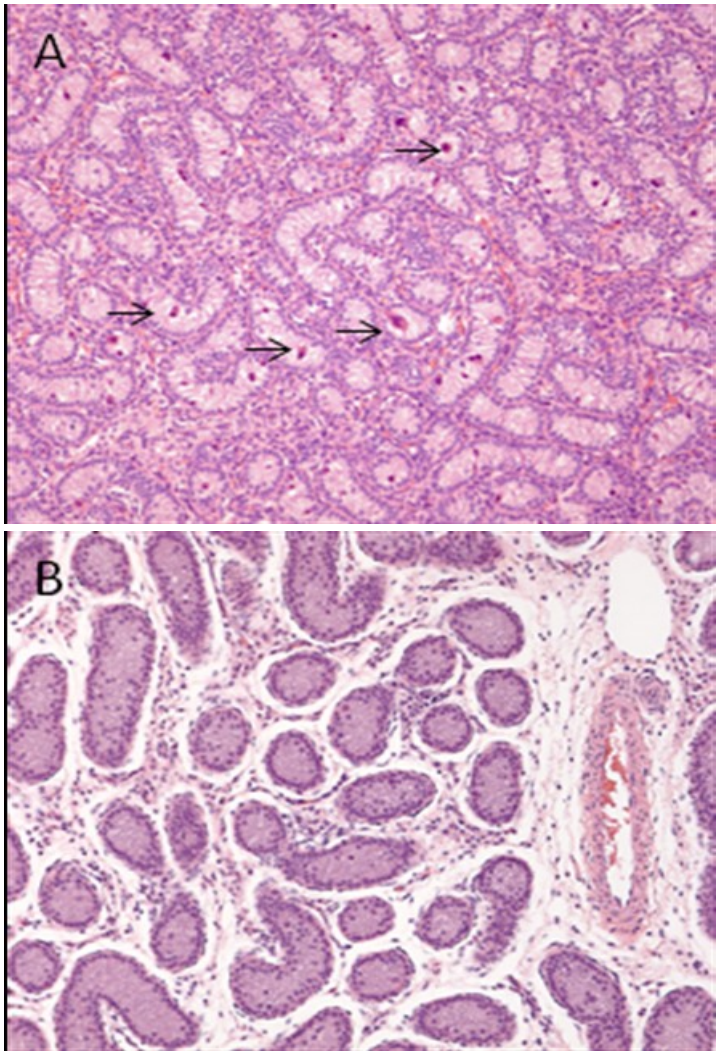

Figure 2. Hematoxylin and eosin staining of developing goat testis. (A)Testis of a 0 month old kid. Major cell types present in the seminiferous tubules are Sertoli and germ cells; however, there are few testicular macrophages (→) that are characterized by their irregular nuclear shape and dark nuclear staining. (B) Testis of a 2 month old kid. Cell types present in the seminiferous tubules are similar to that of the 0 month old; however, tubules have become larger and there is an increase in vascularization. (C, D, and E) Buck kids ages 4, 6 and 8 months of age, respectively. Unlike the testis of 0 and 2 month old buck, the seminiferous tubules of the 4, 6, and 8 month old have a lumen; in addition, the tubules contain cells that represent the stages of spermatogenesis. All representative photomicrographs are shown at 10x objective and width of field (840μm).

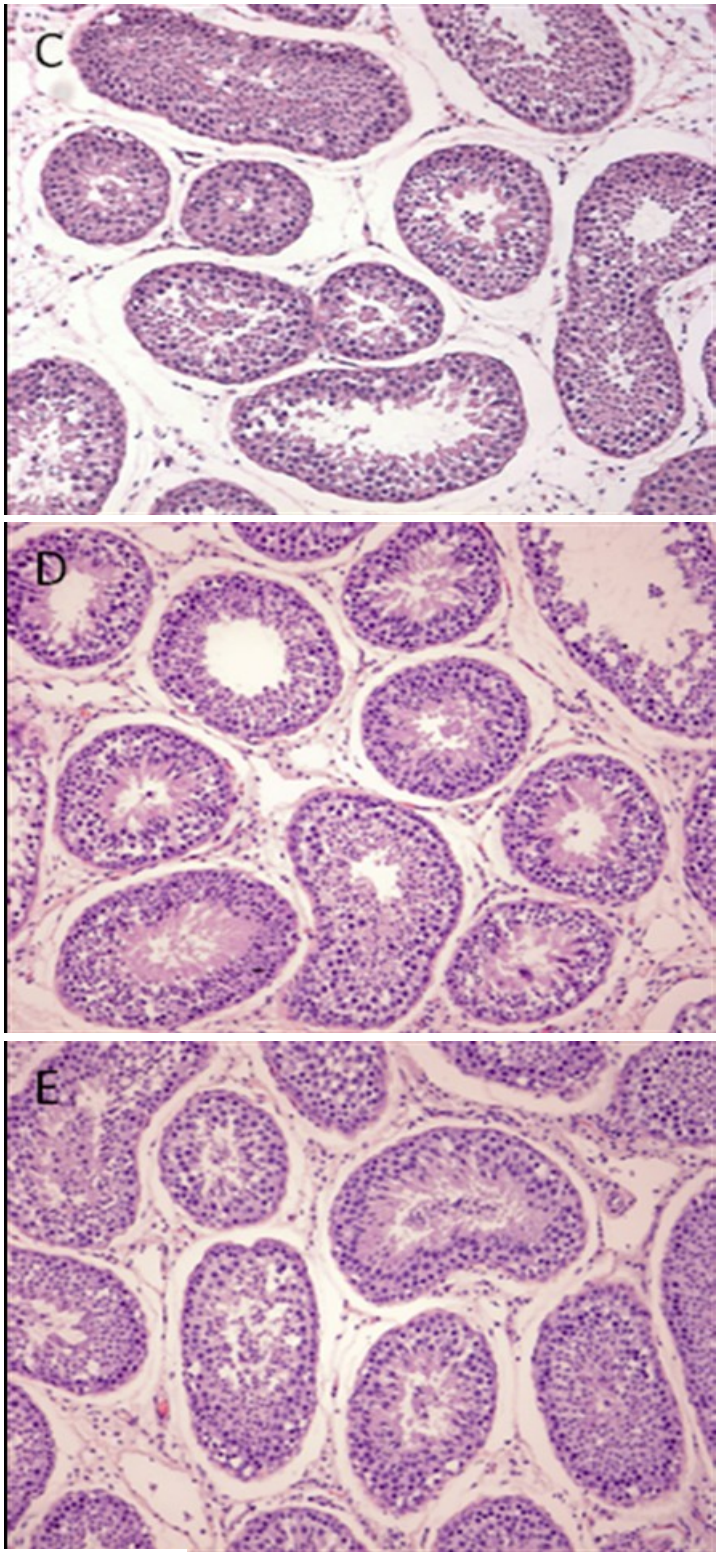

Figure 2. Continued.

Northern blots confirm differential expression of SOX9, HSPA8, STRA8, PRM1 and ODF2

Northern blots were used to confirm the differential expression of SOX9, HSPA8, STR8, PRM1, and ODF2 genes between developing buck testes. Representative Northern blots are depicted in Figure 3. Both SOX9 (3.9 kb) and HSPA8 (2.3 kb) mRNA concentration appear to be greater in bucks ages 0 and 2 month of age. The expression of SOX9 peaked ($p < 0.05$) at 2 months of age, but declined after (Table 2). This was probably due to the number of dividing germ cells increasing at 4 months of age. There was no significant difference between HSPA8 expression at 0 and 2 months of age. However, there was a significant ($p<0.05$) drop in expression of HSPA8 when comparing 0, 2 and 4 months to 8 month old bucks (Table 2). In contrast, STRA8 (1.8 kb), PRM1 (580b) and ODF2 (2.5 kb) mRNAs had no hybridization signals during 0 and 2 months of age, but showed strong expression in 4, 6, and 8 months of age (not shown).

In situ hybridization confirms and localizes differential by expressed genes in developing testis

Representative bright and dark field views of SOX9, HSPA8, STRA8, PRM1 and ODF2 mRNAs are shown in Figures 4, 5, 6, 7, and 8, respectively. SOX9, HSPA8 STRA8, PRM1 and ODF2 cDNA localization of goat mRNA in developing testis by in situ hybridization indicates a similar pattern as seen in northern blots. Hybridization signals for both SOX9 (Figure 4) and HSPA8 (Figure 5) mRNA appear stronger in younger animals due to the high concentration of Sertoli cells and spermatogonia in seminiferous tubules. In contrast, there were intense signals for STRA8 (Figure 6), PRM1 (Figure 7) and ODF2 (Figure 8) mRNAs in older animals indicating sexual maturity. In older animals, hybridization signal for STRA8 was located in both basal and adluminal compartments of the seminiferous tubule; however, there was a high concentration of granules in type B spermatogonia located in the basal compartment. Round and elongated spermatids and spermatozoa had intense hybridization signal for PRM1. Hybridization signals for ODF2 were found in the adluminal compartment of the seminiferous tubules with intense signaling in the round and elongated spermatids and spermatozoa.

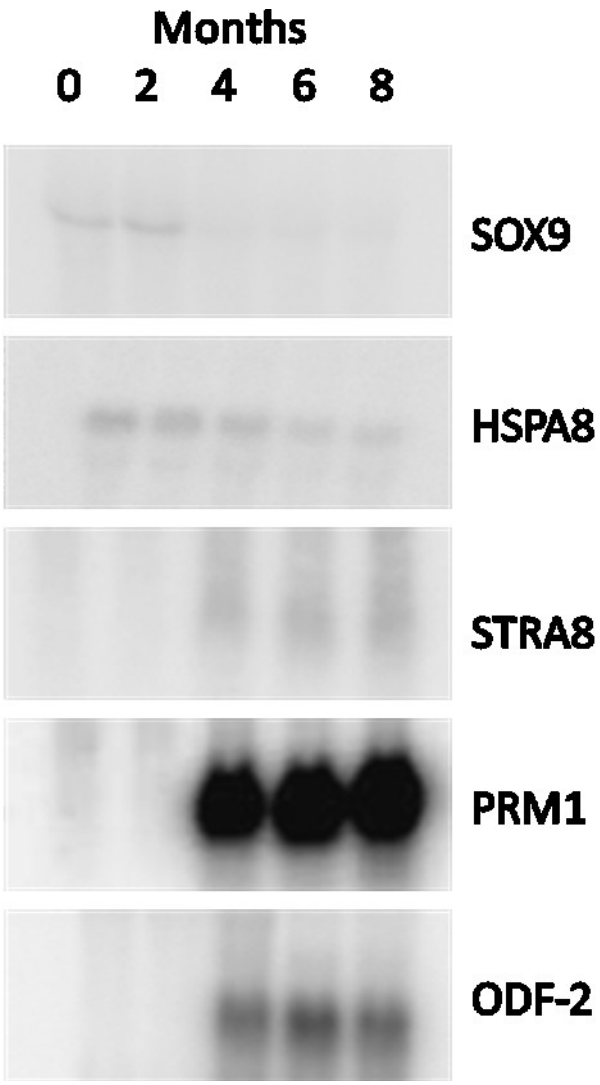

Figure 3. Representative Northern blot analysis of mRNA in developing goat testes ages 0, 2, 4, 6, and 8 months of age. mRNA was isolated from developing testis was hybridized with Sry like HMG box gene 9 (SOX9), heat shock protein A8 (HSPA8), stimulated by retinoic acid gene 8 (STRA8), protamine (PRM1), and outer dense fiber (ODF2) mRNA on separate blots at 3.9 kb, 2.3kb, 1.18 kb, 580 b and 2.5 kb, respectively.

Table 2. Northern blot quantification for SOX9 and HSPA8 mRNA expression in the developing goat testes ages 0, 2, 4, 6, and 8 months of age.

Gene	0	2	4	6	8
	mean ± S.D.	mean ± S.D.	mean ± S.D.	mean ± S.D.	mean ± S.D.
SOX9	76951.78 ± 8022.05[b]	97231.18 ± 14353.18[a]	34061.83 ± 1296.30[c]	23447.75 ± 5829.34[c]	20441.35 ±4429.34[c]
HSPA8	82882.17 ± 9447.08[a]	90058.57 ± 21493.52[a]	52551.17 ± 3889.22[b]	38536.88 ± 8576.55[b, c]	21263.82 ± 6610.58[c]

Values within a column without common superscript differ ([a,b, c] $P < 0.05$).

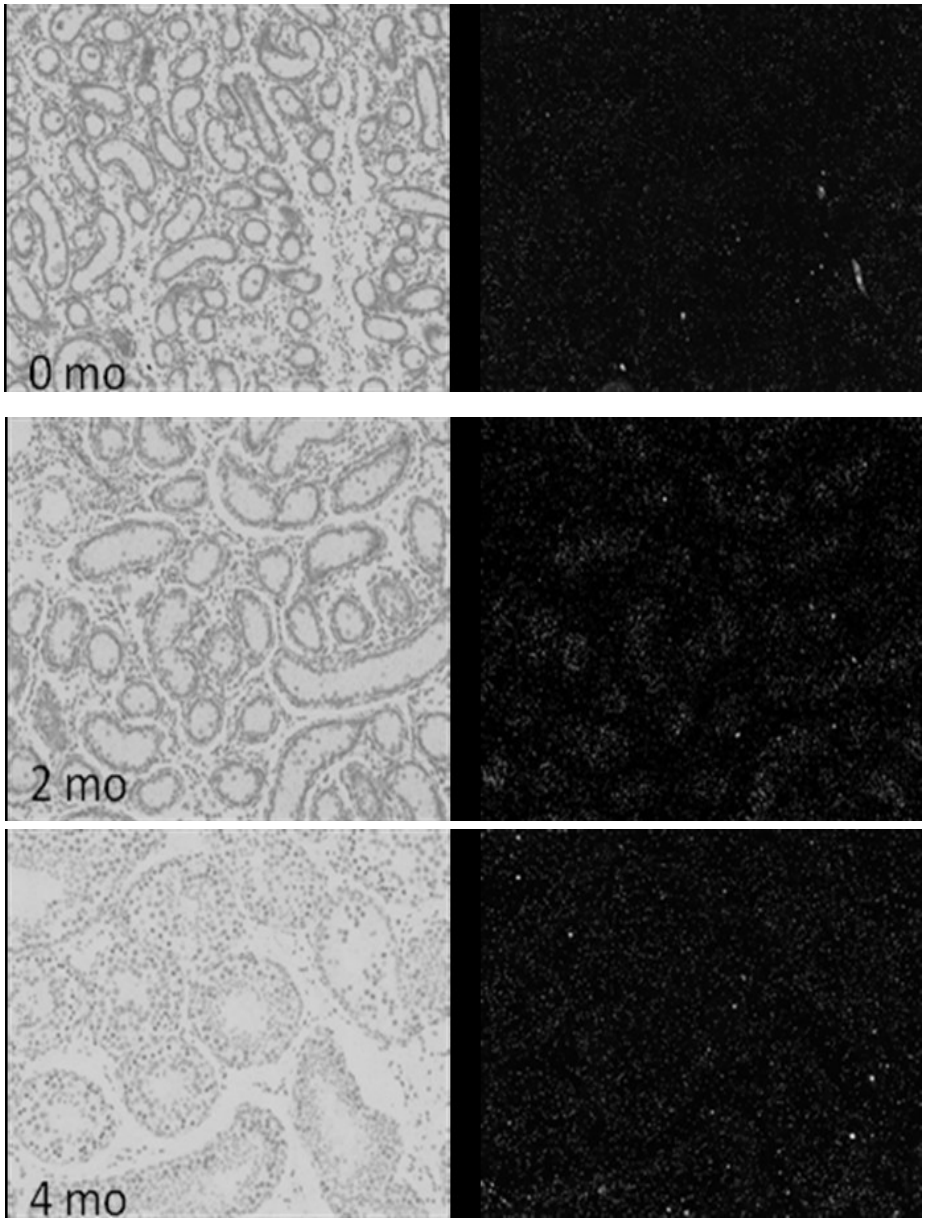

Figure 4. In situ hybridization analysis of SOX9 mRNA in goat testis. Corresponding bright-field and dark-field images from different Months (mo) of the age is shown. Representative sections for the various age groups were hybridized with radiolabeled sense cRNA probe (Sense) serves as a negative control. Scale bar represents 100 μM.

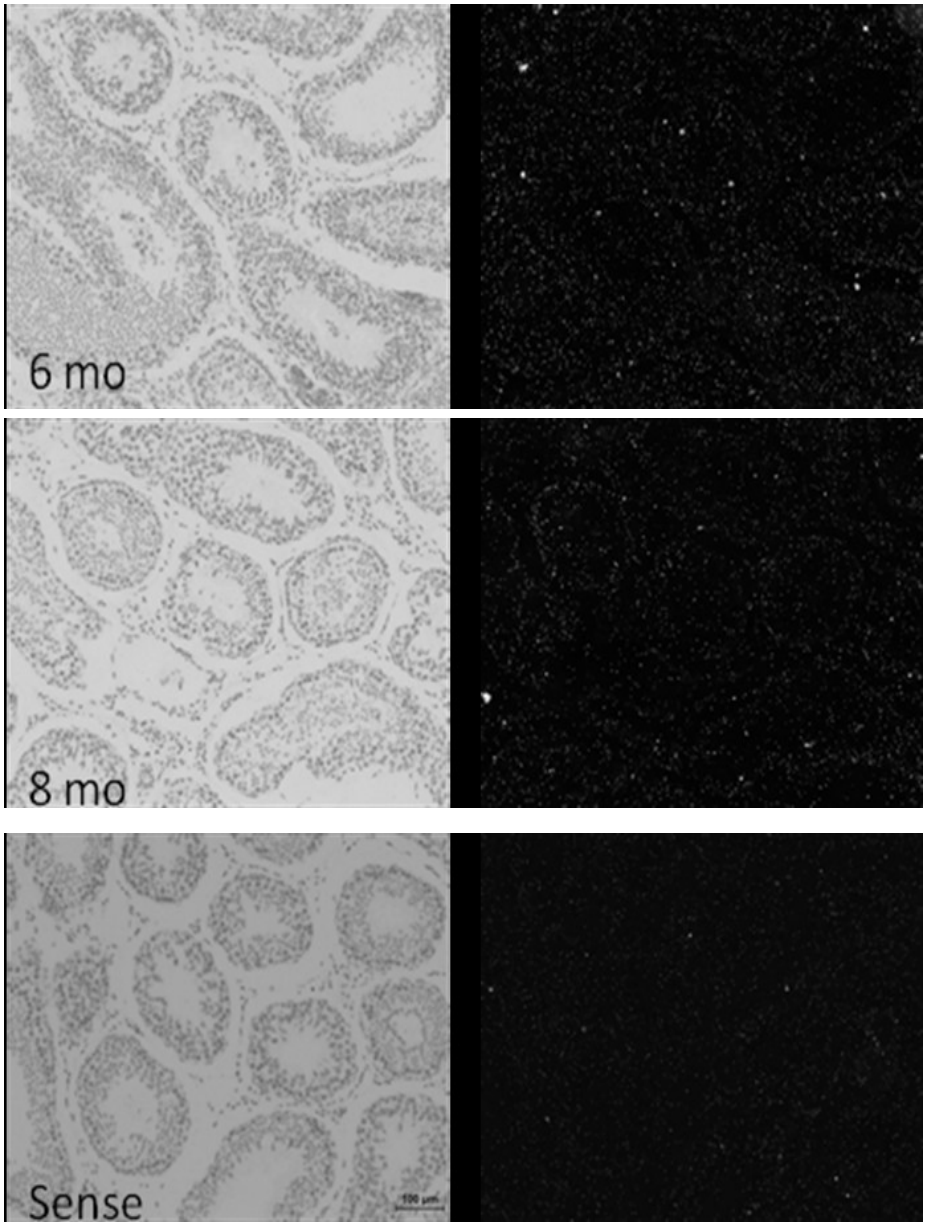

Figure 4. Continued.

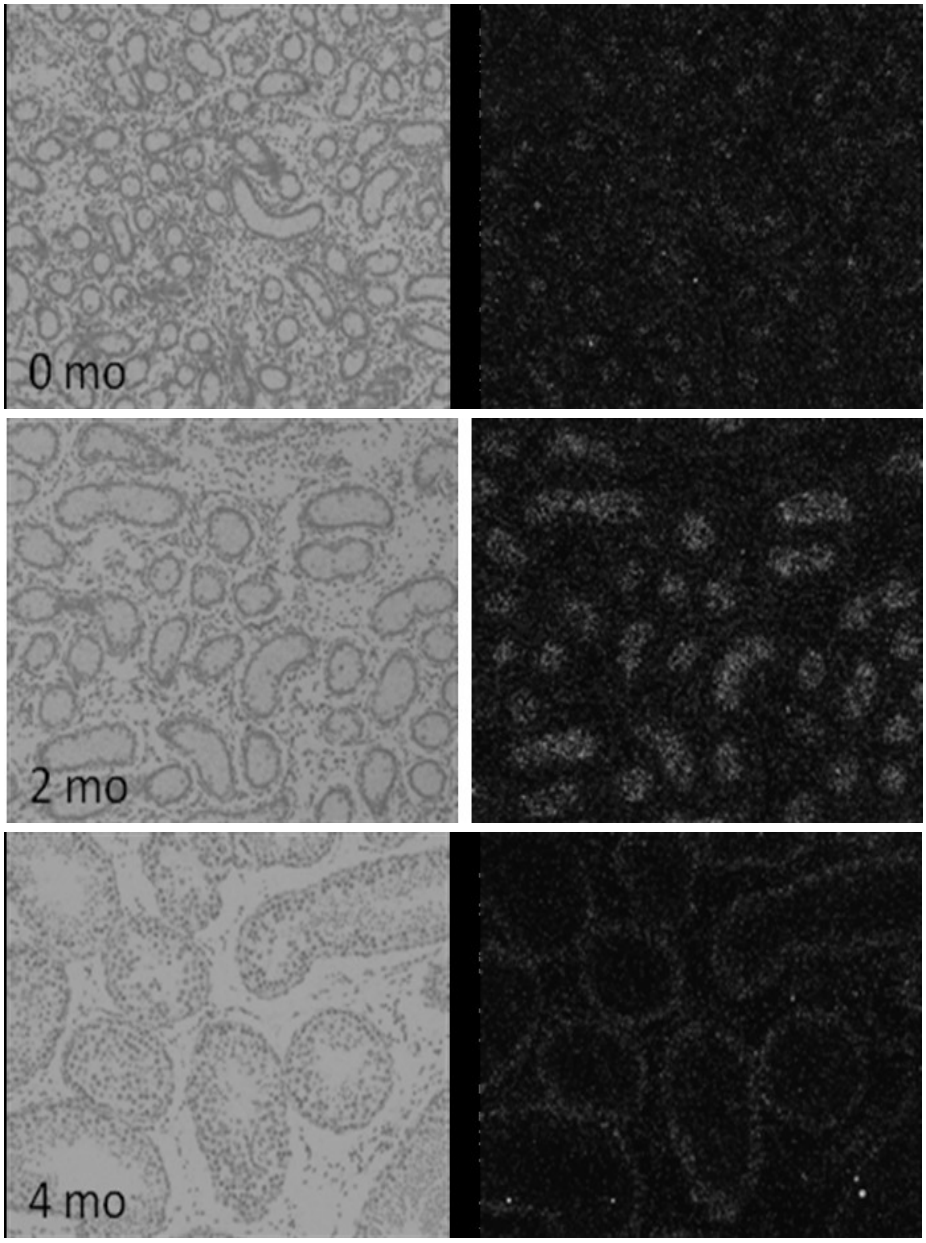

Figure 5. In situ hybridization analysis of HSPA8 mRNA in goat testis. Corresponding bright-field and dark-field images from different Months (mo) of the age is shown. Representative sections for the various age groups were hybridized with radiolabeled sense cRNA probe (Sense) serves as a negative control. Scale bar represents 100 μM.

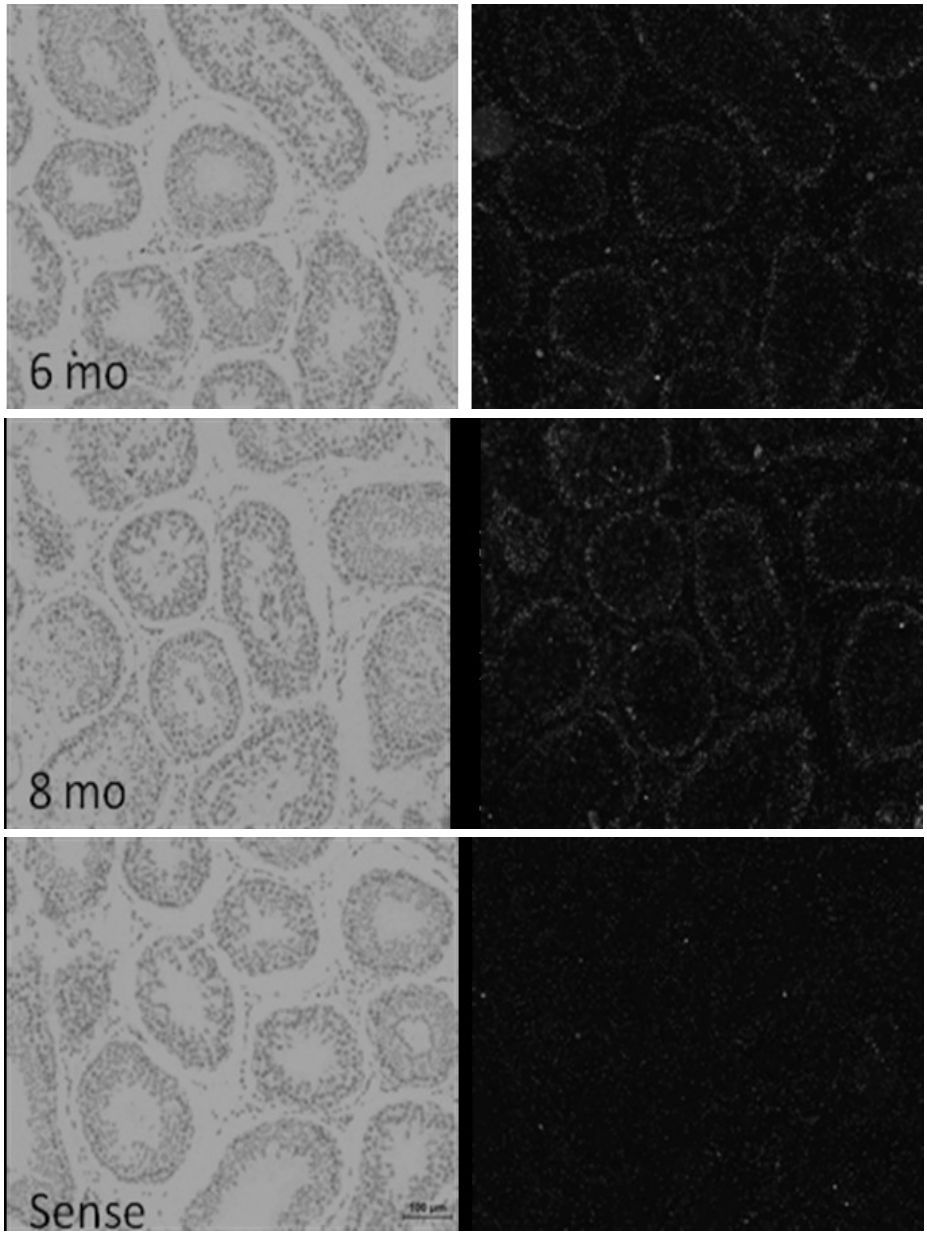

Figure 5. Continued.

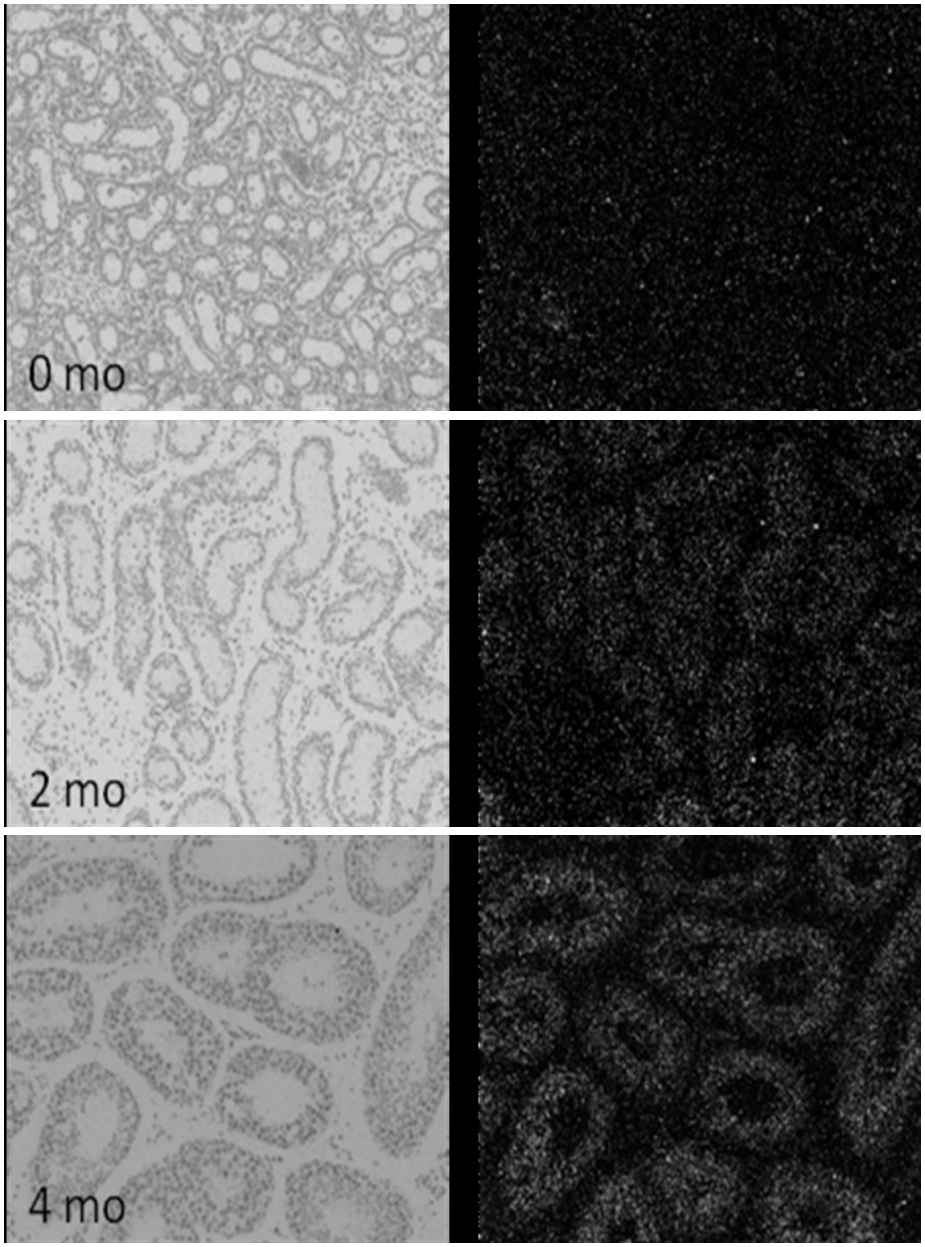

Figure 6. In situ hybridization analysis of STRA8 mRNA in goat testis. Corresponding bright-field and dark-field images from different Months (mo) of the age is shown. Representative sections for the various age groups were hybridized with radiolabeled sense cRNA probe (Sense) serves as a negative control. Scale bar represents 100μM.

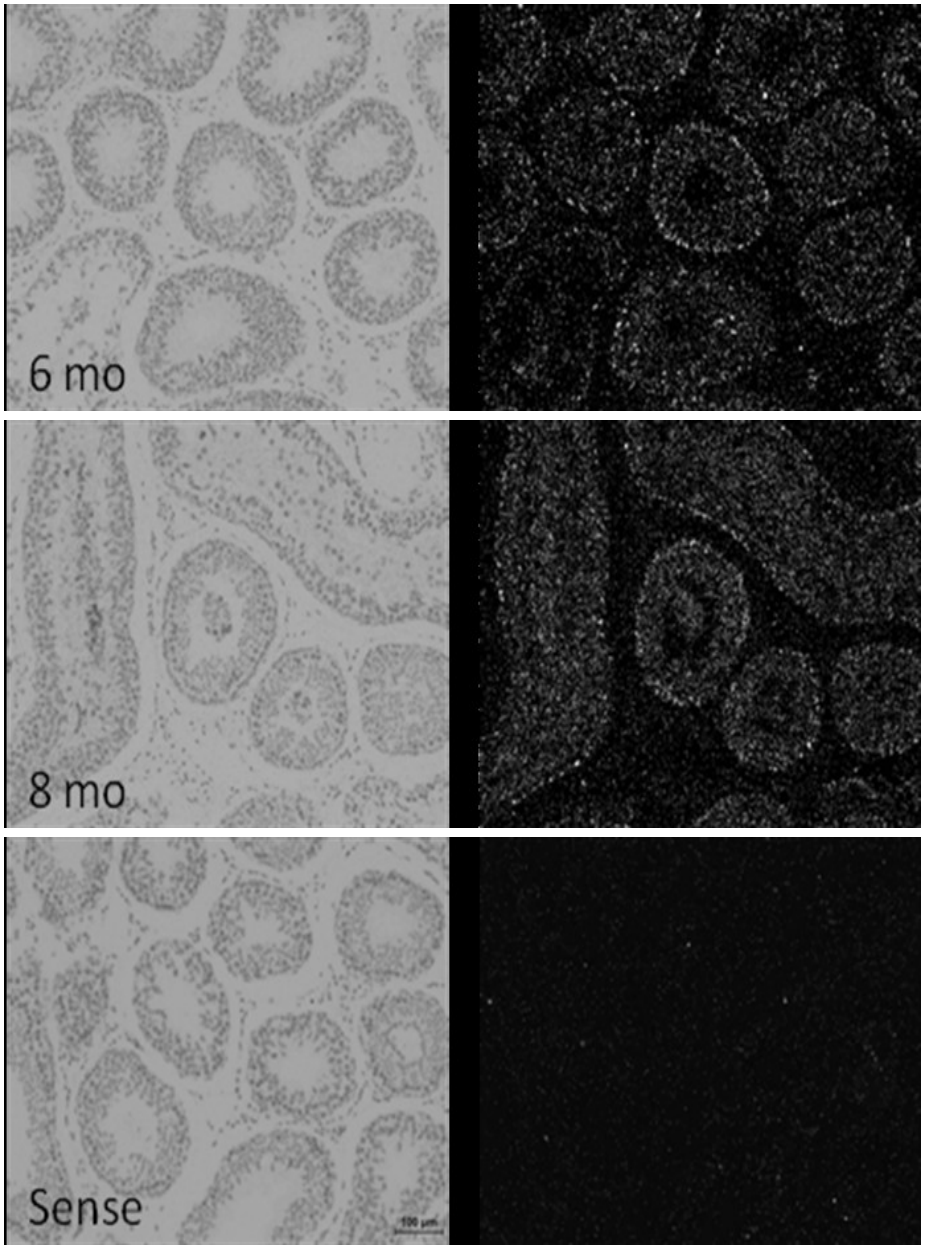

Figure 6. Continued.

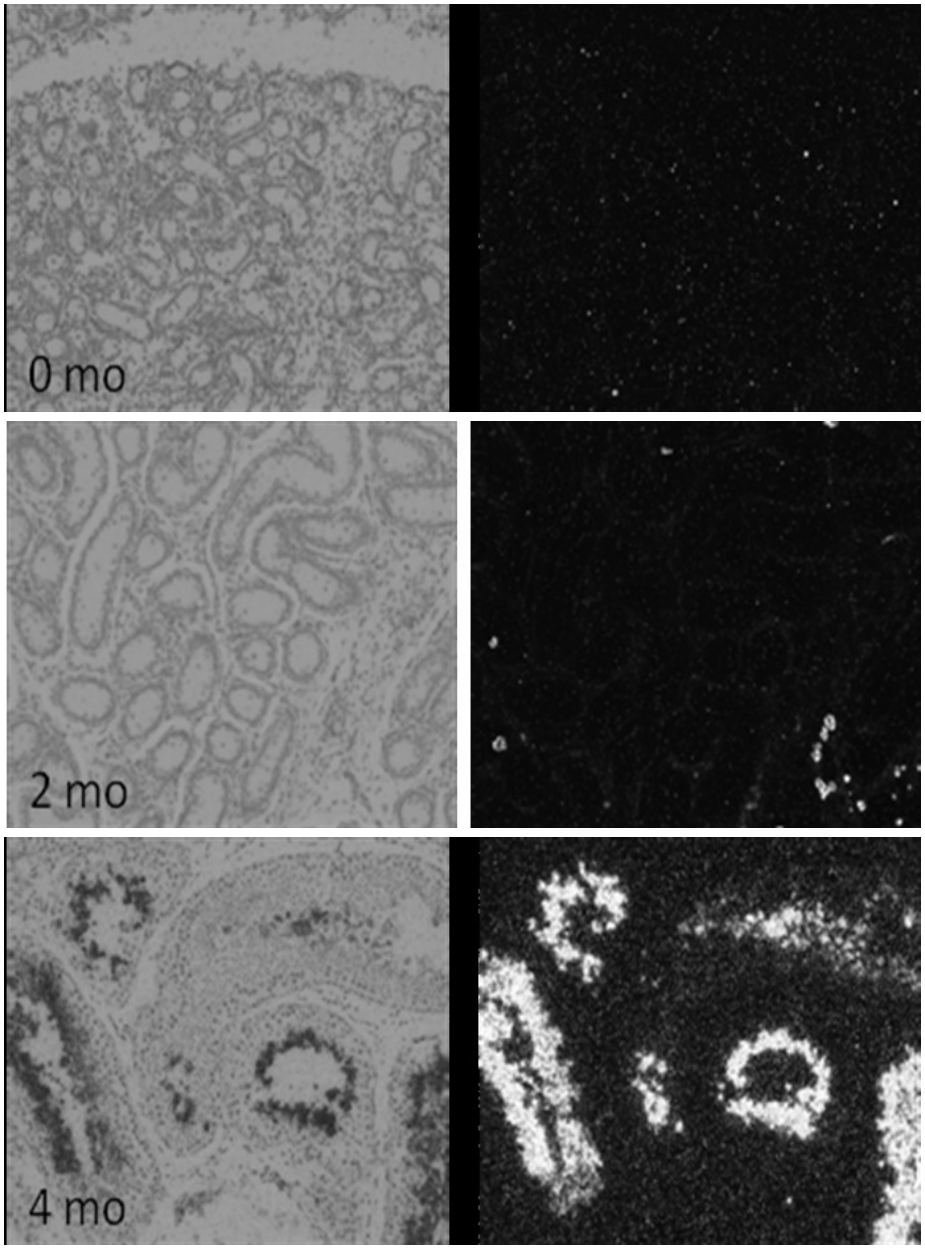

Figure 7. In situ hybridization analysis of PRM1 mRNA in goat testis. Corresponding bright-field and dark-field images from different Months (mo) of the age is shown. Representative sections for the various age groups were hybridized with radiolabeled sense cRNA probe (Sense) serves as a negative control. Scale bar represents 100μM.

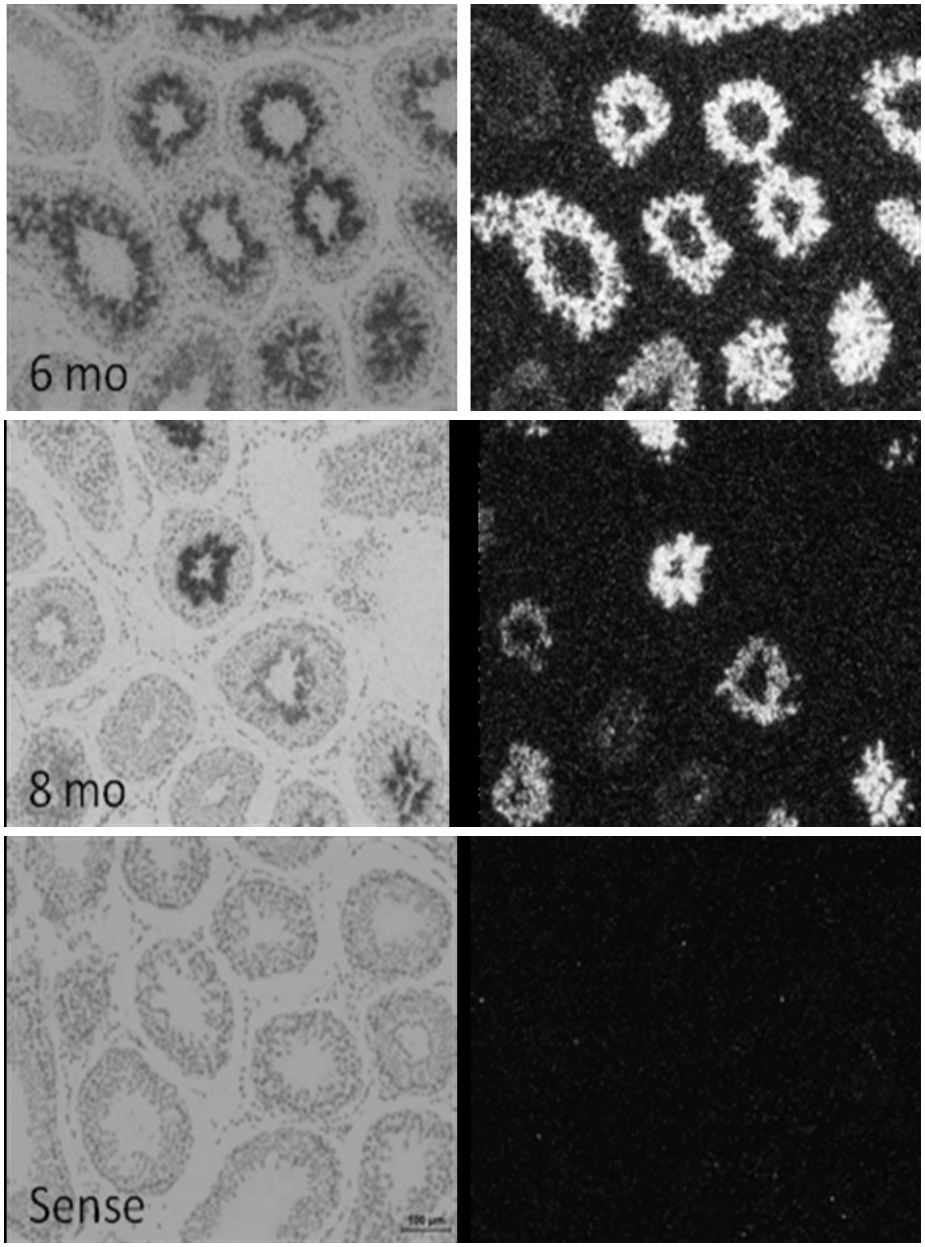

Figure 7. Continued.

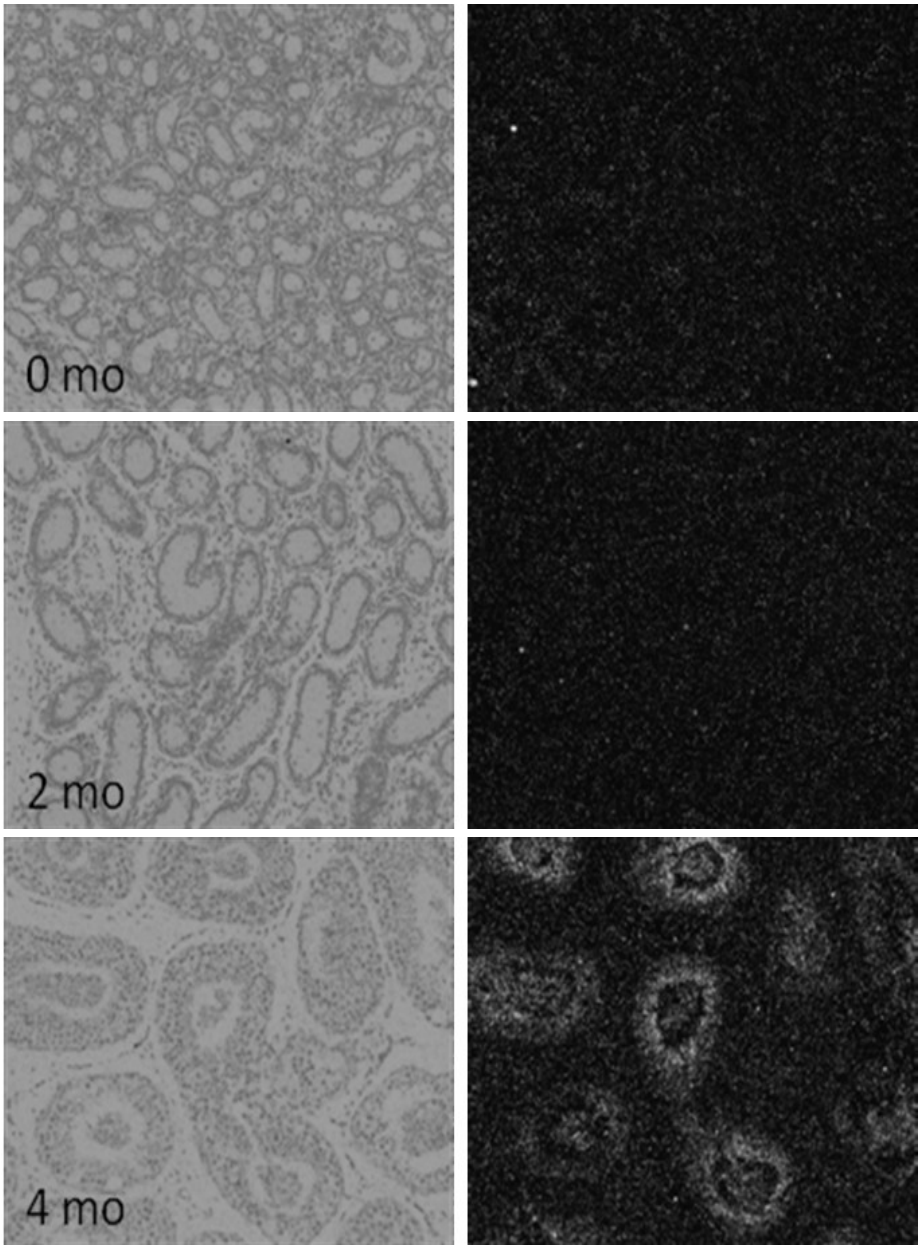

Figure 8. In situ hybridization analysis of ODF2 mRNA in goat testis. Corresponding bright-field and dark-field images from different Months (mo) of the age is shown. Representative sections for the various age groups were hybridized with radiolabeled sense cRNA probe (Sense) serves as a negative control. Scale bar represents 100μM.

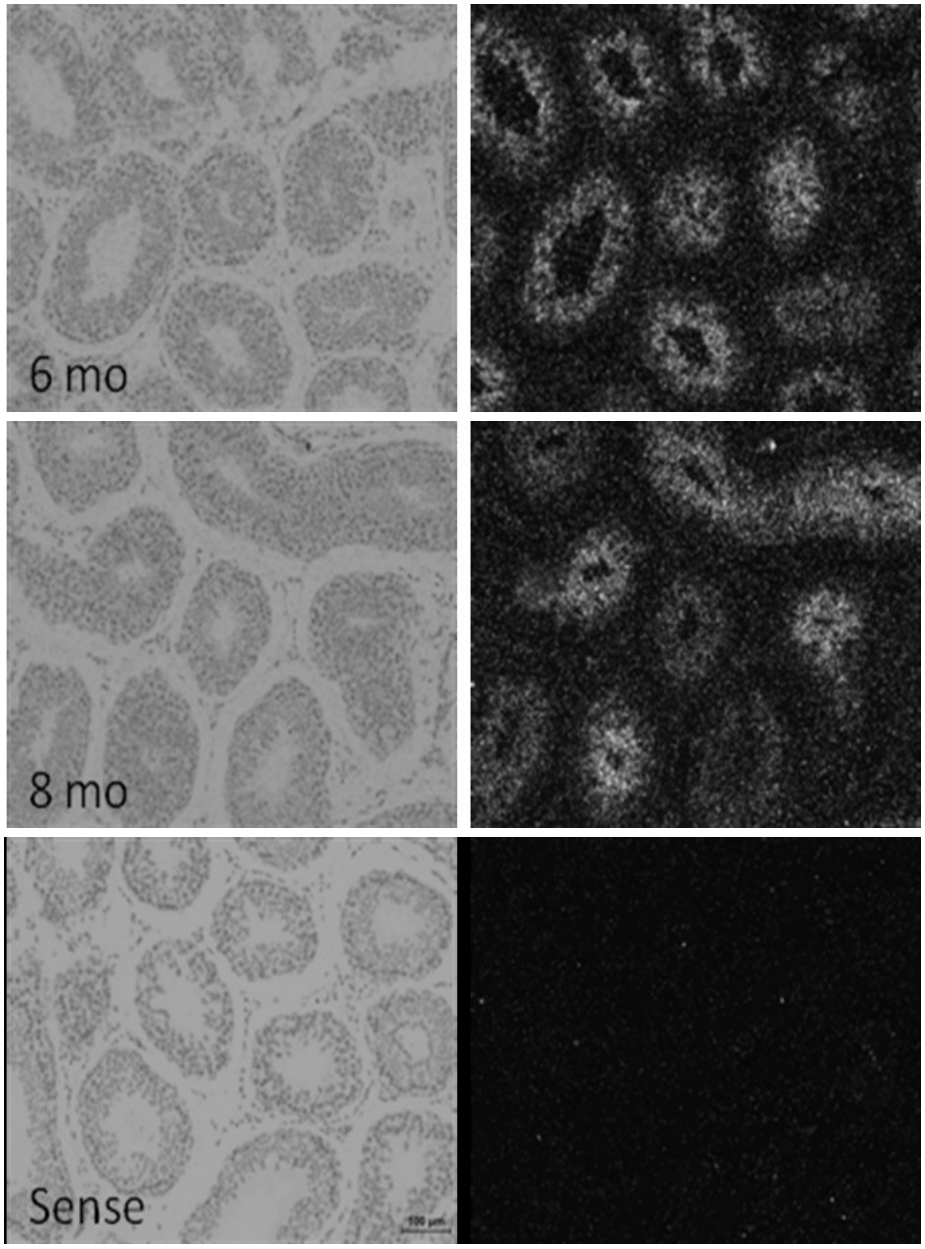

Figure 8. Continued.

Discussion

To our knowledge, this is the first report that associates the histomorphology of the developing testis with the timing of seminiferous tubule gene expression in the buck. Bucks were castrated at 0, 2, 4, 6 and 8 months of age. The results revealed that the 2 month old testes weight was 8 times greater than 0 month old goats; in addition, testes weigh of 4 month olds were 5-times greater than 2 month old goats. Herrera-Alarcón et al. (2007) reported different results in the first 2 months (birth to 9 weeks of age) with rams where there was a slow increase in testis weight. However, they noticed a 5.4-fold increase in testicular weight between 2 and 4 months (9 to 12 weeks). Our results showed a significant difference between the testes weights of 4 and 6 months and 6 and 8 months of age where 6 month old goats had larger testes. Implications from these results would seem that testes weight appeared to have met their capacity by 6 months, which is similar to what was previously reported by Schanbacher (1979) where there was an absence of additional increase in testicular size and spermatogenic function between 6-7 months and 13 – 14 months of age despite the increase in body weight after puberty. Though our experiment did not take into account the body weight of the bucks, it is well documented that body weight correlates to the onset of puberty and testicular function; however, after puberty is met, testicular development is influenced by photoperiod in seasonal animals (Schanbacher, 1979).

Communication between the somatic and germ cells within the testis is essential for proper development and function. There are a multitude of molecules and cells that are a part of this network where the silencing of one will lead to the abnormal organ

function. In this study, we targeted 5 genes (SOX9, HSPA8, STRA8, PRM1 and ODF2) that are expressed in cell types located in the seminiferous tubules. These genes have been studied in humans, rodents, bovine and equine; however, there is limited amount of information about their presence in the goat testis.

Sertoli cell marker, SOX9, is responsible for skeletal development, gonadal sex reversal and seminiferous cord formation (Foster et al., 1994; Wagner et al., 1994). SOX9 gene expression is upregulated shortly after SRY (sex determining region on the Y-chromosome) expression; moreover, it coincides with Sertoli cell development (Marshal and Harley, 2000). The role of SRY in sex differentiation is only linked to mammalian testes development; however, SOX9 is a major sex determining factor in reptilian, amphibian and avian species (Takase et al. 2000, Pask et al. 2002, Swain and Lovell-Badger, 1999). Barrionuevo and Scherer (2010) reported that the ablation of the *Sox9*gene at embryonic day 14 (E14) leads to late onset of sterility at 5 months in mice. After testis formation, *Sox9* gradually declined and was weakly detectable in the 2-d old postnatal rat testis (Fröjdman et al 2000). Fröjdman et al. (2000) reported that 15-d old rats have a strong level of *Sox9* protein in testes and the presence of *Sox9* remains into adulthood. Our results show that the SOX9 genes expression was localized in the seminiferous tubules of developing testis. Northern blot results showed higher expression in bucks 0 and 2 months of age, which corresponds with the increase in tubule size.

The HSPA8 gene was previously reported as expressed in the late pachytene spermatocytes with decreasing expression in stages associated with spermatid elongation

in rodents (Krawczyl et al., 1988). In this study, the expression of the HSPA8 gene had similar Northern blot results to that of SOX9, where expression levels were higher in buck testes at 0 and 2 months of age than those that were 4, 6, and 8 months of age. Results from in situ hybridization revealed HSPA8 mRNA signal was strong in towards the basal compartment of the seminiferous tubules of 4, 6 and 8 month old bucks, where the primary cell types are type A and B spermatogonia. Moreover, HSP8A expression can be seen in 1º spermatocytes with little to no expression in later stages.

Expression of STRA8, PRM1 and ODF2 genes are associated with cell that have committed to undergoing meiosis and spermiogenesis (Hoyer-Fender et al., 1998; Dadoune, 2003; Horowitz et al., 2005; Carrell et al., 2007; Mark et al., 2008). Zhou et al. (2008) noted that peak expression of the STRA8 gene was at the onset of meiosis with the highest levels of both mRNA and protein in preleptotene/early leptotene spermatocytes. Mice with a STRA8-null mutation undergo premeiotic DNA replication, display cytological features for the initiation of recombination and express numerous meiotic genes; however, there is a prolonged asynapsis and heterosynapsis that are important for chromosomal pairing (Mark et al., 2008). Both northern blot and in situ data confirms the presence of STRA8 mRNA in older bucks. Caprine STRA8 mRNA signal strength was high throughout the seminiferous tubules in older bucks especially in areas where mature germ cells are located. During the transition into round spermatid, the exchange of histones for protamines is essential for sperm chromatin condensation (Carrell and Liu, 2001). PRM1 mRNA was expressed only in the testis of mature animals. Signal strength was localized to the adluminal compartment where round and

elongated spermatids are located. The prominent component of the sperm tail is the outer dense fibers (ODF), which is unique to the spermatozoa (Hoyer-Fender et al., 1998; Salmon et al. 2006; Hüber et al., 2008). ODF2 was first described as the major protein in the mammalian sperm tail (Hoyer-Fender et al., 1998). Though it is the major protein, it is not directly associated with motility, but is involved in the elasticity and stability of the sperm tail (Salmon et al., 2006). ODF2 mRNA was isolated in post meiotic spermatids and spermatozoa. These results imply that expression of STRA8, PRM1 and ODF2 genes are essential to the germ cell commitment to spermatogenesis.

Many genes that are important to testicular function have been identified by microarray (Yu et al. 2003; Ing et al.2004). This study focused on genes that have been previously studied and linked to male infertility. Results from the present study imply that expression of SOX9, HSPA8, STRA8, PRM1 and ODF2 genes may play an important role in testicular development and function. In conclusion, expression of these 5 genes can be used as a useful tool (via biopsies) to access proper testicular development and as an indicator of spermatogenesis. Further studies are needed to compare seasonality and factors that will affect buck fertility.

CHAPTER IV

SPERMATOZOAL RNA PROFILE OF MATURE BUCK (*CAPRINE HIRCUS*) SPERM COLLECTED DURING THE BREEDING AND NON-BREEDING SEASON

Introduction

Sperm assessment has been based on visual analysis, such as sperm count and motility, for diagnosing infertility in males. The benefits of this method are that it is easy, inexpensive and useful for onsite evaluation. However, in the case of idiopathic infertility, this method is obsolete and shows that there are still unanswered questions about the processes that are related to fertility.

The mammalian process of producing a normal, motile spermatozoon is a complex process where the diploid spermatogonia needs to be capable of both self renewal and recruitment to undergo nuclear, cellular and structural modifications resulting in the haploid spermatozoa (Lele and Wolgemuth, 1998). During the early stages of spermatogenesis, there is a high level of transcriptional activity in the spermatocytes which is followed by a gradual decline and a short surge at the stage of round spermatid (Dadoune et al., 2004). It was also reported that there is a transcriptional shut off upon chromatin compaction and spermatid elongation (Grunewald et al., 2005). In addition as the spermatid elongates, it begins to shed its cytoplasmic droplet thus leading to the hypothesis that majority of the transcripts are removed with the cytoplasmic droplet, and any remnant mRNA present in the ejaculate are due to the retained cytoplasm or somatic cell contamination (Miller and Ostermeier,

2006). Earlier studies by Pessot et al. (1989) using RNase-colloidal gold and development of new techniques such as reverse transcription-polymerase chain reaction (RT-PCR) and microarray allow scientists to detect specific transcripts in human (Ostermeier et al., 2002), bovine (Lalancette et al., 2008) and rodent (Pessot et al., 1989) spermatozoa, which have been linked with sperm quality and are important to embryonic development. Therefore, the objective of the present study was to characterize specific mRNAs in buck ejaculate that can be linked to sperm quality, and if so, these mRNAs can be used as potential markers for fertility.

Materials and Methods

Animal and sample preparations

Samples were collected from the Prairie View A&M University International Goat Research Facility (Prairie View, Texas) during October 2008 and April 2009 to signify peak and non-peak breeding season months, respectively. Semen was collected via electro ejaculation from eight Boer and Alpine bucks (n=8), age >1 year. All samples were cooled on ice and centrifuged at 1500 x g for 15 min at 4ºC. The seminal plasma was removed, and sperm pellets were frozen in liquid nitrogen and stored at -80ºC until later processing.

RNA isolation

Total RNA was isolated from sperm samples collected from goat bucks ages >1 year of age using Tripure Isolation Reagent (Roche, Mannheim , Germany) according to manufactures recommendations. To determine the concentration, 2 µl of total RNA was subjected, in duplicate, to a NanoDrop-1000 spectrophotometer (NanoDrop

Technologies, Inc., Wilmington, DE). Five µg of total RNA was treated with DNAse. A master mix of 4µl of 5x1st Strand, 2µl of 0.1 M of DTT, 4µl RQ1 DNAse and 9.5µl DEPC-treated H_2O was added to each sample. Samples were incubated for 30 min at 37˚C. Then, the enzyme was heat-killed for 15 min at 70˚C and samples cooled on ice. Fifty µl of Depc-treated H_2O and 70 µl of PCI (RNA grade Phenol: Chloroform: Isoamyl alcohol, 25: 24:1, vol:vol:vol) was added, vigorously vortexed and centrifuged for 4 min at RT. The PCI upper phase product was removed. Fifty µl of CI (Chloroform: Isoamyl alcohol, 29:1, vol:vol) was added to the CI upper phase product, vigorously vortexed and centrifuged for 4 min at RT. The upper phase was removed. RNA in the upperphase was precipitated with PPT (100% EtOH and sodium acetate (NaAc),) and 10 µg/µl glycogen was overnight at -20˚C. Samples were centrifuged for 20 min at 4˚C. The supernatant was discarded, and the pellet washed in 70% EtOH. Samples were spun, supernatant discarded and the pellet was air-dried for 5 min. Pellets were reconstituted with NaCit. To determine the concentration, 2 µl of total RNA was subjected, in duplicate, to a NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Inc. Wilmington, DE). Following each extraction and DNAse treatment, RNA was pooled and subjected to RT-PCR for protamine 1 (PRM1) gene where the primers flanked an intron. This was performed on RNA to check for genomic DNDA (gDNA) contamination.

Microarrays

Total RNA samples from goat sperm appeared to be of similar quality on Bioanalyzer 2100 as that reported by Ostermeier et al. (2002), Bergstrom Lucas (2009)

and Bissonnette et al. (2009). Amplified cDNA was generated using the TransPlex® Complete Whole Transcriptome Amplification kit (WTA2) protocol (Sigma-Aldrich, Inc.; St. Louis, Missouri). An input mass of 300 ng from total RNA derived from goat sperm collect in Oct and April was used in each reaction. QIAquick PCR Purification kit (Qiagen technologies, Valencia, California) was used to purify cDNA products, and yields were quantified by NanoDrop-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE). The Agilent Genomic DNA ULS Labeling kit (Agilent, Santa Clara, California) was used to chemically label 1.65µg of cDNA with ULS-Cy3 dye for 30 min at 85ºC. Cy3-labeled samples were purified using Agilent KREApure columns (component of the Agilent Genomic DNA ULS Labeling kit). The purified cDNA samples were combined with Agilent 10X Blocking Agent and 2X Hi-RPM GE Hybridization Buffer (component of the Agilent Genomic DNA ULS Labeling kit). Before array hybridization, samples were denatured at 95ºC for 3 min, and Agilent-CGH*block* was added. Samples were hybridized to the Agilent Bovine Gene Expression 4x 44K Microarrays (Agilent). Hybridization was carried out at 20 RPM at a temperature of 65ºC for 17 hr. Arrays were washed according to the procedures outlined in the Agilent One-Colored Microarray-Based Gene Expression Analysis manual (Agilent). Scanning and image analysis were performed using the Agilent Microarray Scanner. Featured Extraction Software (version 9.5) was used for data extraction from raw microarray image files. Differentially expressed genes between breeds and based on seasonality were identified with GeneSpring GX (v. 10, Agilent) software and detected

call were made by employing the Agilent default flag settings for Agilent One-Color microarray.

Real time PCR preparation and analysis

Fifty ng of DNAse treated RNA was reverse-transcribed with random octomer primers and oligo dT primers. Samples and primers was heated at 65˚C for 5 min and cooled to RT. Then, a mixture of 5X 1st strand buffer, 0.1 M DTT, RNAsin, 10 mM dNTP and Superscript II were added to the samples. Samples were incubated at 37˚C for 1 hr and terminated at 90˚ for 5 min. cDNA was stored at -80˚ until further processed.

For real-time PCR (qPCR), the ABI PRISM 7900 HT (Applied Biosystems, Foster City, CA) was used to quantify the expression of transcripts. The amplification reaction had a final volume of 15 µl which contained 14.5 µl of master mix (*Power* SYBR® Green Master Mix (Applied BioSystems, Roche, Branchburg, NJ), Ambion PCR grade H_2O, and 3 µM of forward and reverse primers) and 0.5µl cDNA product from ejaculated sperm RNA. Primers for SPAM1, GK2 and MYCBP2 (Table 3) were generated to validate their change in expression during the course of both peak and non-peak breeding seasons (Figure 2). In addition, three candidate reference genes (glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 16S rRNA and protamine1 (PRM1)) were used as normalizers. Mean C_T values of each sample were transformed into raw, non normalized quantities. The expression data was analyzed using the geNorm algorithm (Vandesompele et al. 2002) which determines reference gene stability and produces a normalization factor based on the geometric mean of the expression levels.

Table 3. Real time PCR primers. Real time PCR primers designed based on Agilent ® bovine microarray probe.

Gene Symbol	Forward Primer	Reverse Primer	Accession
GK2	5’ GGTTACAGGTCAGCCTTCTGAAA	5’ TATAAAAAAACCCAAGGGCAGACT	XM_591790
MYCBP2	5’ TGCAATACTGAGTGTGCCTCATT	5’ ACAGTGGTTTATTGACATGTACATTCC	NM_001192817
SPAM1	5’ ACGTGTGTATGGCAGAAGACATTT	5’ GGAGGAATGATCACTGGGTTGT	NM_001008413

GK2 ,Glycerol Kinase2; MYCBP2, Myc-binding protein 2; SPAM1, Sperm adhesion protein 1

Results

The purity of pooled samples was checked by RT-PCR using the PRM1 gene as a target to detect the presence of genomic DNA (gDNA) contamination (Figure 9). The presence of gDNA in samples would produce an amplicon of 340 bp (Lanes 2, 3, and 4). No gDNA contamination was detectable in samples collected during October and April in both Boer and Alpine goats. Since microarray studies require micrograms of RNA, Alpine sperm RNA collected during both October and April

Figures 10 and 11 represent the quality of ejaculated buck sperm during the month of October and April. Sperm RNA profile was similar to that of bovine spermatozoa reported by Gilbert et al. (2007). In comparison to the standard, the RNA profile of goat sperm lack the presence of the two major peaks that correspond to 18S and 28S rRNA. The majority of the RNA appears short in length and fragmented. To recover loss from degradation and fragmentation, the formalin-fixed paraffin embedded (FFPE) tissue protocol was used for cDNA amplification and labeling for microarray analysis (Lucas and Lin, 2009) since RNA profile of sperm resembled that of FFPE tissue.

Due to the lack of available caprine commercial microarrays, samples were hybridized to an Agilent bovine microarray containing 44k genes. Other studies have utilized cross-species hybridization (Gilbert et al., 2009). Of the 44k genes present, 21,535 transcripts were detected above background. With initial cutoff set at fold change (FC) of 2 and $p \leq 0.05$, 1, 318 genes appeared different between October and April. To eliminate the likelihood of false positives, the cut off was set to FC of 3 and $p \leq 0.01$ which narrowed the list of genes to 50 transcripts (Table 4).

For microarray validation, specific genes (sperm adhesion molecule1 (SPAM1), glycerol kinase 2 (GK2) and MYC binding protein 2 (MYCBP2)) were chosen based on the array expression profiles here and those previously reported in humans with teratospermia (www.ncbi.gov/geoprofiles). Microarray results revealed that SPAM1 mRNA concentrations was 5.56 fold higher in April ($p \leq 0.01$), while GK2 and MYCBP2 mRNA concentrations were 4.56 and 3.58, respectively, higher in October ($p \leq 0.01$). Real time primers were designed around the microarray probe sequence. The qtPCR confirmed the pattern of expression for all three genes, but due to variation between individual samples, differences were not significant at $p < 0.05$.

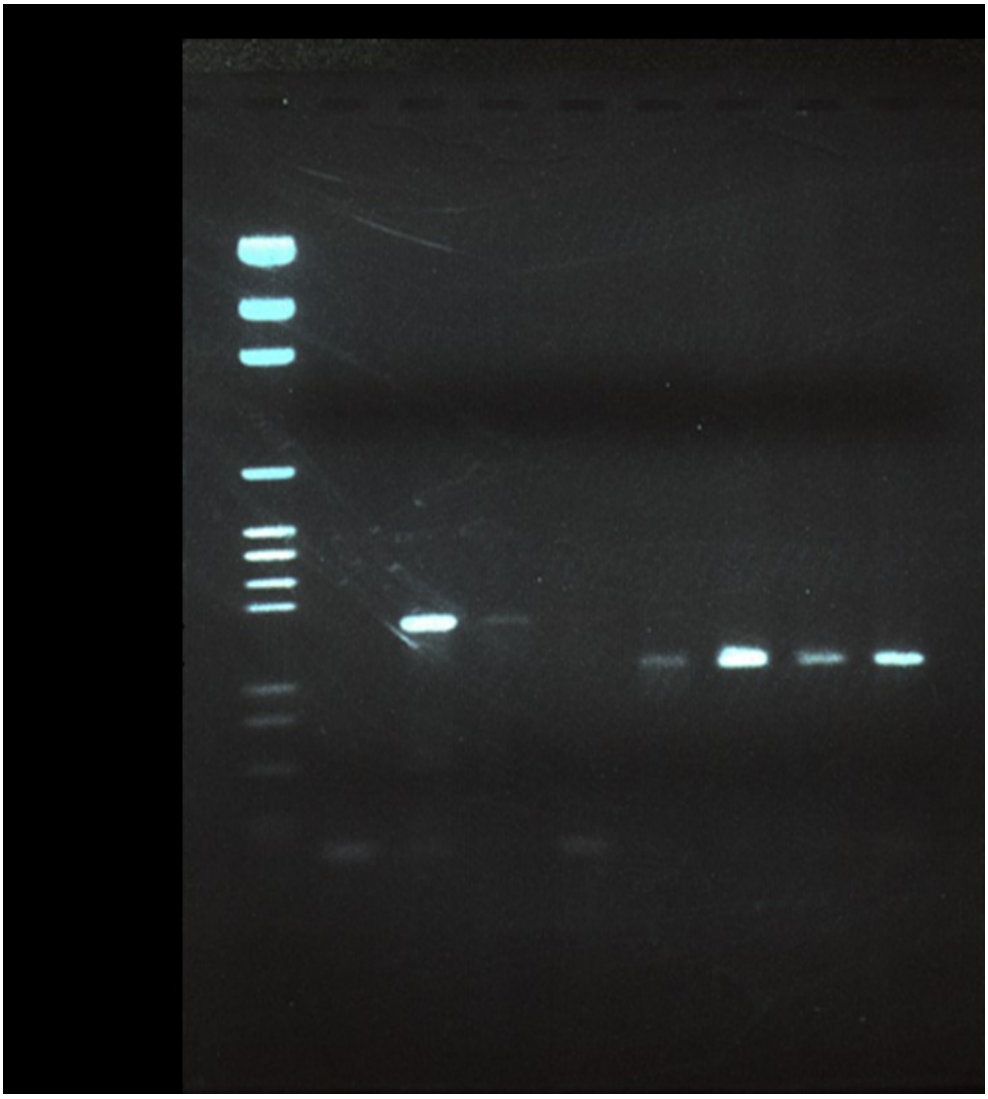

Figure 9. Verification of contamination in ejaculated sperm RNA preparations. Genomic contamination was verified by RT-PCR. Results from the sperm RNA extraction for gDNA contamination revealed an absence of gDNA in pooled sperm RNA. Lane M: ; 1: negative control; 2: 10 ng gDNA; 3: 1 ng gDNA; 4: 0.1 ng gDNA; 5: 100 ng pooled Boer sperm cDNA collected in October; 6: 100 ng pooled Alpine sperm cDNA collected in October; 7: 100 ng pooled Boer sperm cDNA collected in April; 8: 100 ng pooled Alpine sperm cDNA collected in April.

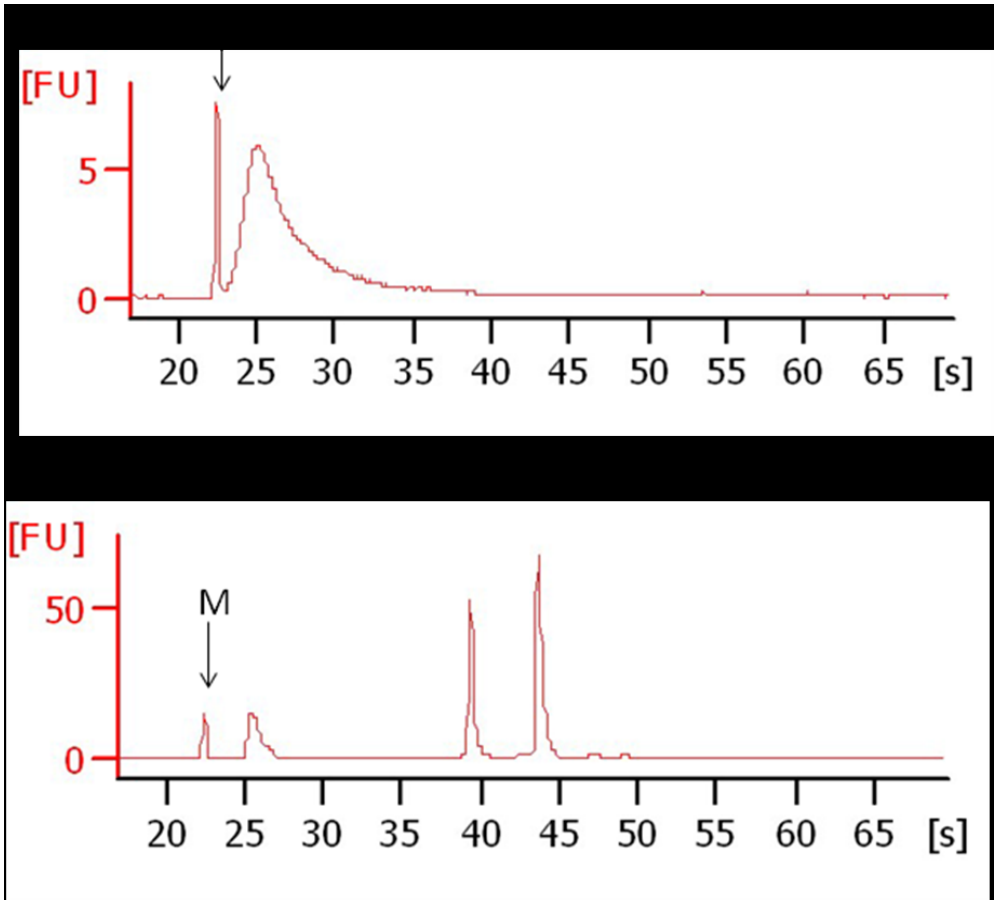

Figure 10. Total RNA microelectrophoretic profile of sperm collected during October. (A) microelectrophoretic profile of sperm and (B) 200 ng standard; M, marker; FU, fluorescence; S, seconds.

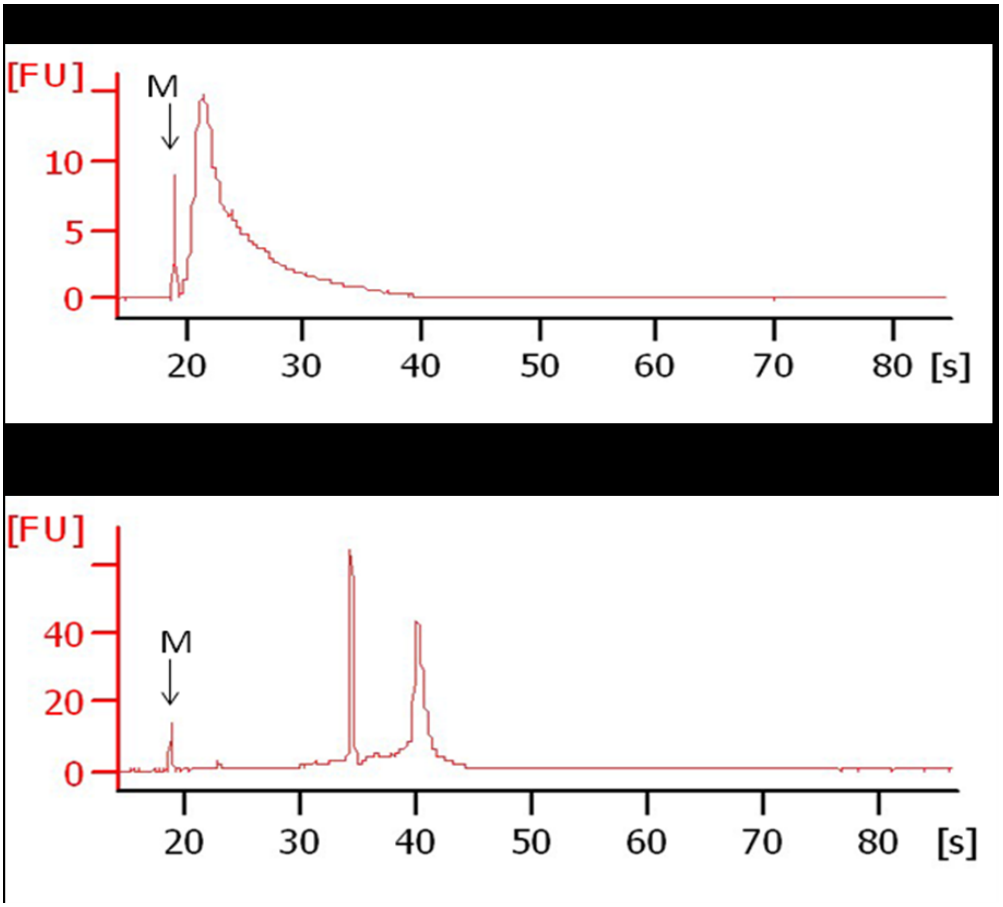

Figure 11. Total RNA microelectrophoretic profile of sperm collected during April. (A) microelectrophoretic profile of sperm and (B) 200 ng standard; M, marker; FU, fluorescence; S, seconds.

Table 4. Differentially expressed genes in buck sperm collected during April and October. Fold change is indicated at the left and is relative to genes expressed in April vs. October.*

mRNA concentrations greater in April than October	
Enzyme and Inhibitors	
42.18	Aldehyde dehydrogenase 16 family, member A1
Cytoskeleton and Adhesion Molecules	
5.56	**Sperm Adhesion Molecule 1**
3.88	Wiskott-Aldrich Syndrome-like
3.66	Similar to Pecanex-like 3
Signaling Molecules	
4.34	Ubiquitin-conjugate enzyme E2 D1
3.37	Calcium Homeostasis modulator 2
3.10	BMP and activin membrane bound inhibitor homolog
Transport	
5.87	Similar to Nuclear RNA Export Factor 2
Cell Cycle	
3.42	Centromere protein I
Unknown function	
10.10	Similar to Uncharacterized protein KIAA0552
3.12	Bos taurus chromosome 12 open reading frame 45 ortholog
mRNA concentrations greater in October than April	
Enzymes and Inhibitors	
4.56	**Glycerol Kinase 2**
4.26	Methyltransferase 5 Domain Containing 1
3.58	**Myc-binding Protein 2**
3.54	Aminoglycoside Phosphotransferase Domain Containing 1
3.46	PREDICTED: Homo sapiens Phospholipid Scramblase family, member 5
3.35	Similar to Beta-galactosamide alpha-2,6-sialytransferase
3.17	Proteasome 26S subunit, non-ATPase 1
3.15	Staphylococcal Nuclease and Tudor Domain Containing 1
3.12	Ceramide kinase-like transcript variant 2
3.11	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 2
3.06	KN-252-lymph (lysine-(k) specific demethylase 1B)
Cytoskeleton and Adhesion Molecules	
3.22	Engulfment and cell motility 2

Table 4. Continued.

	mRNA concentrations greater in October than April
Signaling Molecules	
3.87	Mitogen-activated protein kinase kinase kinase 7 interacting protein 3
3.76	RAS-GEF domain containing family member 1C
3.68	Calcium/Calmodulin-dependent Protein Kinase ID-like
3.45	Interleukin 1 receptor accessory protein-like 1
3.40	Zinc Finger Protein 432
3.37	Ephrin Receptor (EphA7) variant
3.12	Enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase
3.06	M-phase phosphoprotein 6
Transport	
5.61	Synaptogyrin 3
4.99	Similar to Glutamate Receptor 6 isoform 1 precursor
4.61	Similar to Probable Phospholipid-transporting ATPase IF
4.16	Similar to Zinc transporter ZIF2
3.39	Similar to GPI deacylase
3.23	Exocyst complex component 5
3.19	NADH dehydrogenase (ubiquinone) Fe-S protein 3
3.14	Solute Carrier Family 37 (glycerol-6-phosphate transporter) membrane 4
Cell Cycle	
3.56	Family with Sequence Similarity 65, Member B
Unknown Function	
5.26	Unidentified transcripts on BTA2 position 41134753-41133427
4.88	Bos taurus chromosome 12 open reading frame 29 ortholog
4.03	Hypoxia Inducible Domain Family Member 1D
3.46	Transmembrane protein 150C
3.30	Transmembrane protein 144
3.27	Unidentified transcript on BTA11 position 53896241-53898321
3.16	Similar to Down syndrome cell adhesion molecule
3.12	Olfactory receptor 16-like
3.12	Similar to Reelin
3.11	Similar to Coiled-coil domain containing 44

*Differentially expressed genes ($p<0.01$) found in sperm collected from mature bucks during the months of April and October with an absolute Fold Change of 3.0 or higher.

Discussion

The long time theory is that the only contribution of sperm to the developing embryo is the paternal genome. Pessot et al. (1989) stained rat testis with RNAse-colloid gold and noticed that there was a high density of gold particles in the nucleus of elongated spermatids. Almost 30 years later, the topic of sperm containing RNA has been well documented (Ostermeier et al. 2002; Lalancette et al., 2008b; Garrido et al., 2009). The goal of this study was to identify transcripts that are differentially expressed in goat sperm during peak (October) and non-peak (April) breeding season. To ensure that the collected sample represented sperm RNA, samples were analyzed via microelectrophoregraph which indicated no presence of intact rRNA from epithelial cells. Microelectrophoretic profile of sperm demonstrated that sperm lacks the two major rRNA (18S and 28S), which is consistent with the profiles of sperm taken from other species (Ostermeier et al., 2002; Grunewald et al, 2005; Gilbert et al. 2007).
Since mature spermatozoa are translationally inactive, the 18S and 28S rRNAs should not be present because they are essential to protein assembly. Gur and Breitbart (2006) used RT-PCR to detect the presence of 18S rRNA in bovine spermatozoa; in addition, any translational activity is due to mitochondrial ribosomes while any cytoplasmic ribosomes are inactive. Therefore, any cytoplasmic rRNA in sperm would be detected in very low levels.

Sperm RNA are small in size which is not a confirmation of the absence of full length transcripts (Gilbert et al. 2007). Based on the microelectrophoregraph, sperm RNA appeared degraded. Gilbert et al. (2007) addressed the question on whether sperm

contained 5' or 3' degraded RNA. They found the presence of short 3' end RNA. Sperm RNA truncation made these samples difficult to analyze via normal microarray protocol. Agilent developed a protocol that optimizes the hybridization of truncated RNA to the array (Lucas and Lin 2009).

With this knowledge, the current research sought to examine the change in buck sperm transcripts as it relates to season. Sperm collected from mature Alpine ejaculates was analyzed on a 4 x44K Agilent bovine array produced 21,535 transcripts above background. From those genes, 1,318 genes had a fold change (FC) of 2 at $p \leq 0.05$. Gilbert et al. (2007) found 1,117 transcripts in bovine spermatozoa transcript using human arrays. This amount is lower than what was found in this and previous studies (Ostermeier et al., 2002; Zhao et al. 2006) which does not account for low detection rates but rather higher stringency ($p \leq 0.05$ vs. $p \leq 0.01$). Though this is the first study whose purpose is to look at breeding season transcript variation in goat sperm, the number of detected transcripts is similar to previous studies of other species.

For microarray validation, three genes (SPAM1, GK2, and MYCBP2) were chosen based altered expression levels found in males with teratozoospermia. Of the three genes, one (SPAM1) has been directly linked to fertility (Baba et al 2002, etc.). The expression of three genes was confirmed in mature bucks. The results for SPAM1gene expression being higher in April (non-peak season) were interesting. Highly conserved in mammals, SPAM1gene expression is not only localized in the testis and sperm cells, but have also been reported in the epididymus (Deng et al., 2000), accessory organs (Zhang et al., 2004), female reproductive tract (Zhang et al., 2003);

chondrocytes, synoviocytes and dermal fibroblasts (El Hajjaji et al. 2005). In the sperm, SPAM1 is the protein that allows for the interaction with the oocyte cumulus matrix and the secondary binding of the acrosome-reacted sperm to the zona pellucida (Hunnicutt et al 1995; Primakoff et al. 1985). Mice lacking the SPAM1 gene are fertile but have a reduced ability to disperse the cumulus mass (Baba et al., 2002). During spermatogenesis, the SPAM1 gene is expressed in the haploid cells. Zheng et al. (2001) revealed that both the mRNA and protein of SPAM1 is compartmentalized suggesting that SPAM2 is not evenly distributed among spermatids. Based on this knowledge, one would expect SPAM1 expression to be greater in the breeding season; since sperm quality and quantity decreases during the breeding season (Karaginnidis et al., 2000). It is plausible that the high level of SPAM1 during April was due to compensation for the decline in sperm numbers. Known for its regulation of glycerol uptake and metabolism, GK2 is a testis specific glycerol kinase. Though the expression of this gene has not been reported in sperm, high levels of the protein have been linked to asthenoospermia (Bharadwaj Siva et al., 2010). In the human, MYCBP2, an E3-ubiquitin ligase, is a regulator of DNA transcription. One can imply that the increased expression of GK2 and MYCBP2 genes during October is important in preparing the sperm for fertilization.

From previous studies, microarrays have been utilized to identify differences in spermatozoal RNA of fertile and infertile bulls (Lalancette et al., 2008; Feugang et al., 2010) and men (Lambard et al., 2004; Garrido et al., 2009). Before determining the variation in spermatozoal transcriptome between fertile and infertile bucks, this study sought to address the difference in breeding season. The transcripts found represent

different cellular functions and biological processes that are occurring during spermatogenesis. And though this study did not look at fertility, it would prove interesting to subject the buck sperm to a Percoll gradient and analyze the different density fractions during the peak (October) and non-peak (April) breeding seasons. Overall, this study has provided a unique analysis of spermatozoal mRNA which in the future may help identify genes that cause male factor infertility.

CHAPTER V

SUMMARY AND CONCLUSION

Summary

Detailed information about the genes associated with testicular growth, onset of puberty and spermatogenesis maybe the key to understanding male fertility. Clinical tests to screen for fertility include testis biopsies, analysis of sperm number, morphology, motility, chromatin quality and acrosomal integrity of semen. From testes development to spermatogenesis, there are multiple factors that have caused fluctuations in semen quality. To our knowledge, this is the first report that associates the histomorphology of the developing testis with the timing of seminiferous tubule gene expression in the buck; moreover, it is also the first to look at the seasonal changes in the sperm transcriptome.

In the first experiment, bucks were castrated at 0, 2, 4, 6 and 8 months of age. In the developing testes, there was an 8-fold increase of weight in the first 2 months. The 5.4-fold change in weight between 2 and 4 months corresponds to the start of puberty (Herrera-Alarcón et al., 2007).There was an absence of additional increase after 6 month of age. Schanbacher (1979) reported that after 6 or 7 months change in testicular weight was influence by other factors such as photoperiod will cause changes in weight.

To correspond to the histological changes, five genes (SOX9, HSPA8, STRA8, PRM1 and ODF2) that are expressed in cell types located in the seminiferous tubules. Northern blot results showed high levels of SOX9 and HSPA8 being expressed during 0

and 2 months of age, which correspond to tubule formation and increase in Sertoli and germ cells. After 2 months, the expression of these two genes begins to decline but remains present well into adulthood. This phenomenon is similar to reports in rodents (Krawczyl et al., 1988; Fröjdman et al 2000).

An opposite result was seen with the expression of STRA8, PRM1 and ODF2, where there was no signal during the first 2 months of development. These three genes are associated with germ cell commitment to meiosis and spermiogenesis (Hoyer-Fender et al., 1998; Dadoune, 2003; Horowitz et al., 2005; Carrell et al., 2007; Mark et al., 2008). The signal for STRA8 was high in cells that were committed to meiosis (Zhou et al.,2008). Problems with this gene would cause prolonged asynapsis and heterosynapsis that are important for chromosomal pairing and continuous premeiotic DNA replication (Mark et al., 2008). The strongest of all signals was observed in cells expressing PRM1 and ODF2.

In the second study, the objective was to identify transcript that are differentially expressed during breeding and non-breeding season. Studies have shown that the sperm transcriptome may provide insight to the event involved in spermatogenesis (Ostermeier et al., 2002). In order to ensure that samples represented sperm RNA, samples were subjected to microelectrophoregraph which reveal a profile similar to FFPE RNA. From previous reports with bovine sperm, sperm RNA is truncated (Gilbert et al., 2007). By using the FFPE Agilent protocol, sperm cDNA array hybridization was optimized (Lucas and Lin 2009). One thousand three hundred and eighteen genes were above background ($FC \geq 2$; $p \leq 0.05$), which was similar to results previously been reported in

bovine (Gilbert et al., 2007) and humans (Ostermeier et al., 2002; Zhao et al. 2006). Though significance was not found, qPCR was able to confirm the expression of SPAM1 (April), GK2 (October) and MYCBP2 (October).

Conclusion

There are a finite number of genes that interact with each other in order to make viable sperm capable of fertilizing the oocyte. This study sought to analyze genes that are expressed in the testes during specific stages of development and look at the seasonal transcriptome profile of ejaculated caprine sperm. Results from the study were comparable to that found in humans and rodents. In conclusion, findings from both studies provides a unique analysis of the developing testes and spermatogenesis, and through further studies aid in identifying genes that affect male fertility.

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