Abstract

STEWART, KARA ROSKI. Effects of FSH Administration During the Neonatal Period on Adult Sperm Production in Boars. (Under the direction of Dr. William Flowers.)

This study was designed to test the effects of neonatal FSH administration on sertoli cell mitosis and adult sperm production in boars. The treatment groups received 100µg/kg body weight of FSH (I.M.) every other day from either day 4-22 (early) or day 22-40 (late) after birth. Volumes of FSH were corrected weekly for increases in body weight. Four boars from each treatment group were castrated on days 21, 42, 98 and 330. The testes were evaluated for GATA-4 and PCNA activities and germ cell development. Blood samples were collected weekly through 9 weeks of age and then monthly after 59 weeks of age and evaluated for testosterone, estrogen, FSH, LH and IGF-1 concentrations. Four boars from each treatment group remained intact and were used for weekly semen collection until 77 weeks of age. Volume, concentration and total numbers of spermatozoa were recorded weekly and motility, mobility, and morphology analysis were performed monthly. All data was analyzed using SAS and proc GLM or proc Mixed, where appropriate. Boars in the early treatment group had an higher testosterone concentrations at 77 weeks of age compared to the late treated boars and the control boars ($p \leq 0.0001$). There were no differences among treatment groups in the mass of the seminiferous tubules ($p=0.4055$) or the number of sertoli cells per organ ($p=0.7010$). Only boars in the FSH treated groups had sertoli cells undergoing proliferation at day 98. The sperm concentration per ejaculate was higher for the early treatment group compared to controls or the late treatment group ($p=0.06$). The early treatment group also had increased numbers of primary spermatocytes as adults ($p=0.0005$). There were no effects of treatment on estimates of semen quality. Overall these results

indicate that neonatal FSH treatment increased the length of time over which sertoli cell proliferation occurs during sexual maturation which resulted in an increase in the production of spermatozoa in adults.

Effects of FSH Administration During the Neonatal Period on Adult Sperm Production In Boars

by
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Biography

Kara Stewart was born July 28th, 1979 to Kim Kercheval and Richard Roski in Cleveland, Ohio. Her parents already had one daughter, Katie. When she was two, her mother and sister moved to Carmel, Indiana. Her father re-married Debbie Roski and blessed Kara with another sister, Courtney, and a brother, Justin. The author attended grade school and high school in Carmel, Indiana.

In August of 1997, Kara followed in many of her family's footsteps and began working on her bachelor's degree in Animal Science at Purdue University in West Lafayette, Indiana. In August of 2002, Kara moved to Raleigh, North Carolina to where she entered a graduate program in the Animal Science Department at North Carolina State University. Kara earned her Master's degree in Reproductive Physiology in December of 2004 under the direction of Dr. William Flowers. In May of 2005, Kara returned to North Carolina State University to pursue her Ph.D. in Animal Science.

While attending graduate school, Kara met and married Corey Stewart. They share their home with four rescued dogs and are expecting their first baby in July of 2008.

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Sexual and Gonadal Development

Sexual development begins at the level of the chromosome. The male can either deliver an X or a Y chromosome to the oocyte. The Y chromosome is responsible for male sexual development. In early embryos, primordial germ cells originating from the yolk sac migrate to the hindgut and reside in the urogenital ridge (Cupp and Skinner, 2005). The urogenital ridge is comprised of three segments of coelomic epithelium; the pronephros is the most anterior portion, the mesanephros is the middle segment and the metanephros is the posterior segment (Capel, 2000). The urogenital ridge develops at 18-20 days post coitum (d.p.c.) in the pig and 10-11 d.p.c. in the rat (Cupp and Skinner, 2005). The indifferent gonad develops as a paired structure from a thickening in the epithelium of the mesoderm (Capel, 2000). It contains three cell types, the germ cells, the coelomic epithelial cells and the interstitial cells of the genital ridge (George and Wilson, 1994). This indifferent gonad is similar between males and females therefore sexual differences are not evident at this time. The bipotential gonad can be identified in the pig at 21 d.p.c. and at 11.5-12.5 d.p.c. in the rat (Cupp and Skinner, 2005, Colenbrander et al., 1982a). About the same time, the mesonephric duct forms from the pronephros and the Müllerian duct forms from the mesonephros. In male sexual development, the mesonephric duct forms the epididymis and the vas deferens. In female sexual development the müllerian duct will give rise to the oviducts, uterus, upper vagina and cervix (Senger, 2005, Capel, 2000). There are a few genes thought to be responsible for the formation of the mesonephric and müllerian ducts, Wilm's

Tumor 1, Pax2 and Wnt (Capel, 2000).

The area of the Y chromosome thought to be responsible for male sexual development is the SRY gene (Koopman et al., 1991). The SRY gene is thought to begin a cascade of events leading to male sexual development. In the rat, expression of the SRY gene is maximal at 11.5 d.p.c. in the coelomic epithelial cells correlating with an increase in proliferation of these cells. These epithelial cells give rise to the sertoli cells (Morrish and Sinclair, 2002, Cupp and Skinner, 2005). These pre-sertoli cells will aggregate with the primordial germ cells and form the testicular cords (George and Wilson, 1994, Cupp and Skinner, 2005) which are visible at 26 d.p.c. in the pig (Pelliniemi, 1975, McCoard et al., 2002) and 13.5-14 d.p.c. in the rat (Cupp and Skinner, 2005).

Jost (1947) removed the indifferent gonad from developing rabbit fetuses and found that all rabbits developed as female. It was found that secretions from the testis were needed for male sexual development. A peptide hormone was needed to regress the müllerian duct and a steroid hormone to maintain the Wolffian duct (Jost, 1953, Jost 1972). This peptide hormone is called Müllerian Inhibiting Substance (MIS) or anti-müllerian hormone (AMH). MIS is produced by the sertoli cells at 26 d.p.c. in the pig and 11.5 d.p.c. in the rat (McCoard et al., 2002, Chapel, 2000, Morrish and Sinclair, 2002). The concentration of MIS increases until after birth (Colenbrander et al., 1982a). The Sox-9 gene, which is up-regulated by SRY, seems to be involved in the production of MIS in the presence of Steroidogenic Factor 1 (Cupp and Skinner, 2005). The Dax-1 gene is also down-regulated in male mice at 12.5 d.p.c.

and therefore may also play a role in the production of MIS and male differentiation (Cupp and Skinner, 2005). The transcription factor, GATA-4 is also expressed in sertoli cells compared with ovarian cells at this time and may play a role in MIS gene expression (Cupp and Skinner, 2005). At this time primordial germ cells are located within the lumen of the testicular cords and are surrounded by sertoli cells (Pelliniemi, 1975, Colenbrander et al., 1982a). At 28 d.p.c. in the pig, P450c-17, a steroidogenic enzyme, can be detected in the testis suggesting differentiation of the leydig cells (McCoard et al., 2002). Other enzymes involved in steroid hormone pathway increase such as hydroxysteroid dehydrogenase (George and Wilson, 1994). Leydig cells produce steroid hormones, namely testosterone, that are responsible for differentiation of the wolffian ducts into the epididymis, vas deferens and seminiferous tubules (Morrish and Sinclair, 2002).

Testosterone from the testis produced during fetal development has profound effects on the brain. The hypothalamus is inherently female. The testosterone from the testis gets converted into estrogen by the brain and blocks the formation of a gonadotropin releasing hormone surge center in the hypothalamus. In the developing pig, hypothalamic control of gonadal development does not appear to begin until 70 d.p.c. as determined by in utero decapitation of fetuses. It was found that before 70 d.p.c. decapitation did not disturb the developing populations of sertoli and leydig cells (Van Vorstenbosch, et al., 1984). Gonadal receptors for FSH are present around 28 d.p.c. in the pig; however, the pituitary does not produce FSH until 65 to 70 d.p.c. (Allan and Handlesman, 2005). Luteinizing hormone becomes detectable in the pig around 80 d.p.c. (Colenbrander et al., 1982a). Pituitary LH and FSH are seen between 16 and 17 d.p.c. in the rat which coincides with an increase in the

LH and FSH receptor concentration (Cupp and Skinner, 2005). Therefore the early fetal period of gonadal development is not under hypothalamic control. The testosterone and estradiol produced during this time provide strong negative feedback on the hypothalamus to decrease GnRH production.

The testis will continue to grow and develop during the late fetal and neonatal periods. The testis will descend into the scrotum between 80 and 90 d.p.c. in the pig. The sertoli and leydig of the testicle which began to divide and grow in utero will continue to divide during the postnatal period. The developing gonocytes will enter mitotic arrest during the later fetal period and not resume mitosis until puberty.

Puberty can be defined in many ways. One of the most common is the period of time when a boar is capable of successful reproduction. This would include: the ability to produce viable spermatozoa capable of fertilization; possessing the desire to mount a sow during natural mating or a dummy for artificial insemination; ability to extend the penis; and ejaculate. As puberty approaches, a desensitization of the negative feedback of testosterone and estradiol on GnRH secretion from the hypothalamus occurs. Therefore the boar begins to produce higher concentrations of GnRH and the subsequent release of LH and FSH from the pituitary. In addition there is an increase in the amount of testosterone produced. The net result of the increase in these hormones is an increase in sexual behaviors and spermatogenesis.

Sexual Behaviors in Pigs

Differences in the behaviors of male and female pigs can be seen as early as 1 month of age where male pigs can be seen mounting other pigs more readily than females (Ford, 1990). In the swine industry, semen is collected from a post-pubertal boar that is trained to mount a dummy sow, extend and ejaculate as a result of manual stimulation from a trained technician. In order for the boar to be able to be trained to collect, they must first express a sexual desire, or libido, to mount the dummy. Then they must be physically capable of thrusting behaviors, extension of the penis and copulation. Hemsworth and Tilbrook (2007) reviewed the sexual behaviors of male pigs and reported that 49-50% of culled boars were due to problems with sexual behaviors or poor semen quality. Of additional importance is the notion that there are different levels of sexual behaviors among boars that possess a sexual desire. In other words, some boars have a higher libido than others. There is conflicting evidence as to whether measures of libido correlate with an increase in sperm production and other measures of semen quality (Levis and Reicks, 2004).

Sexual behaviors in pigs are under the hormonal control from the gonadal steroid, testosterone (Hemsworth and Tilbrook, 2007). In early development, before sexual differentiation, the brain is inherently female and will be masculinized via androgens during critical points in development (Ford and D'Occhio, 1988). Defeminization of sexual behaviors occurs as a result of an increase in testosterone concentrations around the time of puberty in the pig (Ford, 1990). There appears to be a threshold concentration of testosterone that must be achieved in order for sexual behaviors to be seen. However, concentrations above this threshold do not result in an increase in libido (Hemsworth and Tilbrook, 2007).

Testosterone may not be acting alone in regulating sexual behaviors in the male. Estrogens may also play a role. Hemsworth and Tilbrook (2007) reviewed the effects of estrogen and testosterone on re-establishing sexual behaviors after castration. Testosterone was found to completely restore sexual behaviors in the male where as estrogen only partially restored sexual behaviors and only over a short period of time. Prostaglandins have also been shown to affect sexual behaviors in the boar. Prostaglandins were successful at stimulating sexual behaviors in immature boars, but similar results have not been found when treating adult boars (Estienne and Harper, 2004, Estienne et al., 2007).

Libido can also be affected by social environment during development. In 1979, Hemsworth and Beilharz raised boars with and without contact with other pigs. They found that boars that had no visual or physical stimulation with other pigs had decreased libidos compared to social pigs. This decrease in libido was greater depending on the age at which social restriction began, the earlier restriction began resulting in a greater decrease in libido. Exposure to females can also increase libido in the boar during pre-pubertal development as well as post-pubertally (Hemsworth and Tilbrook, 2007).

As stated earlier, the boar has to not only have a sexual desire, but must also be capable of mounting the dummy, extending his penis and successfully ejaculating. Management practices must also attempt to decrease the occurrence of penile injuries that would deter a boar from extension of the penis and ejaculation. The boar must also be maintained at a reasonable body condition score allowing his hind legs to support the weight of his body during collection as well as to support the thrusting behaviors prior to extension of the penis.

Testicular Function in the Adult

In order to completely understand spermatogenesis in the adult, an understanding of the anatomical and functional characteristics of the testicle is needed. The adult mammalian testis serves two basic functions. The first is to produce steroid hormones and the second is to produce spermatozoa, both being regulated by the hypothalamus and pituitary gland. The neurons of the hypothalamus produce a protein hormone called gonadotropin-releasing hormone (GnRH). GnRH is produced in a pulsitile fashion in the male (Senger, 2003). GnRH travels through the hypothalamo-hypophyseal portal system and acts on the anterior lobe of the pituitary gland stimulating the pulsitile release of gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (Senger, 2003). These gonadotropin hormones act on the gonads to stimulate testicular function in pigs (Esbenshade et al., 1990).

The parenchyma of the testis can be divided into two main compartments, the interstitial compartment and the tubular or seminiferous compartment surrounded by a capsule called the tunic (Amann and Schanbacher, 1983, O'Donnell et al., 2001). The interstitial compartment consists of lymph cells, connective tissues, blood vessels and leydig cells while the tubular compartment consists of the seminiferous tubules and the cells associated with the tubules, namely the sertoli cells, peritubular cells and germ cells (Senger, 2003). Hormones and nutrients move into and out of the testis through the blood and lymph vessels of the interstitial compartment (O'Donnell et al., 2001). The tubular compartment can be divided into two compartments, the basal and the adluminal compartment (Amann and Schanbacher, 1983). Separating these two compartments is the blood-testis barrier. The

sertoli cells lie on the basement membrane of the seminiferous tubule. Under the basement membrane are the peritubular myoid cells (O'Donnell et al., 2001). Junctional complexes form between adjacent cells forming the blood-testis barrier. These complexes are comprised of proteins from three classes, the occludins, claudins and junctional adhesion molecules (Hess and Franca, 2005). This barrier is impermeable to large molecules and immune cells. The basal compartment is the region that is associated with the basement membrane and the interstitial compartment is adjacent to the lumen of the seminiferous tubule. Therefore the basal compartment of the seminiferous tubule is not in direct contact with the blood or interstitial fluid (Senger, 2003, Amann and Schanbacher, 1983). These compartments are also associated with different cell types. The basal compartment is associated with stem cells and mitotic germ cells or spermatogonia while the interstitial compartment is associated with meiotic spermatocytes and differentiating spermatocytes (Amann and Schanbacher, 1983).

The three main cell types found in the testis are leydig cells, germ cells and sertoli cells. Steroidogenesis takes place in the leydig cells and is stimulated by LH (Amann and Schanbacher, 1983). The leydig cells contain membrane bound receptors for LH. Luteinizing hormone stimulates the conversion of cholesterol into pregnenalone in the mitochondria of the leydig cells, which is the rate-limiting step in testosterone production (Amann and Schanbacher, 1983). The amount of testosterone produced is correlated to the amount of smooth endoplasmic reticulum in the cell (Amann and Schanbacher, 1983). A negative-feedback mechanism exists in the testicle where sustained high levels of LH result in a decrease in the number of LH receptors on the cell and a subsequent decrease in

testosterone production (Senger, 2003). This testosterone produced by the leydig cells is converted to dihydrotestosterone and estrogen by the sertoli cells (Senger, 2003). The leydig cells also produce other steroid hormones depending on the species. In the boar, the leydig cells produce high levels of estrogen (Amann and Schanbacher, 1983).

In 1865, Enrico Sertoli called the sertoli cells of the testicle "mother cells" (Sertoli, 1865, Hess and Franca, 2005). This is because the sertoli cells are responsible for providing the nourishment to developing germ cells by transporting nutrients and regulatory factors across the tight junctional complexes of the blood-testis barrier (Griswold and McLean, 2005). Follicle stimulating hormone stimulates the sertoli cells. The sertoli cells are the only testicular cells containing FSH receptors thereby providing the indirect effects of FSH on spermatogenesis (Amann and Schanbacher, 1983). The response of the sertoli cell to stimulation by FSH is to produce proteins, namely androgen binding protein (ABP) and inhibin (Senger, 2003, Amann and Schanbacher, 1983). Androgen binding protein binds testosterone and plays a role in the transport of testosterone as well as regulating the concentration of free testosterone. Inhibin acts on the pituitary gland to decrease the release of FSH (Amann and Schanbacher, 1983). There are many other proteins produced by sertoli cells. These can be classified into four categories: transport proteins; proteases and protease inhibitors; membrane glycoprotiens; and growth and paracrine factors. All of these proteins are involved in sertoli-sertoli cell interactions or sertoli-germ cell interactions to increase spermatogenesis (Griswold and McLean, 2005). Estrogen from the sertoli cells and testosterone from the leydig cells provide negative feedback on the hypothalamus to decrease GnRH secretion, subsequently decreasing gonadotropin secretion (Senger, 2003).

The structure of the sertoli cell is unique. One of the most distinguishing characteristics of sertoli cells is the large, irregular shaped nucleus and its tripartite nucleolus (Hess and Franca, 2005, de Kretser and Kerr, 1994). The nucleus lies near the basement membrane in young animals and the will migrate toward the lumen at different stages of testis development (Hess and Franca, 2005). The cytoplasm contains many other cellular structures that play a role in sertoli cell function. The basal region of the cell has a greater abundance of organelles than the regions closer to the lumen (de Kretser and Kerr, 1994). Mitochondria are abundant in the cytoplasm suggesting high metabolic activity (Hess and Franca, 2005). Smooth endoplasmic reticulum is also present playing a role in steroid and lipid metabolism as well as protein production (Hess and Franca, 2005, Bardin et al., 1995). The sertoli cells are capable of phagocytosis of germ cells and residual bodies (Hess and Franca, 2005, de Kretser and Kerr, 1994). Therefore, products of phagocytosis are also present in the cytoplasm, the lysosomes and multivesicular bodies (Hess and Franca, 2005). There is a small golgi apparatus with defined membranes, but the sertoli cell lacks any other membrane-bound vacuoles usually present in protein producing cells (Hess and Franca, 2005, de Kretser and Kerr, 1994). The cytoskeleton is made up of elements involved in cellular structure and polarity as well as the interactions of sertoli cells with other cells of the testicle (Hess and Franca, 2005).

At puberty, specialized tight junctions form between adjacent sertoli cells forming the blood-testis barrier. These junctions form from the union of cytoplasmic projections on adjacent sertoli cells (de Kretser and Kerr, 1994). This barrier separates the seminiferous tubule into the basal and adluminal compartments. The blood-testis barrier prevents large

molecules from the blood from entering the adluminal compartment, but not the basal compartment (de Kretser and Kerr, 1994). The barrier's main function seems to be to provide a highly specialized microenvironment for zygotene through pachytene meiotic germ cells (Setchell, 1979, de Kretser and Kerr, 1994). These inter-sertoli junctions are not present at birth, but develop as puberty approaches. In the pig, tight junctions appear at 6 weeks of age (Colenbrander, 1982) and a functional blood-testis barrier is present at 120 days of age (Tran et al., 1981). Developing germ cells migrate toward the seminiferous lumen, and permeate the barrier. It is thought that this is accomplished by breaking the tight junctions and forming new junctions below themselves in order to maintain the integrity of the barrier (de Kretser and Kerr, 1994). Some hormones, including FSH, are impermeable to the adluminal compartment. In 1977, Orth and Christensen radioactively labeled FSH to determine its site of action in the testis. They found that the receptors were located at the basal region of the sertoli cell. The products of sertoli cells, namely ABP and inhibin, are released at a slower rate in the adluminal compartment compared to the basal compartment (Setchell, 1979). The barrier also functions to protect the germ cells from toxins (Setchell, 1979).

Sertoli cells interact with other cells of the testis, some via direct contact and others through regulatory mechanisms. The leydig cell is an example of a cell that is critical for normal function of sertoli cells, but is not in direct contact with them (Skinner, 2005). It is well established that FSH and testosterone are required for spermatogenesis to occur, although each exerts its actions at different stages of development (Sharpe, 1994). Testosterone is a product of the leydig cells resulting from stimulation by LH from the

anterior pituitary. Some of this testosterone is converted to dihydrotestosterone by the sertoli cells and is involved in negative feedback on the hypothalamus (Senger, 2003). The pathway of how androgens produced in the leydig cells stimulate the sertoli cells is still unclear (Skinner, 2005). *In vitro* studies have shown that sertoli cells can also play a role in the regulation of leydig cell function. Follicle stimulating hormone acts to increase the secretion of a substance from the sertoli cells that functions to maintain steroidogenesis in the leydig cell and increase the number of hCG receptors (Bardin et al., 1994, Saez et al., 1986). Bardin et al. (1994) speculated that this substance could be inhibin. Estrogen produced in the sertoli cell may play a role in decreasing steroidogenesis (Skinner, 2005). The *in vivo* effects of the sertoli cell-leydig cell interactions need to be studied further.

Outside the basement membrane are the peritubular myoid cells that also interact with sertoli cells. In the adult, these cells function to cause stage-related contractions to release sperm into the lumen of the seminiferous tubule (Skinner, 2005). *In vitro* co-culture studies of sertoli and myoid cells showed that the interaction of these two cell types was responsible for producing products to maintain the basement membrane integrity (Skinner, 2005). The interaction of these cells also stimulates protein and enzyme production by sertoli cells (Bardin et al., 1994). Buzzard et al., (2003) co-cultured sertoli cells and peritubular cells of rats and determined that activin of peritubular cell origin stimulated proliferation of sertoli cells in an age-dependant manner. This study cultured sertoli cells from 3, 6 or 9 day old rats in the presence of activin for 2 or 8 days. Sertoli cells from 3 day old pups did not respond to treatment with activin when only cultured for 2 days, but did increase in proliferative activity when treated for 8 days. Cells from the older pups were responsive to activin after 2 and 8

days of culture. Therefore, sertoli cells in the 3 day old rat pup requires an increase in the length of exposure to activin in order to have proliferation stimulated compared to older pups. Peritubular cells also appear to differentiate in response to indirect stimulation by sertoli cells (Skinner, 2005).

The developing germ cells are embedded within the sertoli cells in highly organized arrangements. As the developing germ cell changes morphologically, the structures of the sertoli cell also change (Bardin et al., 1994). The changes occur in the nucleus, lipid volume, enzyme secretion and protein secretion of the sertoli cell (Bardin et al., 1994). Most of these changes are cyclic and occur with specific stages of the seminiferous cycle (Bardin et al., 1994).

The sertoli cells produce a vast array of proteins, proteases, growth factors and paracrine regulators that function in regulating androgen secretion and spermatogenesis. One of the first proteins discovered in rats was androgen-binding protein (ABP). Androgen binding protein has a high affinity for binding androgens, namely 5α-dihydrotestosterone and testosterone with a lesser affinity (Courot, 1980). ABP production is regulated by FSH and testosterone (Courot, 1980, Sharpe, 2005). ABP is transported from the testis to the epididymis where its androgen binding abilities decrease or diminish completely as it travels from the caput to cauda epididymis (Courot, 1980). This transport is controlled by FSH (Courot, 1980). Secretion of ABP is regulated also by the presence of spermatids, therefore depending on the stage of the seminiferous cycle (Sharpe, 1994, 2005). The patterns of secretion vary greatly among species (Sharpe, 1994). Pigs have not been shown to produce ABP in the testis (Courot, 1980).

Other proteins important in the movement of metals to the adluminal compartment have been identified. These are transferrin which transports iron; and ceruloplasmin which transports copper (Bardin et al., 1994). Transferrin production was stimulated by FSH and testosterone (Bardin et al., 1994). The sertoli cell production of specific proteins needs to be elucidated. The enzyme plasminogen activator is also produced by sertoli cells and thought to play a role in the release of spermatids into the lumen of the seminiferous tubule or the migration of germ cells through the epithelium (Bardin et al., 1994). This enzyme production is stimulated by FSH and increases 10-fold at the stage when primary spermatocytes are present in the seminiferous epithelium (Monet-Kuntz and Fontaine, 1989).

Sertoli cells produce growth factors that are responsible for the growth and development of the germ cells. These growth factors also regulate growth and maintenance of the other cells of the testis (Bardin et al., 1994). Some examples of these growth factors are insulin-like growth factors I and II, transforming growth factor alpha and beta, fibroblast growth factor, the neurotropin family of growth factors, cytokines, stem cell growth factor and glial cell-derived growth factor (Skinner, 2005).

The paracrine function of the sertoli cells also plays an important role in regulating germ cell development. Inhibin and activin are protein hormones produced by the sertoli cells (Ying, 1987). Inhibin is made up of an alpha and a beta subunit connected by disulfide bonds whereas activin is made up of two beta subunits (Ying, 1987, Skinner, 2005). Inhibin inhibits FSH secretion from the sertoli cells while activin stimulates FSH secretion (Ying, 1987).

Overall, the testicle is a unique organ consisting of specialized cells for the production of steroid hormones and spermatozoa. Many of the functions of the testicle are regulated by the hypothalamus and pituitary. In addition, all of the cells within the testicle communicate and interact with each other in order to for these two processes to occur. The actual process of producing viable spermatozoa can be better understood with an understanding of the anatomical location and function of the different cell types of the testicle.

Spermatogenesis

Spermatogenesis is the process of continuously producing mature spermatozoa that are capable of fertilizing the female oocyte. This process takes place in the seminiferous tubule (de Kretser and Kerr, 1994, Senger, 2003). Spermatogenesis can be divided into three phases: mitotic stem cell renewal; meiotic reduction of chromosomal number; and spermiogenesis (de Kretser and Kerr, 1994). There are four categories of spermatogonia found in the boar: undifferentiated spermatogonia; differentiated spermatogonia; intermediate spermatogonia; and B spermatogonia (Frankenhuis et al., 1982). In order for spermatogenesis to be a continuous process in the male, stem cell renewal must take place (de Kretser and Kerr, 1994). Mitotic stem cell renewal has been reviewed by de Kretser and Kerr, 1994, and involves the random dividing of undifferentiated stem cells (As) to produce replicates of themselves. These paired spermatogonia (Apr) divide and are joined by intracellular bridges, and called aligned spermatogonia (Aal). These aligned spermatogonia are capable of differentiating into A_1 spermatogonia. Six mitotic divisions follow resulting in

A_2-A_4 spermatogonia, intermediate spermatogonia (I) and B spermatogonia (B) (Senger, 2005). The final mitotic division of the B spermatogonia results in two primary spermatocytes. Meiosis consists of two divisions of the diploid primary spermatocytes (2N) into haploid secondary spermatocytes (2N). These cells then divide again resulting in haploid spermatids (N) (de Kretser and Kerr, 1994). These meiotic divisions all occur in the adluminal compartment whereas the mitotic divisions occur in the basal compartment of the seminiferous tubule (Sharpe, 1994). Without germ cell death and apoptosis, each diploid primary spermatocyte would give rise to 4 haploid spermatids and this population will represent the number of spermatozoa that the sertoli cells should release into the seminiferous tubule lumen (Senger, 2003, Sharpe, 1994).

The final phase of spermatogenesis is differentiation or spermiogenesis where a spermatid undergoes morphological changes to become a mature and motile spermatozoa (de Kretser and Kerr, 1994). This phase can be sub-divided into four phases. Senger (2003) describes these phases as the golgi phase, the cap phase, the acrosome phase and the maturational phase. In the golgi phase, small vesicles of the golgi apparatus of the developed spermatid will fuse resulting in a large acrosomic vesicle. Also during this phase, the longitudinal and transverse centrioles migrate to the opposite end of the cell from the acrosomic vesicle. The cap phase follows the golgi phase. Here the acrosome flattens over the nucleus forming a cap. Also, the axoneme elongates from the longitudinal centriole forming the axial filament of the tail. The acrosome continues to elongate around the nucleus in the acrosomal phase. The nucleus also elongates down toward the elongating tail. Small microtubules will form called manchette and attach to the end of the acrosome and extend

down into the plasma membrane. The neck of the spermatozoa forms from the transverse and longitudinal centrioles. Dense fibers attach to the outside walls of the axial filament (de Kretser and Kerr, 1994). A reduction in cytoplasmic volume also occurs during elongation (Sharpe, 1994). Finally the maturation phase occurs where the manchette microtubules form the postnuclear cap under the acrosome. Mitochondria aggregate below the neck of the spermatozoa and form the mid-piece of the tail (Senger, 2005). The entire spermatozoa remains covered in a plasma membrane. DNA transcription in these elongated spermatids will decrease and then stop under the genomic control of the gamete (Dacheux et al., 2005). The final step in the process of spermatogenesis is called spermiation where the mature spermatozoa is released into the seminiferous tubule lumen (Senger, 2003). Just prior to this time, remaining cytoplasm of the sperm is released in the form of a residual body and is degraded by the sertoli cell (Sharpe, 1994).

The process of spermatogenesis can be functionally divided into stages along the seminiferous tubule. The stages are based on different cellular associations in a cross-section of the seminiferous tubule. In any give cross-section, there are four to five germ cells. There association with each other and their location within the seminiferous tubule are used to distinguish the various stages of spermatogenesis (Amann and Schanbacher, 1983, Swierstra, 1968, Sharpe, 1994, Senger, 2003). There are 12 stages where the cell types appear similar. These are the stages of the seminiferous cycle (Amann and Schanbacher, 1983, Frankenhuis et al., 1982). These stages are based on the stage of acrosome development and nuclear shape of the developing germ cell and the function of the sertoli cell at that point (Frankenhuis et al., 1982, Sharpe, 1994). The length of the stage is constant ending when the

types of germ cells present have developed into the cells characteristic of the following stage (Sharpe, 1994). Due to the consecutive nature of the cycles, in a cross-section of the same area of the tubule over time, all stages in the cycle would be identified. The length of the entire cycle is the summation of all stages in the cycle. In the boar, the length of the seminiferous cycle is 8.6 days (Swierstra, 1968). It takes 4.5 cycles to complete spermatogenesis in all species assuming the starting point is stem cells dividing into A spermatogonia (Sharpe, 1994). Therefore spermatogenesis in the boar takes 34-48 days (Swierstra, 1968). Spermatogenesis in the seminiferous tubule occurs in waves down the tubule. This refers to the sites where spermiation, or release of spermatozoa into the lumen, is occurring along the tubule at any given moment in time. There is a distance between each spermiation site down the tubule remaining constant (Senger, 2003).

Testosterone and FSH are both required for spermatogenesis to take place. However, the hormones are required at different stages of the seminiferous cycle. The effects of testosterone withdrawal have been extensively studied in rats. One approach is to eliminate testicular testosterone production. The most popular methods to accomplish this are hypophysectomy, administration of ethane dimethane sulfonate which destroys the leydig cell population, immunoneutralization of LH or administration of GnRH analogs (Sharpe, 1994, 2005). Each of these methods eliminates testicular testosterone production via a different mechanism. Testosterone withdrawal is faster using some and slower in others. In the rat, the result of testosterone withdrawal is a loss of stage VII and VIII germ cells within 3-5 days (Sharpe, 1994, 2005). This germ cell loss can partially be reversed by high-doses of testosterone or by administration of LH (Sharpe, 1994). These stages coincide with the time

when the androgen receptor concentration is the greatest and the FSH receptor concentration is the lowest (Sharpe, 2005, Huhtaniemi and Toppari, 2005). Therefore, testosterone is primarily responsible for the structural and functional changes that germ cells undergo at stages VII and VIII (Sharpe, 1994). The subsequent stages are dependant on the changes in stages VII and VIII (Sharpe, 1994). At the end of the androgen dependant phase, FSH dependency begins. However, in hypogonadal mice which lack LH and FSH, supplemental androgens can maintain spermatogenesis in all stages (O'Donnell et al., 2001). The FSH receptor concentration and FSH binding is highest during stage II through stage XII (Huhtaniemi and Toppari, 2005). Therefore FSH regulates the number of B-spermatogonia as well as maintains spermatid numbers (Sharpe, 1994). There are FSH dependant stages and testosterone dependant stages and both hormones are required for complete spermatogenesis. The absence of FSH in mice results in smaller testes and reduced sperm numbers (O'Donnell et al., 2001). Estrogen has also been implicated in the maintenance of spermatogenesis. Studies in rats have shown that administration of estrogens decreases sperm output probably by increasing LH secretion and/or decreasing testosterone production (Sharpe, 1995). Some studies have suggested that oxytocin, prolactin and insulin also play a role in the regulation of spermatogenesis (Sharpe, 1994), but the mechanisms associated with each need to be elucidated.

In rats and pigs, FSH production also coincides with the period of pre-pubertal sertoli cell mitosis (Allan and Handelsman, 2005, Franca et al., 2000). In rats, the response to FSH stimulation in the adult is activation of a cyclic AMP-mediated pathway and subsequent protein production. In young animals, an additional cascade is initiated by FSH

through the ERK/MAP kinase pathway resulting in additional protein production (Huhtaniemi and Toppari, 2005). Once sertoli cell mitosis has ceased, in the post-pubertal animal, this additional pathway is blocked by FSH (Huhtaniemi and Toppari, 2005). Therefore FSH may regulate spermatogenesis at specific stages through regulation of sertoli cell protein production as well as play an important role in development of the adult sertoli cell population.

Once sperm cells are released into the lumen of the seminiferous tubule, they come in contact with the rete testis. The rete testis is a network of tubules that carry sperm from the seminiferous tubules of the gonad to the efferent ducts and into the epididymis (Setchell, 1970). The spermatozoa come in contact with the rete testis fluid when they are released from the seminiferous tubule and migrate to the epididymis.

As mentioned earlier, just prior to leaving the testicle, the gametes undergo morphological changes known as spermiogenesis. This process is regulated by the gamete's DNA. However once outside of the testicle, transcription of DNA in the gamete stops (Dacheux et al., 2005). At this point, the sperm cell is not motile or fertile. Additional biochemical and morphological modifications must be made in order for the sperm to gain these functions. These changes take place in response to the microenvironment provided by the epididymis.

The epididymis is a long tubule derived from the mesonephric duct consisting of three distinct segments: the caput (head); corpus (body); and cauda (tail). Each has unique characteristics and functions in sperm maturation (Dacheux et al., 2005, Franca et al., 2005).

There are two main cell types located in all three segments of the epididymal duct. These are referred to as principle and basal cells. Similar to the blood-testis barrier formed from adjacent sertoli cells in the testis, the principle cells of the epididymis form tight junctions and a functional blood-epididymal barrier (Dacheux et al., 2005). There are additional cell types that are present in a segment specific manner along the epididymal duct such as the clear, narrow, apical and halo cells. These cells function in endocytosis and acidification of the luminal fluid (Franca et al., 2005). All of these cells work together to form a unique, complex exocrine organ.

As the sperm cells enter the epididymis, they become highly concentrated due to water resorption in the efferent duct and the initial segment of the caput epididymis (Dacheux et al., 2005). As much as 96% of the fluid in boars is absorbed at this point (Pruneda et al., 2005). This concentration allows the sperm cells to come in contact with the epithelium of the epididymis as well as the fluid that is produced from its epithelium. The epididymal fluid consists of hundreds of different proteins, many secreted from the principle cells (Dacheux et al., 2005, Franca et al., 2005). The protein profiles are different for each of the segments along the tubule providing many unique microenvironments for the developing sperm cells (Dacheux et al., 2005). Proteins in the epididymis function to prevent oxidative damage to the sperm membranes; change the protein and lipid composition of the sperm membrane; and provide immunological protection for the sperm cells (Dacheux et al., 2005).

Many of the functions of the epididymis are controlled via hormones. Dihydrotestosterone, produced by the principle cells, is thought to regulate maturation and transport of sperm cells. Estrogens play a vital role in fluid absorption within the tubule as

well as mediating the smooth muscle contractions of the tubule via regulation of oxytocin receptors in the epithelium. Vitamin E may also play a role in maintaining the structure of the cells within the tubule (Franca et al., 2005).

Movement of sperm cells along the tubule is controlled by oxytocin, and the sympathetic nervous system (Franca et al., 2005). The rate at which sperm travel along the tubule can affect fertility of the sperm cells. Acceleration through the caput and corpus is associated with a decrease in the production of normal sperm (Franca et al., 2005). In other words, there is a specific length of time that the sperm need to remain in the first two segments of the epididymis in order to reach their full fertilizing ability. In contrast, the cauda epididymis serves mainly as a storage site for sperm before ejaculation. The length of time spent in the caput and corpus varies between species with boars being 5.4-7.0 days (Franca et al., 2005). In boars, the sperm cells remain in the cauda epididymis for 6.4 days (Franca et al., 2005). The sperm cells are released from the cauda epididymis during ejaculation or, if ejaculation does not occur, they undergo apoptosis and are reabsorbed.

During ejaculation, the sperm cells come in contact with the secretions from the secondary sex glands: the prostate; the vesicular glands; and the bulbourethral glands. The majority of the fluid in an ejaculate comes from these glands (Luke and Coffee, 1994). This fluid contains proteins, enzymes, hormones and many other biological substances (Luke and Coffee, 1994). Although these secretions do not appear to be essential to the sperm's ability to fertilize the egg, they may optimize the conditions that lead to a successful fertilization (Luke and Coffee, 1994). The vesicular glands are well developed, paired structures that provide the majority of the fluid volume of semen in the boar. The boar also has a

disseminate prostate gland and large, pronounced bulbourethral glands. The bulbourethral glands produce a viscous secretion known as the gel fraction which is thought to form a plug in the sow's cervix preventing the loss of semen out the vagina during natural service.

Seminal plasma contains many compounds such as sugars, amines and lipids. The seminal plasma of the boar is also rich in proteins. These proteins serve many different functions including protection of spermatozoa from reactive oxygen species; suppression of the immune response in the female reproductive tract; and binding to and modifying the sperm cell for transport in the female reproductive tract (Strzezek, 2002). A unique group of proteins, called spermadhesins, has also been identified in the boar's seminal plasma. These proteins are required for capacitation, the acrosome reaction , and sperm-gamete interactions (Strzezek, 2002). It is believed that these proteins may have profound effects on fertility. Two proteins have been identified in the pig that may be good predictors of fertility (Flowers, in press). The first protein has a molecular weight of 26 kilo Daltons and a p.I. of 6.2 while the second protein has a molecular weight of 55 kilo Daltons and a p.I. of 4.8 (Flowers, in press).

The secondary sex glands also produce a variety of hormones. Of particular interest are the high concentrations of estrogen found in the boar's seminal plasma. Estrogen is thought to function in the sow's reproductive tract to increase prostaglandin secretion in the female and stimulate myometrial contractions, aiding in synchronizing ejaculation with ovulation (Claus, 1990, Langendijk et al., 2004). In addition, prostaglandins and oxytocin are found in the seminal plasma of the boar and have been shown to increase myometrial contractions in the sow (Luke and Coffee, 1994).

While the function of all of the constituents of the seminal plasma are not known, they are collectively important for sperm function and the reproductive performance of the sow (Flowers and Esbenshade, 1993). Seminal plasma added to spermatozoa during mating significantly increases farrowing rate and litter size in the pig (Flowers and Esbenshade, 1993). In 1996, Henault and Killian found that the penetration of zona-free hamster oocytes by bovine sperm from a bull that was considered to be low in fertility could be improved by washing the sperm in the seminal plasma from a bull that was considered to have high fertility. Collectively, these studies suggest that seminal plasma plays an important role in the function of the spermatozoa including fertilization.

Additional maturation of the sperm takes place inside the reproductive tract of the female. During natural and artificial matings in pigs, semen is deposited into the cervix of the sow. Some of the semen will immediately be lost via retrograde flow resulting from the contractions from the sow's uterus. Some sperm cells are degraded in the sow's reproductive tract by phagocytosis (Rodriguez-Martinez, 2005). In the end, only a small population (hundreds) of sperm cells arrive at the opening to the oviduct, called the utero-tubular junction (UTJ). These cells arrive at this location within minutes of being deposited into the sow. This population of sperm cells is maintained at the utero-tubular junction and is called the sperm reservoir. All of the functions of the sperm reservoir are not known. It may function to identify spermatozoa that have abnormalities. Alternatively, it may act as a "holding tank" that allows stored spermatozoa to begin maturational processes, or function to shield the sperm cells from phagocytosis.

While in the sperm reservoir all of the sperm cells come in contact with the fluid of the sperm reservoir which is rich in proteins and enzymes, specifically, glycosaminoglycans and hyaluronan, and show a reduction in motility (Rodriguez-Martinez et al, 2005, Hunter 2002). Some of the sperm cells come in contact with the microvilla of the ciliated cells of the epithelium. They are orientated such that their heads become embedded in the folds of the epithelium and their tails point towards the lumen of the tubule (Rodriguez-Martinez et al., 2005). Other sperm cells do not come into contact with the epithelium and are left "floating" in the fluid of the sperm reservoir. There is debate as to whether these two populations of sperm cells differ in their fertilizing capabilities. Some research suggests that the cells located in the lumen of the tubule contain abnormalities and are not fertile (Mburu et al., 1996, 1997). Many *in vitro* experiments have shown that the spermatozoa bind to the oviductal cells in response to estrogen (Sirard et al., 1993). *In vitro* and *in vivo* experiments have suggested that the temporary binding of the sperm cell to the epithelium can increase the sperm viability, specifically by delaying capacitation (Smith, 1998) and decreasing the occurrence of polyspermy (Sirard et al., 1993). The ability of a sperm cell to bind the epithelium is increased in cells that have not undergone capacitation (Smith, 1998) and occurs in response to estradiol (Sirard et al., 1993). All of the cells that are maintained in the sperm reservoir are protected from phagocytosis and the sow's immune system. This is important because semen in the sow's reproductive tract, as mentioned previously, contains proteins and will recruit lymphocytes within 30 minutes of deposition (Rodriguez-Martinez et al., 2005).

There is a lot of debate surrounding the mechanism by which sperm cells are released from the sperm reservoir and travel up the oviduct. Some research suggests that spermatozoa are released from the reservoir in a continuous fashion due to a lack of, or desensitization of the binding sites in the epithelium. Other research suggests that when ovulation occurs, a signal is produced that stimulates the release of the sperm from the reservoir (Rodriguez-Martinez et al., 2005, Rodriguez-Martinez, 2001). Some researchers believe that capacitation occurs in the sperm reservoir as a result of a signal after ovulation. The changes that occur to the plasma membrane during capacitation actually cause their release from the binding sites of the sperm reservoir (Smith, 1998). However, some researchers believe that the unstable capacitated sperm cell would be unlikely to travel some 6-8 cm from the sperm reservoir to the site of fertilization (Hunter, 2002). Regardless of the mechanism, sperm are released continuously and slowly into the isthmus of the oviduct around the time of ovulation. This may be a very important mechanism in the pig as it may reduce the number of viable sperm that reach the oocyte and, thus decrease the chance of polyspermy.

As spermatozoa travel through the oviduct they encounter an ever-changing environment. The fluid of the oviduct contains many different proteins and enzymes specific to the location within the oviduct. While in the oviduct, the spermatozoa undergo capacitation, the acrosome reaction and hyperactivation before depositing their nuclear contents into ova. Capacitation was first identified by C.R. Austin in 1951, and described as a process in which the plasma membrane surrounding the sperm cell becomes destabilized (Chang. 1984). Capacitation is necessary for the sperm cell to penetrate the multiple layers of cumulus cells that surround the oocyte and to bind to the zona pellucida, or the outer

membrane of the oocyte (Chang, 1984). The study of capacitation *in vivo* can be difficult because the oviduct is such a small, dynamic organ. Consequently, many *in vitro* studies have been performed to determine the mechanisms by which capacitation occurs. Capacitation is a two-step process whereby proteins originating in the seminal plasma are removed from the plasma membrane followed by a lipid destabilization of the plasma membrane (Tienthai et al., 2004). Studies in humans and rodents have revealed that albumin stimulates the removal of cholesterol from the plasma membrane (Tardif et al., 2003). This results in an increase in calcium uptake by the sperm cell and an increase in the fluidity of the plasma membrane (Rodriguez-Martinez, 2001). However, there is still some debate as to whether albumin plays a critical role in the pig (Tardif et al., 2003). The removal of cholesterol, as well as bicarbonate and calcium, stimulates a protein phosphorylation pathway, involving sperm protein 32 and tyrosine kinase TK-32, which causes destabilization of the plasma membrane (Tienthai et al, 2004, Tardif et al., 2003, 2001).

Once capacitation has been achieved, hypermotility can occur. Hypermotility is characterized by a significant increase in the beating of the tail and a classical figure 8 pattern of movement (Rodriguez-Martinez, 2005). Suggested functions of the hypermotile state are to cause the release of the sperm cells from the lining of the epithelium; allow the cell to travel through the thick mucus of the oviduct; and to assist the sperm in penetration of the ovum (Rodriguez-Martinez, 2005, Suarez and Ho, 2003). It is not known what the exact signal is that initiates hypermotility. *In vitro* experiments in several species have suggested that the small amount of follicular fluid that enters the oviduct at ovulation provides a signal for hyperactivation. Other studies suggest that the signal for hypermotility comes from the

cumulus cells (Suarez and Ho, 2003). The sperm cell has a much greater opportunity for fertilization if hyperactivation occurs.

As spermatozoa approach the ova, the acrosome reaction takes place. The sperm nucleus is covered by a cap-like structure known as the acrosome. The acrosome consists of two layers: the inner acrosomal membrane lying closest to the sperm's nucleus; and the outer acrosomal membrane lying closest to the sperm's plasma membrane. The space in between these two membranes contains a multitude of enzymes (Eddy and O'Brien, 1994). During the acrosome reaction, the outer acrosomal membrane fuses with the plasma membrane in various locations around the sperm's head. This results in an expulsion of the contents of the acrosome (Eddy and O'Brien, 1994). The exact mechanism by which the acrosome reaction is initiated is not known. Some researchers suggest that the spermatozoa contact the zona pellucida which triggers the acrosome reaction. Others believe that the fluid environment of the ampullary region of the oviduct, and possibly progesterone, activate the reaction (Hunter, 2002). Once the acrosome reaction has taken place, the sperm is able to penetrate the zona pellucida and fertilization can occur.

Spermatogenesis is a complex process taking place not only in the reproductive tract of the male, but continuing in reproductive tract of the female. In most animal production arenas, focus is placed on improving the management of the females in the herd to increase reproductive efficiency. However, it is equally important to properly mange the males to optimize sperm production. There are some genetic parameters that cannot be manipulated to increase sperm production. However, there are plenty of management practices that can be utilized to maximize sperm production.

Factors affecting spermatogenesis

There are many factors that can limit spermatogenesis such as the efficiency of sertoli cells; the length of the spertmatogenic cycle; the number of spermatogonia entering meiosis; the number of sertoli cells present in the testes; and many other hormonal and environmental factors.

The sertoli cell functions to provide the environments and nourishment needed for germ cells to undergo the cell divisions and morphological changes associated with spermatogenesis and spermiation. Each sertoli cell can only support a finite number of germ cells during spermatogenesis (Orth, 1988). Therefore the number of sperm cells that complete spermatogenesis are dependant on the number of sperm cells that each sertoli cell can maintain. In 1995, Sharpe reviewed the regulation of spermatogenesis and discussed the differences seen between species in daily sperm production (10^6) per gram of testis where the rabbit was the most efficient at 25 followed by the rat at 24. The boar fell in the middle at 23 followed closely by the ram at 21. The bull was less efficient at 13, but man was by far the least efficient at 4.4.

Sperm numbers can also be affected by the length of the spermatogenic cycle. As discussed previously, spermatogenesis can be divided into stages along the seminiferous epithelium. Spermatogenesis is complete after these stages have cycled 4.5 times. The length of time for 1 cycle to be completed can vary from species to species causing significant differences in the length of time required for spermatogenesis to take place. For example, one cycle in the boar lasts about 8.6 days. This number is then multiplied by 4.5 to

determine that spermatogenesis takes 38.7 days. One cycle is significantly longer in the bull, being 13.5 days resulting in spermatogenesis taking 60.75 days (Franca et al., 2005). The length of spermatogenesis, determined in this manner, for the ram (1 cycle = 10.6days), the rat (1 cycle = 12.9 days) and the stallion (1 cycle = 12.2 days) would be 47.7 days, 48.05 days and 54.9 days, respectively.

Spermatogonial numbers entering meiosis can also affect the number of sperm cells produced by an animal. The number of spermatocytes that enter meiosis is dependant on the number of spermatogonia that are present (Sharpe, 1995). In the rodent and the pig, spermatogonia have additional mitotic divisions before committing to meiosis. Their spermatogonia divide to become B cells which are also capable of meiosis. Not all of the B cells produced enter meiosis so some undergo density-dependant degeneration. However, despite this degenerative process, numbers of B cells entering meiosis is high in these species. This situation is quite different in humans where spermatogonia do not undergo additional mitotic divisions prior to meiosis. In contrast to rats and pigs, humans have fewer numbers of B cells entering meiosis (Sharpe, 1995).

The efficiency of sertoli cells, length of the spertmatogenic cycle and number of spermatozoa entering meiosis are genetically determined and cannot be easily manipulated to increase spermatogenesis. There are other factors that should be mentioned, that have more potential for regulating sperm numbers. However, it is important to first really understand the process involved in the development of sperm cells in order to better understand how management and other factors can affect sperm output. Flowers (1997) describes this entire process by describing three theoretical pools of sperm within the male reproductive tract. The

first is the “resting pool”; the second is the “developing pool”; and the third is the “storage pool”. Sperm cells leave from the resting pool in the testicle every 2-3 days and begin developing. Cells remain in the developing pool for about 50-60 days, where the first 36-46 days are located in the testicle and the remaining 14 days is in the epididymis. This would include the 37.8 days required for spermatogenesis to take place in the testicle as well as the time required for the sperm to mature in the epididymis. Every 2-3 days, new cells move from the developing pool into the storage pool located in the epididymis where they are stored until ejaculation. Flowers (2001) suggested that the main two ways to increase sperm numbers biologically is to increase the frequency or number of sperm cells that leave the resting pool.

Many environmental factors affect spermatogenesis, including photoperiod, temperature, social environment, nutrition and season. It has been established that sows experience a depression in fertility in the summer months (Love, 1978). The wild boar has also been shown to have season fluctuations in fertility being lowest in the summer months. Supplemental lighting has been used experimentally to try and increase sperm concentration in boars. Claus and Weiler (1985) shortened the day length during the summer months using supplemental lighting which resulted in an increase in libido, steroid hormone concentrations and sperm productions. In the summer months, boars typically have a decrease in sperm numbers as well as a decrease in blood and seminal plasma steroid concentrations (Claus et al., 1983). Claus and colleagues (1983) found that implementing a supplemental lighting program during the summer that is representative of the daylength experienced during the autumn months could increase steroid concentrations to levels similar to those seen in

autumn. There is conflicting evidence on the effects of supplemental lighting on spermatogenesis. Some studies suggest that exposing boars to long day lengths decreases the number of sperm in the ejaculate while others did not see these effects (Colenbrander and Kemp, 1990, Flowers, 1997). Brandt and Diekman (1985) examined the addition of supplemental lighting on LH, testosterone and semen quality in boars from 8-35 weeks of age. They were unable to find differences in the LH and testosterone concentrations between boars with and without supplemental lighting. No differences were seen between treatment groups in spermatozoa concentration or motility. The libido scores of 20 week old boars receiving supplemental fluorescent lighting were higher than boars receiving natural lighting. These results are similar to what was found by Hoagland and Diekman (1982) where boars receiving supplemental lighting from December through April had higher libido scores at earlier ages compared to boars receiving natural lighting. Therefore supplemental lighting could decrease the age at which puberty occurs in the boar. Therefore the effects of photoperiod and supplemental lighting need to be elucidated, but it is unlikely that the effects of photoperiod on sperm production would result in a profound increase in sperm numbers (Flowers, 1997).

Another environmental factor that can detrimentally affect spermatogenesis is temperature. In response to an increase in environmental temperature, boars will have an increased respiratory rate and rectal temperature, but only exhibit a small increase in sweating. An increase in abnormal spermatozoa, a reduction in sperm number, and a decrease in fertility result from an acute increase in ambient temperature (Colenbrander and Kemp, 1990, Flowers, 1997, Wettemann and Bazer, 1985). These detrimental effects are

seen immediately. An ambient temperature above 30˚C for longer than 72 hours causes these detrimental effects on spermatogenesis (Flowers, 1997). Motility may also be reduced in response to acute heat stress. It may take up to 2 weeks for a reduction in motility to be seen in the ejaculate and up to 5 weeks for the boar to recover after temperatures are returned to within the boar's comfort zone of 26-29ºC (Wettemann and Bazer, 1985, Flowers, 1997). It is therefore important not to expose boars to a sudden increase in temperature. Exposure to a low ambient temperature does not appear to have detrimental effects on sperm output (Swierstra, 1970).

It is well established that acute exposure to heat stress can have deleterious effects on semen quality. However, few studies have examined the effects of chronic or long term exposure to elevations in temperature. The effects of chronic exposure to high ambient temperatures are seen in the summer months in temperate and semi-tropic climates (Flowers, 1997). Flowers (1997) examined data collected from boar studs in North Carolina from June through September, a period of time where there is an increase in the number of ejaculates that are rejected for poor quality and a decrease in the sperm output of the boars. The average weekly ambient temperature did not exceed 29ºC (the upper limit of the boar's comfort zone) during these months. However, it was noted that 5-6 weeks after the ambient temperature was maintained at 27.5ºC was when the deleterious effects were seen in the ejaculates. Therefore, there is also the possibility that chronic exposure to moderate amounts of heat stress can also have negative effects on semen quality. This may account for some of the seasonal differences in sperm production and sperm abnormalities seen in boars. The wild boar has a distinct mating period from fall to early winter (Colenbrander and Kemp,

1990). Studies have shown an increase in sperm concentration in ejaculates from September through February compared to the spring and summer months (Colenbrander and Kemp, 1990). As the long-day lengths approach (September through March) certain sperm abnormalities increase. Bent tails are the most common, increasing from 38% to 80% (Trudeau and Sanford 1986). This study also observed an increase in the occurrence of cytoplasmic droplets during the periods of increasing day length.

Nutrient intake also influences spermatogenesis. Adequate nutrition will not only stimulate growth in pigs, but also influence the age at which puberty occurs and libido (Foote, 1987). Undernutrition can have detrimental effects on spermatogenesis. Flowers (1997) reviewed nutritional studies and reported that a period of 6-8 weeks of undernutrition will decrease the number of sperm produced in mature boars. Improper energy and protein balances appear to have the greatest detrimental effects on spermatogenesis (Flowers, 1997). Louis et al. (1994a) studied the effects of varying dietary protein and energy concentrations in mature boars on libido and semen characteristics. They tested two protein and two energy concentrations: 7.7 and 18.1 g/d and 6.1 and 7.7 Mcal/day of ME, respectively. They had four treatment groups: high energy and high protein; low energy and low protein; high energy and low protein; and low energy and high protein. They found that the detrimental effects appeared after 7 weeks of decreased dietary intakes of protein and energy. They also reported that decreased dietary protein was associated with a significant decrease in libido and semen volume regardless of the levels of energy. There was also a trend for a decrease in sperm numbers. Inadequate protein and energy intakes do not seem to affect libido except in extreme cases (Colenbrander and Kemp, 1990). Maintaining proper nutrition without

over-conditioning is an important management procedure in order to maintain normal reproductive efficiency in boars.

There is also an interaction between season and nutrition that needs to be considered. As mentioned before, the comfort zone for the pig is between 26-29ºC. The body must function to maintain its temperature within this comfort zone. This process is known as thermoregulation. In a situation where the ambient temperature is above this comfort zone, the boar will increase its respiration rate in order to increase the loss of heat as water vapor from the lungs. The hair on the animal's body will also lie flat against the skin to prevent heat from being trapped against the skin. Pigs do not have enough functional sweat glands to regulate body temperature, so there is not a significant increase in sweating in response to an increase in temperature. In an outdoor situation, boars would also submerge their bodies in water or mud to allow for heat exchange with the water. Most commercial operations use misters which spray water onto the surface of the skin of the pig allowing for heat loss from the pig to the water. In a situation where the ambient temperature is below 26ºC the pig's hair will stand on end allowing for heat to be trapped underneath it to warm the skin or the pig will shiver as a means to produce heat. Both of these situations require energy from the pig to maintain a homeostatic body temperature. This energy would be provided in the diet of the pig. In the summer and especially the winter months, the boar may need to have a higher feed intake to compensate for the energy that is being spent on thermoregulation.

Another factor to consider is humidity. The actual temperature of the air and how hot it really feels can be two very different numbers depending on the amount of humidity in the air. Humidity is a measure of the amount of water vapor in the air. When humidity is high,

there is a large amount of water vapor in the air, therefore evaporation is low. Therefore the rate of heat loss is also lower when humidity is high. When the humidity is high, the boar may have to put more energy into thermoregulation. There would be less energy available for other bodily functions such as maintaining spermatogenesis. Therefore, ambient temperature and humidity need to be monitored (Flowers, 1997).

Boars that are not exposed to other males or females appear to have a decreased libido and accentuation of other seasonal effects. Social restriction in young boars is associated with a decrease in courting behaviors and a decrease in the number of successful copulations with no subsequent detrimental effects on semen quality (Colenbrander and Kemp, 1990). The peak in sperm concentration seen in the winter months is greater in boars that are social compared to socially restricted (Trudeau and Sanford, 1986). This study also revealed that social restriction results in a lower semen volume across all seasons. This should be taken into consideration when managing boars on a commercial unit.

There are other management practices that may need to be considered to optimize sperm production in boars. Several studies have been published discussing the effects of neonatal environment, timing of training for collection and collection frequency on sperm production. The neonatal environment could effect the adult body weight and sperm production in the adult pig. In 2006, Griffith and colleagues performed a study comparing the effects of rearing boars in litter sizes of 6 versus 12 on adult sperm production. It was discovered that boars raised in a litter of 6 were larger at 120 days of age and produced more spermatozoa as adults compared with boars raised in litters of 12. It is thought that boars

raised in smaller litters would have less competition for milk prior to weaning resulting in an increase in adult sperm production.

In gilts, the time when the gilt is physiologically ready for puberty can be significantly different from the time when they actually attain puberty and achieve successful matings (Kirkwood and Aherne, 1985). Therefore, age may not be the best indicator for when the gilt is ready to be bred. It may be that there is a pre-determined weight or body composition that must be achieved in order for puberty to occur in the gilt (Kirkwood and Aherne, 1985). Some researchers believe that there may be a similar situation in the boar where the age at which collection of semen begins could effect sperm output. In 2006, Griffith and colleagues compared the initiation of collection at 160 days (roughly 5 months of age) compared to 190 days of age (roughly 6 months of age). Typically what is seen in a boar's semen production during its life is that the numbers of sperm in the ejaculate increase linearly at younger ages until it reaches a plateau, or the adult sperm output for that boar. Initiation of semen collection at 160 days of age resulted in adult sperm output being achieved at a more advanced age, but the reduction in sperm numbers in the ejaculate was short lived (Griffith et al., 2006).

Collection frequency can have negative effects on sperm concentration. Collections every 24 or 48 hours will reduce the volume and concentration of the ejaculate within 3-4 days (Colenbrander and Kemp, 1990). Sperm present in the ejaculate come from the resting pool of sperm cells located in the epididymis. Not all sperm cells located in the resting pool leave the epididymis during ejaculation. In fact about 50-60% of the sperm in this pool leave the epididymis at a single ejaculation (Bonet et al., 1991). When a boar is collected

frequently, the numbers of sperm cells in the ejaculate decreases because the resting pool is being depleted faster than it is being replenished. Therefore, increasing the frequency of collection cannot speed up the process of development of sperm cells and a subsequent increase in sperm output. From a management perspective, an optimal number of collections per week would need to be determined for the farm to optimize the number of sperm produced each week. Cameron (1985) compared collections 3 days a week (Monday, Wednesday and Friday) to collections every 24 or 48 hours and found an increase in volume, concentration and total sperm per ejaculate associated with collections 3 times per week. It was thought that the boars that were collected only 3 times per week maintained a higher libido compared to the boars that were collected every 24 or 48 hours. Therefore, when selecting a collection frequency that optimizes sperm output, the libido of the boar must be taken into consideration as high collection frequencies may decrease the boar's sex drive and subsequent sperm production.

In addition to a reduction in sperm numbers, intense collection frequencies can act as a stressor to the boar and result in changes in the fluid composition of the epididymis resulting in increases in sperm morphologic abnormalities in the ejaculate (Pruneda et al., 2004). An increase in abnormal sperm cells is also seen when semen collections are not maintained at a consistent time interval. Flower and Seal (2005) examined data retrospectively from a commercial boar stud. These boars were routinely collected 1.5 times per week except during the summer months when the collection frequency is increased to make up for a decrease in sperm numbers during this time of year. The boars that had an increase in the number of collections were either collected at a consistent 3 times per week or

a variable 2-4 times per week. It was found that an increase in the number of collections increased the number of ejaculates discarded for poor quality. This effect was enhanced when the collection consistency was variable compared to consistent. A subsequent controlled study was performed and showed similar results where 3 collections per week with variable consistency of collection resulted in negative effects on the motility and morphology of the spermatozoa (Flowers and Seal, 2005).

Selection for increased testis size appears to be correlated with an increase in sperm production (Huang and Johnson, 1996). Selection for testis size can increase the sperm numbers by $14.6x10^9$ most likely due to an increase in LH production between 42 and 84 days of age (Huang and Johnson, 1996, Schinckel et al., 1984). Lunstra and colleagues (2003) crossed Meishan boars and white-cross boars to produce offspring that varied significantly in the weight of their testis. Boars with small testis had a smaller population of sertoli cells and a delayed onset of puberty as measured by the onset of sperm production. Boars with large testis had a greater number of sertoli cells resulting from an increase in the length of time, but not the rate of proliferation. Unfortunately, this author did not measure gonadotropins concentrations to determine if there was a difference between hogs with large and small testis. Testis size is responsive to selection and can be utilized in management practices to increase the reproductive function of boars.

Finally, another factor that can affect sperm numbers is the number of sertoli cells present in the testicle. Sertoli cell proliferation occurs in the fetal and neonatal periods in the rat and the pig (Orth, 1982, McCoard et al., 2003). Once sertoli cell proliferation ceases, the cells can no longer divide. Therefore sertoli cell proliferation in the fetal and neonatal

periods is representative of the adult sertoli cell population (Orth, 1988). Each sertoli cell can only support a finite number of germ cells through the spermatogenic cycle (Berndtson and Thompson, 1990). Therefore the number of sperm that can be produced depends on the number of sertoli cells that proliferate in the fetal and neonatal periods (Orth, 1988).

Johnson et al. (2003) used knockout mice to determine the roles of FSH and androgens in sertoli cell proliferation at different stages of development. They found that fetal and early neonatal proliferation is dependent on testosterone, but not FSH. However, testosterone and FSH were required for proliferation in the later neonatal period. The mouse develops more rapidly than the rat or the pig (Cupp and Skinner, 2005). In the rat, FSH regulates sertoli cell proliferation in the late fetal period as determined by in vitro studies with cultured sertoli cells in the presence and absence of FSH (Orth, 1984).

In the pig, FSH is at high concentrations from 80 d.p.c. until 3 weeks after birth (Colenbrander et al., 1982a) being maximal at birth (Franca et al., 2000). McCoard et al. (2003) reported maximal numbers of proliferating sertoli cells to be at 90 d.p.c. in the pig. This suggests that sertoli cell proliferation is not dependent on the concentration of FSH in the testes. The length of time that sertoli cell replication takes place can vary from species to species. In rodents, replication takes place from 11.5 d.p.c. until 15-17 days after birth with puberty occurring at 30-40 days after birth (Franca et al., 2005, Sharpe, 1995, Meachem et al., 2005). Proliferation of sertoli cells begins in the fetal period in all mammals, but the postnatal proliferation can be longer in non-rodent species where puberty does not occur until a later time. For example, in the boar, bull and ram, proliferation can continue for 6-10 weeks postnatally because puberty occurs at 170-190 days, 10-17 months and 5-12 months,

respectively, in these species (Sharpe, 1995). In 2000, Franca and colleagues reported that postnatal sertoli cell proliferation occurred in two distinct phases in the Piau pig. The first phase, and most predominant phase, occurred from birth until one month of age and was classified by a 6-fold increase in sertoli cell number (Franca et al., 2000). The second phase came just prior to puberty (2-4 months in the piau pig) and was associated with an increase in hypertrophy rather than hyperplasia. This is probably indicative of the time when sertoli cell maturation is taking place and the cells begin to produce proteins and other substances needed for nourishing germ cells during spermatogenesis. In all mammals, sertoli cell proliferation appears to cease when the blood-testis barrier (BTB) is formed and does not continue after puberty (Franca et al., 2005). In the pig, the BTB is formed around 120 days after birth (Tran et al., 1981). If there is actual mitotic activity occurring just prior to the formation of the BTB, it is minimal compared to the rate of sertoli cell mitosis in the fetal and early postnatal periods. It is this postnatal period of sertoli cell mitosis that holds the best opportunity for manipulating the rate of cell division and adult sertoli cell numbers.

It has been well established in the literature that FSH plays an important role in sertoli cell development in the rodent. Follicle stimulating hormone functions to increase the rate of sertoli cell replication during the neonatal period (Sharpe, 1995). In 1996, Meachem and colleagues injected rats with FSH (200I.U./kg s.c. daily) beginning at birth and ending at 5, 10, 15 and 20 days after birth. They found that the FSH treatment resulted in an increase in sertoli cell number. It was decided that the FSH treatment increased the rate at which mitosis was taking place and not the length of time over which replication was occurring.

In 1996, Ford and colleagues examined the relationship between FSH and adult testis size. They first determined that the FSH concentrations were 5 times higher in Meishan boars compared to white cross boars despite having significantly smaller testicles and daily sperm production. Crossbred boars (Meishan, White cross, ¾ Meishan x ¼ White cross and ¼ Meishan x ¾ White cross) were then selected for blood sampling and castration based on the greatest amounts of differences in FSH concentrations. Boars with high FSH concentrations had lower adult testicular weight and daily sperm production. The boars that had increased FSH concentrations as adults also had increased FSH concentrations at 2 and 8 weeks of age, coinciding with the period of sertoli cell replication in the boar. This implies that high FSH concentrations during the period of sertoli cell replication in the boar does not result in an increase in adult testis size, contrary to what has been seen in rodents. In contrast, Swanlund and colleagues (1995) administered FSH (100µg/kg body weight i.m. daily) to boars from 8 to 40 days after birth. After castration at 100 days of age, the FSH-treated boars had an increase in length, but a decrease in the diameter of the seminiferous tubules compared with untreated controls. This study indicates that FSH might play a similar role in pigs as rodents; however, sertoli cell number was not enumerated in this study. In 2004, Bagu and colleagues administered FSH to bull calves during the neonatal period which resulted in an increase in testicular size (measured in scrotal circumference being ≥28cm) and a subsequent increase in sertoli cell number at 56 weeks of age. Taken collectively, these studies would suggest that FSH treatment during the critical neonatal period could increase the rate of mitosis in sertoli cells.

However, other studies do not show similarities between pigs and rodents in FSH's role in sertoli cell function. One, in particular, examined differences in hormonal and testicular characteristics between Large White and Meishan boars. The Meishan breed is a Chinese breed of pig known for its high prolificacy. Meishan boars reach puberty at 56-84 days of age and have a lower mature body weight compared with domestic breeds which reach puberty at 120-180 days (Lunstra et al., 1997). During the postnatal period, Meishan boars had higher concentrations of FSH and LH compared with the Large White breeds; however, the adult testis size and sertoli cell numbers were not different (Lunstra et al., 1997). Meishan boars still produced more spermatozoa than the Large Whites due to the fact that they saw reduced germ cell losses and increased sertoli cell efficiencies (Lunstra et al., 1997).

In 2003, McCoard and colleagues looked at the testicular composition of Meishan and white-cross boars at 60 days post coitum through 25 days post partum. They found maximal rates of sertoli cell mitosis (measured as Ki-67 uptake of sertoli cells) occurred at the fetal age of 90 days post coitum in white-cross boars. This developmental period did not coincide with the peak of FSH concentrations which occurred at birth. It is important to note that sertoli cell mitosis was still occurring during the neonatal period in these animals, but the rate of proliferation was declining. Similarly, in 2001, Ford and colleagues found that boars with similar concentrations of FSH during the first 3 weeks after birth had different size testicles at 220 days of age. Taken collectively these studies indicate that FSH does not enhance sertoli cell proliferation in pigs by the same mechanism of action as it works in rodents. However, none of these studies actually administered FSH. Consequently, FSH

probably is involved in sertoli cell proliferation in the pig, but may not be the most potent stimulus as it appears to be in the rat.

Unilateral castration results in an increase in FSH concentrations and a subsequent hypertrophy in the remaining testicle in many species including the ram, rat, lamb, boar and bull (Sharpe, 1995). However this hypertrophy is only seen when castration is performed on prepubertal animals. Kosco and colleagues (1989) implied that unilateral castration resulted in an increase in sertoli cell number, but their study only reported an increase in the relative mass of the seminiferous tubule which was computed by dividing the mass of the tubule by body weight. In 2003, Lunstra and colleagues performed unilateral castrations on boars on days 1, 10, 56 and 112 and counted sertoli cells using brightfield microscopy and GATA-4 staining, a known marker for sertoli cells (McCoard et al., 2001). Their results showed that unilateral castration did cause hypertrophy of the seminiferous tubules or an increase in sperm production when the castration was performed on days 1 and 10. There was an increase in the mass of the seminiferous tubules. However, this increase was not due to an increase in sertoli cell number at any of the castration ages, but rather to an increase in the size of the sertoli cell (mass). Unilateral castration and subsequent hypertrophy of the remaining testicle has been used as a model to study the mechanisms by which the compartments of the testicle mature and develop. However, this may not be a good model for studying sertoli cell mitosis in the boar because unilateral castration during the neonatal periods did not increase sertoli cell proliferation despite an increase in FSH concentrations (Lunstra et al., 2003).

Other hormones may also play a role in sertoli cell mitosis. Induction of a hypothyroid state, via administration of 6-propyl-2-thiouracil (PTU), from birth to 25 days has been shown to increase testis size and daily sperm production in rodents (Sharpe, 1995, Cooke and Meisami, 1991, Cooke et al., 1991). The neonatal hypothyroidism caused a delay in the maturation of the sertoli cells allowing them to proliferate over a longer period of time compared with FSH which increased the rate of mitosis during the same period of time (Sharpe, 1995). In the rat, the hypothyroidism during the neonatal period is accompanied by a decrease in FSH concentrations which lasts into adulthood. Treatment of cultured sertoli cells from neonatal rats with triiodothyronine (T3) inhibits mitosis and differentiation (Cooke et al., 1994). Humans suffering from juvenile hypothyroidism present similar symptoms as observed in the rat. However, enumeration of sertoli cells in humans with this condition has not been undertaken (Sharpe, 1995).

A possible mechanism in humans for thyroid hormone has been proposed from work with cDNA libraries. A novel thyroid hormone receptor was identified in the testis of humans. The rationale is that thyroid hormone may function to increase the mRNA for GLUT1 and increase glucose transport in cells (Sharpe, 1995). GLUT-1 is a transmembrane glucose transporter that functions to transport glucose into the sertoli cell. Germ cell development requires that sertoli cells uptake glucose for proper energy metabolism. In the rat, FSH has been shown to increase glucose transport into the sertoli cells necessary to maintain germ cell development (Sharpe, 1995). Therefore it is thought that thyroid hormone may be increasing the normal metabolic activities of the sertoli cell.

In the pig, few studies have been conducted to examine the direct effects of an induced hypothyroid state during the neonatal period. Klobucar and colleagues (2003) administered 0.1% PTU in the drinking water of boars beginning at 21 days of age until 20 weeks and did not see an increase in sertoli cell proliferation. However, the timing of the PTU treatment is imperative. In 1992, Cooke and colleagues determined that PTU administration must begin within 4-8 days after birth in order to have its effects on sertoli cell mitosis in rats. A similar situation may be true in rams where administration of PTU from 16-24 weeks of age had no effects on pubertal development and testis size (Chandrasekhar et al., 1985). Hyperthyroidism from 16-24 weeks caused a decrease in testicle size whereas treatment from 6-12 weeks showed an increase in scrotal circumference and advancement of puberty (Chandrasekhar et al., 1985, Fallah-Rad et al., 2001). Consequently, it is likely that the study conducted by Klobucar et al, missed this critical window and the fact that they observed no differences is not surprising.

In 2003 McCoard and colleagues compared the thyroid function of Meishan and white cross boars. They determined that Meishan boars underwent a period of hyperthyroidism (increased T3 concentrations) from day 105 post coital until birth compared to the white cross boars. They also found that in both breeds, the thyroid hormone receptor (THRβ1) concentration in the sertoli cells decreased from 105 days post coital to 7 days after birth (McCoard et al., 2003b). During this time, sertoli cell proliferation was still occurring, but the rate of proliferation was decreasing (McCoard et al., 2003a). Taken together, these studies indicate that thyroid hormone plays a role in sertoli cell mitosis in the boar, but further research is needed to determine the critical windows during which it is most active.

Other hormones most likely play a part in sertoli cell mitosis and differentiation. In rats, a deficiency in growth hormone results in a decrease in testis size, despite having normal sperm production (Sharpe, 1995). In the pig, neonatal treatment with growth hormone resulted in an increase in seminiferous tubule lumen formation (Swanlund et al., 1995). This could be reflective of an early maturation of sertoli cells, even though sertoli cells were not measured in this study. Further research is also needed to look at the roles that inhibin and activin play in the development of the sertoli cell populations. Inhibin is a hormone produced by the testis and regulates FSH secretion from the pituitary gland. Inhibin may also have additional roles in mediating cell divisions in the testicle (De Jong, 1988). Mice that lack the α-subunit of inhibin develop tumors in the gonads. This suggests that sertoli cells are capable of a self-regulation of cell division via inhibin acting in an autocrine fashion to decrease cell division (Sharpe, 1995). It has also been suggested that leydig cells could also play a role in the regulation of sertoli cell mitosis. In the rat, neonatal hypothyroidism as well as neonatal hemicastration studies have resulted in leydig cell numbers increasing with sertoli cell numbers (Sharpe, 1995). Leydig cells also produce β-endorphin which can bind to opiate receptors on the sertoli cell membrane to possibly decrease proliferation in the sertoli cells (Sharpe, 1995).

Measuring Semen Quality and Quantity

The use of artificial insemination has increased the need for accurate measures of a boar's fertility. With artificial insemination, one boar can inseminate 6000-8000 females in a year. Therefore it is important to know which boars are the most fertile. Measuring an

individual boar's fertility can be difficult. It is easier to address semen quality and quantity, separately. Sperm quantity can be assessed by counting the numbers of spermatozoa that are produced in an ejaculate (Flowers, 2001). The greater the number of sperm produced, the more sows that are able to be inseminated. Sperm numbers or concentration is most commonly measured visually via a heamocytometer or an electronic cell counting device (Colenbrander and Kemp, 1990).

Semen quality refers to the number of sperm in an ejaculate that possess the ability to penetrate the egg (Flowers, 2001). The best *in vivo* method to measure semen quality is its ability to produce live offspring that result from a mating. However, measuring a boar's fertility in this manner is difficult because a poor result could be the fault of the sow and not the boar. Many different tests have been designed to measure the quality of the semen. However, there is not yet one test that can accurately predict the fertility of an individual boar.

Typically, motility, acrosome morphology and sperm morphology are used to predict semen quality (Flowers, 2001). Motility is measured visually; using a swim-up method like percoll gradients; or using computerized image analysis (Colenbrander and Kemp, 1990). Motility generally is believed to be a good indicator of semen quality as it may be some what indicative of the spermatozoa's ability to penetrate the egg, however the relationship between motility and fertility is not well defined in the pig (Flowers, 1997). Flowers (1997) studied the effectiveness of motility as an estimate of fertility. Twelve boars were collected weekly and semen analysis was performed on each ejaculate. A portion of each ejaculate was then used for *in vitro* fertilization and the remainder of the ejaculate was used to breed 5 sows via

artificial insemination. All estimates of semen quality were divided into groups of 10% each (100-90%, 90-80%, etc.). Ejaculates with motility greater than 60% did not differ in the *in vitro* sperm penetration rates or in the *in vivo* estimates of fertility, farrowing rate and number of pigs born alive. In addition, this estimate of fertility could not be improved when additional parameters were considered such as acrosome integrity and sperm cell morphology. Therefore motility should be considered a qualitative estimate because there is a critical value above which no differences in fertility can be seen (Flowers, 1997).

Motility may be useful when using semen that has been extended and stored for artificial insemination. Xu and colleagues (1996) reported that motility decreased from day 1 to day 7 in extended semen. The motility at after 7 days of storage was correlated with *in vitro* sperm penetration rates. Juonala and colleagues (1998) examined the relationship between the motility of extended semen after 7 days of storage on litter sizes in 106 boars used for artificial insemination. They found a positive relationship between motility after 7 days of storage and litter size. While motility may not be the best predictor of a boar's fertility, it can be a useful technique for estimating quality in stored semen.

A high occurrence of morphological abnormalities is associated with a decrease in fertility in the boar (Colenbrander and Kemp, 1990). There are many tests that can measure a specific function of the sperm or a specific abnormality. However, none of these tests have been shown to have a strong correlation to estimating fertility of an individual boar. Some of these tests for abnormalities include tests for intact sperm plasma membranes, presence of cytoplasmic droplets, presence of tail abnormalities, sperm chromatin structure, protein concentration in the plasma membrane, enzyme release, mucus penetration tests, measures of

sperm capacitation abilities, metabolic activities tests, sperm-oviduct binding assays and *in-vitro* fertilization tests (Colenbrander and Kemp, 1990, Flowers, 1997, Flowers, 2001).

There is additional evidence in pigs and other species that components of the seminal plasma play an important role in the fertilizing abilities of sperm (Flowers, 1997). Seminal plasma has also been shown to influence the amount of time that spermatozoa spend in the oviduct. Mburu and colleagues (1996) examined the number, distribution and membrane integrity of spermatozoa found in the utero-tubular junction before, during and after ovulation. They found that ovulation was able to influence the number, distribution and membrane integrity of the sperm cells in the utero-tubular junction. There were also differences between boars in the number and distribution of sperm cells in the oviduct around ovulation. Flowers (1997) reported that spermatozoa from boars that sire the majority of piglets in a litter as determined by paternity testing of the piglets is retained in the oviduct at a higher concentration 24 hours after mating compared to boars that only sire a few piglets in the litter. It is thought that components of the seminal plasma effect the retention of spermatozoa in the oviduct.

In order for successful fertilization to occur, spermatozoa must be present in the oviduct before ovulation occurs. Therefore the best time for insemination is 12-24 hours before ovulation occurs (Waberski et al., 1994, Kemp and Soede, 1996). This ensures that sperm cells are present in the oviduct when ovulation occurs. However, components of the seminal plasma may also induce ovulation to better time the presence of the spermatozoa in the oviduct with ovulation. Claus (1990) suggested that the large amounts of estrogens present in the seminal plasma were responsible for inducing the LH surge and ovulation

during mating. Waberski and colleagues (1995) infused the uterine horns of sows with seminal plasma and found that ovulation occurred 10.7 hours earlier compared to saline infused horns. Therefore there is good evidence that seminal plasma functions to induce ovulation.

Flowers and colleagues (unpublished, review in Flowers, 1997) mixed the seminal plasma from a boar that was known to sire the majority of piglets in a litter with the sperm rich fraction of an ejaculate of a boar that was known to not sire many piglets in a litter. The opposite was done as well where the seminal plasma from a "low fertility" boar was mixed with the sperm rich fraction of a "high fertility" boar. They found that the seminal plasma played a role in the boar's ability to sire more piglets in the litter. Therefore, the components of the seminal plasma responsible for affecting fertility needed to be researched.

Proteins present in the seminal plasma can vary greatly from animal to animal. This could provide the differences seen in fertilizing abilities between animals. In 1996, Henault and Killian found that the penetration of zona-free hamster oocytes by bovine sperm from a bull that was considered to be low in fertility could be improved by washing the sperm in the seminal plasma from a bull that was considered to have high fertility. Two proteins have been identified in the seminal plasma of the pig that may be good predictors of fertility (Flowers, in press). The first protein has a molecular weight of 26 kDa and a p.I. of 6.2 while the second protein has a molecular weight of 55 kDa and a p.I. of 4.8 (Flowers, in press). Similar proteins have been identified in the bull. There are two proteins thought to be associated with high fertility bulls, the first with a molecular weight of 26 kDa and a p.I. of 6.2 and the second with a molecular weight of 55 kDa and a p.I. of 4.5 (Killian et al., 1993).

This 55kDA protein was later sequenced and found to be 86% homologous to bovine osteopontin-K precursor (Cancel et al., 1997). Osteopontin is a protein that serves many functions in the body including remodeling of mineralized tissue, cell migration, calcium regulation and recruitment of cells involved in the immune response (Denhardt and Guo, 1993), however the function of this protein as related to fertility is unknown. There were two additional proteins identified that were associated with bulls having a lower fertility (16 kDA, p.I. 4.1 and 16 kDa, p.I. 6.7) (Killian et al., 1993). In 1999, Brandon and colleagues identified a 72 kDa protein with a p.I. of 5.6 to be associated with increased fertility in the stallion. They were also able to identify three additional proteins associated with a decreased fertility (75 kDa, p.I. 6.0; 18 kDa, p.I. 4.3; 16 kDa, p.I. 6.5).

It should be reiterated that some semen characteristics are influenced by the frequency in which the boar is collected. Collections every 24 or 48 hours compared to 3 times per week results in a decrease in volume and number of spermatozoa in the ejaculate (Cameron, 1985). The incidence of masturbation increases if the boar is not collected at regular intervals. Boars have the ability to masturbate resulting in a release of small amount of sperm from the epididymus. These sperm typically die and are wasted. In addition, motility and morphology of the spermatozoa can be negatively affected if the consistency of semen collection is not consistent (Flowers and Seal, 2005). Therefore maintaining boars on a regular, consistent collection frequency will decrease the amount of wastage in the ejaculate and optimize the number of spermatozoa produced (Kemp et al., 1991).

The presence of semen in the sow's reproductive tract has been shown to induce an immune response in the sow. This response is important in order to remove excess sperm

cells and bacteria from the reproductive tract to optimize the environment for fertilization to occur (Rozeboom et al., 1998). This immune response consists of the recruitment of leukocytes and polymorphonuclear neutrophilic granulocytes from an increase in the expression of pro-inflammatory cytokines (Rozeboom et al., 1998, Robertson et al., 2002, O'Leary et al., 2004). The recruitment and retention of leukocytes in the sow's reproductive tract following mating is increased by the presence of seminal plasma, suggesting there are factors in the seminal plasma regulating this immune response (O'Leary et al., 2004). The seminal plasma has been thought to have immunosuppressive constituents for protecting the sperm cells from rejection by the female reproductive tract as well as possibly increasing embryo viability (O'Leary et al., 2004). Transforming growth factor beta (TGFβ) has been suggested to play a role in the immunosuppressive actions of the seminal plasma by increasing immune tolerance in the female (Robertson et al., 2002). TGFβ is present in the seminal plasma of the boar at relatively high concentrations (28-495ng/ml TGFβ; 96-144ng/ml TGFβ2) (Robertson et al., 2002).

Other proteins and cytokines that have been studied are heat shock protein 70 (HSP70) and tumor necrosis factor alpha (TNFα). Heat shock proteins are a class of proteins that are produced when a cell encounters a rise in temperature and other insults. These proteins provide that cell with a defense against the environmental insults. Heat Shock Protein 70 was identified in germ cells in the mouse even when heat shock was not applied (Eddy, 1999). Heat Shock Protein 70 has also been identified in the ejaculated sperm cells of the boar (Huang et al., 2000). It has been suggested that the presence of HSP70 could correlate with semen quality estimates in the pig (Huang et al., 2000, Turba et al., 2007),

however further investigation is required. TNFα is a pro-inflammatory cytokine that has been shown in humans to decrease sperm motility (Estrada et al., 1997). However, the opposite has been suggested in pigs where the presence of TNFα in the ejaculate was positively correlated with motility and negatively correlated with morphological abnormalities (Turba et al., 2007). However results from this study are not definitive and require further investigation into the role of TNFα in semen quality.

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Effects of FSH Administration During the Neonatal Period on Adult Sperm Production In Boars

Introduction

There are many management strategies that have been adopted in the swine industry to improve the reproductive efficiency on a farm. Most of these strategies focus on improving the reproductive performance of the sows, with very little attention focused on improving the reproductive performance of the boars. However, improvement in the reproductive performance of the boars can also improve the reproductive efficiency of the farm (Flowers, 1997). The use of assisted reproduction in the swine industry has been increasing over the past 20 years. Since 1990, the numbers of sows bred by artificial insemination has grown in the United States as well as in North Carolina. Artificial insemination began to be adopted as a reproductive technique for use in the swine industry in North Carolina in the early 1990's. In 1991, fifty percent of the sows bred in the state were bred artificially. By the year 2001, almost 100% of the sows were bred by artificial insemination. The adoption of artificial insemination across the United States has shown a similar trend where in 50% of the sows were bred artificially by 1998 and 75% in 2001 (Flowers, unpublished data). With artificial insemination, one boar can inseminate 6000-8000 females per year. The number of females that each boar can breed depends on the number of sperm produced by that boar. One way to increase the reproductive efficiency of a boar is to increase the number of spermatozoa that a boar produces. In a commercial boar stud, a typical boar is used for semen collection from puberty until about 18 months of age. Therefore this increase in the number of spermatozoa produced would need to occur at a young age in order to improve efficiency on the farm.

There are many genetic and environmental factors that can affect the number of spermatozoa produced in the testicle. One of these factors is the number of sertoli cells located in the seminiferous tubules. The sertoli cells are responsible for providing the nourishment for the developing spermatogonia within the testicle. Each sertoli cell can only support a finite number of germ cells through development. Therefore spermatogenesis is limited by the number of sertoli cells within the testicle (Berndtson and Thompson, 1990). Sertoli cells undergo the greatest increase in cell number during the first month after birth (Franca et al, 2000). The number of sertoli cells present in the testicle at puberty is representative of the adult population because no further cell divisions take place after puberty (Orth, 1988). Manipulation of the mitotic activity of the sertoli cells during this neonatal period provides an opportunity to increase adult sperm production.

Many studies have been performed in rodents to manipulate mitotic activity of the sertoli cell. Treatment with FSH, hemicastration and induction of a transient hypothyroid state during the neonatal period have all been shown to increase sertoli cell mitotic activity in the rat (Sharpe et al., 2003). Very little research has been done in pigs to determine if sertoli cell mitotic activity can be stimulated in a similar fashion as the rodent. One study induced a transient hypothyroid state in weaned pigs and did not see any effects on testis development (Klobucar et al., 2003). However, treatment did not begin until 3 weeks of age, which may have been too late if sertoli cells are only undergoing mitosis for the first month of life. Swanlund and colleagues (1995) administered FSH from 8-40 days of age in boars and found a seven-fold increase in the length of the seminiferous tubule at 100 days of age. However, this study did not enumerate sertoli cells in the testicle. Therefore, the question remains as to

whether this seven-fold increase in the length of the seminiferous tubule is a result of an increase in sertoli cell number within the testicle. All boars were castrated prior to pubertal development. Therefore the effect of neonatal FSH treatment on the number of spermatozoa in the ejaculate of mature boars remains to be elucidated. Each sertoli cell can support a finite number of germ cells through development. Therefore an increase in sertoli cell number should correlate with an increase in germ cell numbers in the testicle as well and a subsequent increase in the number of spermatozoa present in the ejaculate of sexually mature boars. Therefore, the objective of this study was to determine whether FSH administration during the neonatal period could increase the rate of mitotic activity of sertoli cells in the boar and a subsequent increase in the number of sertoli cells per organ. A second objective was to determine whether FSH administration during the neonatal period could increase the numbers of spermatozoa present in the ejaculate of a sexually mature boar.

Materials and Methods

Animals

The study was conducted at the North Carolina State Swine Educational Unit, a 120 sow farrow-to-finish operation located at North Carolina State University in Raleigh, North Carolina. The eighty-five boars used in this study were offspring of Yorkshire x Landrace x Large White sows bred to Hampshire x Duroc x Pietran boars and were expected to exhibit 100% heterosis. All of the boars were obtained from 14 sows that were bred between December 26 and December 31, 2005 and farrowed between April 15th and April 24th, 2006. All experimental animals were negative for porcine reproductive and respiratory syndrome, mycoplasma hyopneumonia, parvovirus, leptospirosis, hemophilus parasuis, streptococcus suis, swine influenza and transmissible gastroenteritis.

Experimental Design and Data Collection

The goal of this study was to determine the effects of FSH administration during the neonatal period on sertoli cell mitotic activity and adult sperm production in boars. Follicle stimulating hormone was administered during the neonatal period for differing lengths of time. Measures of body weight and testicle size were taken throughout the study. Blood samples were also obtained to measure serum hormone concentrations. Castrations were performed at 14, 21, 42, 98 and 330 days of age on randomly selected boars from each treatment group for morphometric analysis. Semen was collected in a randomly selected group of adult boars and used for analysis of semen quality. Figure 1 is a graphical depiction

of the experimental design. Appendix A contains a detailed list of all boars used and their disposition during the study. Specific details about the design of the study including the animals used and analytical procedures are included in the following sections.

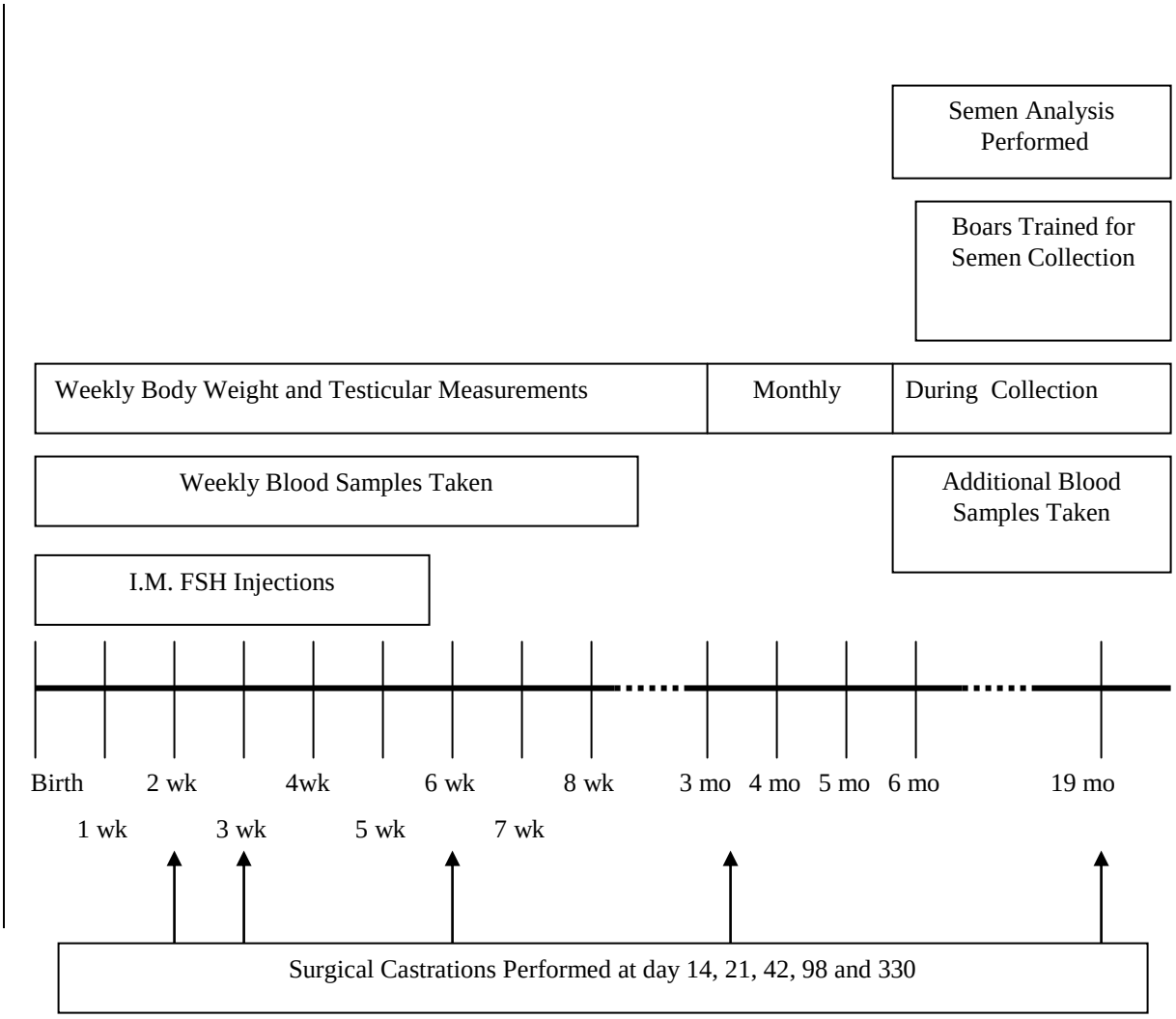

Figure 1. Graphical depiction of the experimental design beginning from the birth of the piglets until termination of the study at approximately 20 months of age.

Sows were moved into a farrowing room on December 20, 2005 which was 4 to 7 days prior to their estimated farrowing date. The farrowing room contained 12 individual,

bow-bar farrowing crates and was equipped with a side-wall baffle ventilation system with an evaporative cooling cell. The bow-bar farrowing crates were 1.5 m wide by 2.5 m long. Each crate had an airplane slat flooring pattern with cement slats underneath the sow; TriBar®, an expanded metal, behind the sows; and Tenderfoot®, a plastic coated wire, in the piglet area. Two heat lamps were also placed at varying heights in each crate as additional heat sources for the piglets. While in the farrowing barn, sows were fed a corn and soybean diet ab libitum two times per day that met or exceeded NRC recommendations for protein, energy, vitamins and minerals for lactating sows (N.R.C., 1998). Estimated daily average feed intake of sows during lactation was 7.1 $\pm$ 1.2 kg.

Within 48 hours after birth, all boars were processed. Processing included ear notching for permanent identification, administration of iron and penicillin, tail docking and clipping needle teeth. After processing, the boars were assigned to treatment groups. They were crossfostered so that each sow nursed at least three boars from her own birth litter and at least one boar from a different sow. For example, if a particular sow's litter contained six boars, then three of these boars remained; three were crossfostered off; and at least one boar was crossfosterd on. There was one litter in which no crossfostering of boars occurred. Female piglets were randomly crossfostered based on body size. This strategy resulted in 14 litters with between 10 and 12 piglets each. Litters consisted of an average of 6.08 ± 0.29 boars and 5.00 ± 0.25 gilts. After farrowing, one sow had a rectal prolapse and her pigs were removed. The five boars that she was nursing were fostered onto sows that were at a similar stage of lactation.

After crossfostering, boars within each litter were randomly assigned to receive one of three treatments. Treatment group one was called the "early" treatment and consisted of boars that received FSH injections from day 4 through day 22 of age. Since injections were given every other day, this resulted in 10 FSH injections given to each boar. Treatment group two was called the "late" treatment and consisted of boars that received FSH injections from day 22 to 40 after birth. Since injections were given every other day, this also resulted in 10 FSH injections per boar. The dose of FSH (pFSH; catalog number F2293, Sigma, Saint Louis, MO) administered intramuscularly at each injection was 100µg per kilogram of body weight. Boars were weighed at birth and then again at weekly intervals during lactation. The total amount of FSH administered to each one was adjusted accordingly. The third treatment group consisted of the control boars that received intramuscular injections of saline of a comparable volume to the FSH administered to the treated boars. These boars received injections from day 4 to 40 after birth.

Preparation of FSH for injections was as follows. The FSH was first reconstituted with 0.87% sterile saline. A solution with a final concentration of 2.5 mg/ml was made by adding 20 ml of saline to a vial containing 50 mg FSH. Doses for individual boars were removed from the diluted FSH solution with either tuberculin (1 ml) or 3 ml syringes depending on the volume required. Each syringe was equipped with a 22 gauge, 1 inch needle. Injections were given in the neck approximately 3 to 4 cm lateral from the top line in alternate fashion between the right and left sides. The FSH solution was kept at 4ºC for one week and then any unused portion was discarded.

While they were housed in the farrowing rooms, body weights, testicular size and blood samples were taken when the boars were 4, 7, 14 and 21 days of age. Body weight was obtained by placing boars in a plastic container on an electronic scale (5000 Series; T5IP; Ohaus; Pine Brook, NJ) and taking a reading at a point when their body movements were minimal. Two measurements were taken to determine testicle size: length and width. Measurements were taken with digital calipers (Fisher Scientific; Atlanta, GA). Testicular height was taken by measuring from the top to the bottom of the right testicle. Testicular width was taken by measuring across both testicles from left to right. All testicular measurements were taken by the same technician through the entire study and recorded in centimeters.

For blood sampling, boars were placed on their backs in a V-trough and restrained manually. Samples were collected via venipuncture of the jugular vein using 1 or 1.5 in, 20 gauge needles depending on the size of the piglet (Becton Dickinson; Franklin Lakes, NJ). The needle was inserted into jugular furrow and a volume of 1 to 2 mL was collected. Blood samples were stored in a cooler at about 4ºC for less than 6 hours, then centrifuged at 2700 x g at 4ºC. Serum was decanted and then placed into a plastic tube and stored at -20ºC for subsequent analyses.

Four boars from each treatment group were randomly scheduled to be castrated at 14 and 21 days of age. Boars were weighed immediately prior to surgical castration. A surgical plane of anesthesia was achieved with a single intramuscular injection of a mixture of ketamine (2.2 mg/kg, Fort Dodge; Overland Park, Kansas), telasol (4.4 mg/kg, Fort Dodge; Overland Park, Kansas) and xylazine (4.4 mg/kg, Fort Dodge; Overland Park, Kansas). Once

a boar was lacking a response to skin pinching and palpebral stimulation, he was rolled on his back and lifted onto the operating table.

Each boar's scrotum and surrounding skin was first cleaned with hot water to remove any fecal material. The area was next cleaned with an iodine and soap mixture (1N Iodine Solution; Fisher Scientific; Atlanta, GA) by scrubbing the entire area with a circular motion radiating outward from the center of the scrotum. This was repeated three times. After the final cleaning, the iodine / soap mixture was removed with sterile gauze and a 1% solution of iodine (1N Iodine Solution; Fisher Scientific; Atlanta, GA) was applied. A 2 cm incision was made in the center of the caudal surface of each testis. The testicles and epididymi were carefully separated from the tunica albuginea and other membranes with blunt dissection. Testes were then rotated counterclockwise 360^{o}. This caused the spermatic cord to compress around itself. A 7.6 cm angiotribe was placed just underneath each testis and epididymis. The spermatic cord was ligated with a series of interlocking surgeon's knots made with 1-O absorbable suture (1 Chromic Gut; Ethicon, Summerville, NC) placed just underneath the ventral surface of the angiotribe. A scalpel blade (No. 22, Butler Co.; Columbus, Ohio) was used to remove the testes and epididymi by cutting on the dorsal surface of the angiotribe. The inguinal canal and surrounding membranes were closed using continuous interlocking sutures with 1-O chromic gut. The tunica albuginea and other connective tissue layers were closed using a continuous, mattress stitch, while the skin of the scrotum was closed using a series of interrupted mattress stitches. Once removed, the right testicle was submerged into a glass jar containing Bouins fixative solution (Fisher Scientific; Atlanta, GA). Between 10 and 15 punctures were made in the testicle using an 18 gauge needle to increase the uptake of

Bouins solution by the tissue. The samples remained in the Bouins solution until embedded for morphometric analysis. The left testicle was placed into a plastic bag and stored on ice for approximately 2-3 hours. It was then placed at -22ºC for long term storage. Details of these procedures are described in subsequent sections.

Boars were placed in an isolated, clean and warm environment and allowed to recover from anesthesia. Rectal temperatures, heart rate, and palpebral responses were monitored every 30 minutes until the boars were conscious and ambulatory at which time they were returned to their respective litters. The entire procedure from administration of anesthetics to recovery typically took between 30 and 45 minutes.

On May 11th, 2005, boars were weaned at 21.45 ± 0.23 days and moved into one room of the nursery barn. Nursery rooms were equipped with side-wall baffle ventilation systems and consist of six pens on either side of a centrally located hallway. Each pen is 1.82 x 1.82 m with 0.91 m of feeder space and 4 nipple waterers. The boars were randomly assigned to nursery pens based on body weight at weaning. This created a situation in which boars from each of the treatment were housed together in one of 9 pens with 6 to 9 boars per pen. All boars were fed a standard 23% protein starter diet consisting of milk byproducts for 7-10 days. Following this diet, they received a corn and soybean meal based diet in which the protein percentage gradually decreased to 18%. These diets met or exceeded N.R.C. recommendations for growing pigs between 7 and 35 kg (N.R.C., 1998). Fifty-five boars were housed in the nursery with 17 being control boars, 18 boars belonging to the early treatment and 20 boars belonging to the late treatment group. The pigs remained in the nursery for six weeks. Body weights (Mosdal Seed Carts; Broad View, MT), testicular size,

and blood samples were collected weekly via procedures identical to those described previously.

At 42 days of age, four animals in each treatment were randomly chosen for surgical castration. Procedures before, during and after surgery were identical to those described previously with the following three exceptions. A 5 to 10 cm incision was made in the skin on the scrotum. In addition to using an angiotribe (12.7 cm) to affect hemostasis, visible blood vessels were ligated with a series of surgeon's knots using 1.3 cm umbilical tape (Ethicon; Summerville, NC). Finally, skin and connective tissue layers were closed with 3-O absorbable suture (3-O Catgut Chromic; Braun; Melsungen, Germany) using Ford's continuous, interlocking stitch pattern.

At 63 days of age (9 weeks) the boars were moved into the finishing barn. This occurred on June 29, 2006. This barn was a curtain-sided environmentally controlled building. The floors were completely slatted with a flush, underslat ventilation system. This barn was equipped with misters and cooling fans programmed to activate when ambient temperatures reach 25.5ºC to provide supplemental cooling for the boars. The boars were randomly assigned to one of nine 1.84 x 2.84 m pens and housed in groups of three to five. Each pen was equipped with a two-hole feeder with 0.91 m of feeder space and with 2 nipple waterers located in either the southwest or northwest corner of each pen. Boars were fed a corn and soybean meal based diet ad libitum formulated to meet the NRC requirements for growing boars (N.R.C., 1998). Crude protein was adjusted according to the age of the boar in the following manner: 9-12 weeks – 18%; 12-18 weeks – 16%; and 18-23 weeks – 14%. Monthly weight (Ezi Weigh 2; Tru Test; Mineral Well, Texas) and testicular measurements

were taken as described previously while the boars were in the finishing phase. The only exception to this was that a cloth tape was used to obtain the height and width for testicular size estimates rather than digital calipers. Blood samples were not taken at this stage. At 98 days of age, four boars in each treatment were randomly chosen for surgical castration. Procedures were identical to those described for boars castrated at 42 days of age except the size of the initial incision was increased by approximately 0.5 to 1.5 cm in order to accommodate the increased size of the testicles. At 161 days of age, boars were vaccinated for erisyphelas, parvovirus, and leptospirosis with commercially available vaccines.

At 175 days of age (25 weeks; October 13, 2006) 18 (6 from each treatment group) of the remaining 32 boars were moved into individual pens in the gestation barn. All boars in each treatment group were ranked based on body weight. The heaviest and lightest boars as well as 4 or 5 intermediate boars in each treatment group were selected to begin training to mount a collection dummy. The gestation barn was a curtain-sided building with underslat ventilation. The crates were 2.43 x 1.07 m. This barn was also equipped with misters and cooling fans programmed to activate when the ambient temperatures reached 25.5ºC providing additional cooling for the boars. Boars were fed a 14% corn and soybean meal diet formulated to meet the NRC requirements for boars (N.R.C., 1998). The boars were fed between 2.3 and 2.9 kg based on body weight and condition.

At 182 days of age (26 weeks; October 16, 2006) 20 boars began training for semen collection. The collection pen was 2.43 x 3.65 m and housed a dummy sow (Minitube of America, Inc., Verona, WI). The dummy sow was 0.3 m wide and 1.21 m long. During the training period, the dummy sow was kept at a height of 0.76 m. The dummy sow was

strategically located in the pen so that the boars could move freely on either side and behind the dummy, but not in front of it. Six boars from each treatment group were randomly placed into two treatment groups for training (two additional boars were also trained for replacement purposes). The first treatment group trained to collect 5 consecutive days per week while the second treatment group only trained 2 days per week. Each day of training, boars were removed from their crates and moved into the collection pen. Each training session was 5 minutes in duration, with two exceptions. The first was if the boar was actively interacting with the dummy after the 5 minute period had expired. In this situation, the training session was extended until the boar did not make contact with the dummy for 1 min. The second exception was if the boar mounted and was collected before the 5 minute period was over. In this situation, the boar was collected and returned to his respective crate. For each training session, the behavior of the boars was given a numerical score based on the following criteria:

0 – No interest or contact with the dummy during the training session

1 – Sniffs and rubs against the dummy but does not chew on, bite or charge it

2 – Aggressively interacts with the dummy – bites, chews on and charges it

3 – Mounts the dummy, but does not extend penis

4 – Mounts the dummy, extends penis, but does not collect

5 – Mounts the dummy, extends penis and is collected

Following the first successful collection, boars were collected for two additional consecutive days. They were then given at least a three day rest period and placed on a weekly collection schedule.

After 5 weeks of training, a control boar and a boar from the early treatment group were removed from the training regimen. One boar was lame and could not mount the dummy and the other had a penile injury. Two other boars (one control boar and one boar in the late treatment group) had not mounted the dummy sow and were considered training failures. These boars were replaced with four additional boars from the same treatment groups and were subjected to the same training regimen as previously described. Once 20 boars were trained to collect, they were all placed on a weekly semen collection regimen. One half of the boars were randomly assigned to Tuesday collections and the other half to Thursday collections.

All boars were collected using the gloved-hand technique (Almond et al., 1998). Semen was collected into a plastic bag (Minitube of America, Verona, WI). This semen collection bag was placed into a plastic thermos that was pre-warmed to 37ºC. The weight of the thermos and the plastic bag were recorded as a pre-weight. The top of the thermos was covered with a milk filter (IMV International, Eden Prairie, MN) piece of cheese cloth which was secured in place using a rubber band. After collection, the milk filter and rubber band were removed and the weight of the thermos containing the ejaculate was measured. The pre-weight was subtracted from the total weight after collection and the volume of the ejaculate was recorded as the difference between these two weights. The number of sperm cells per milliliter was measured using a SpermaCue® (Minitube of America, Verona, WI).

Semen volume, concentration of spermatozoa per mL, and total number of spermatozoa per ejaculate were collected weekly on each ejaculate. The semen was then placed into a water bath at 37ºC. The semen remained in the water bath until it was placed into a styrofoam cooler for transportation back to the laboratory at North Carolina State University, which was roughly 7 miles from the North Carolina State University Swine Educational Unit.

Between 182 and 539 days of age body weights (Ezi Weigh 2; Tru Test; Mineral Well, Texas) and testicular measurements were collected every month and blood samples were collected at 413, 483 and 539 days of age. Body weights and testicular measurements were obtained as described previously. Blood samples were collected via venipuncture of a marginal ear vein during collection with an infusion set (21 gauge, 0.75 in needle; Vaccutainer Blood Collection Set; Becton Dickinson; Franklin Lakes, NJ) connected to a 3 mL syringe. During the same time period boars were collected weekly and various estimates of semen quantity and quality were recorded (see subsequent sections for more detail). At 330 days of age, 1/7, 1/7, and 2/6 boars in the early, late, and control groups were selected at random for surgical castration. An additional 3/3, 3/3, and 2/4 boars in the early, late, and control groups that were not trained for collection were also castrated. Procedures used for surgical castration were identical to those described for 42 and 98 day-old boars except incisions through the scrotum were between 13 and 18 cm long and an emasculatome (HIFA; Poland) with a 15 cm head was used to initiate homeostasis.

After surgical castrations at 330 days of age, there were 4 boars in each treatment group that were maintained on a weekly collection schedule until they were 539 days of age. Semen depletion tests were performed on all boars at 448 and 546 days of age. This

consisted of collecting the boars daily for 5 consecutive days; allowing them to rest for 3 days; and then returning them to their weekly collection regimen. The study was terminated when the boars were 553 days of age. All procedures performed on the boars including the surgical castrations were approved by the N.C.S.U. Institutional Animal Use and Care Committee (06-035-A).

Analytical Procedures

Specific details of procedures used for analyses of semen quality, testicular histology, and seminal plasma and endocrine profiles are included in the following sections.

Semen Evaluation

Upon arrival at the laboratory, the semen was placed into a water bath pre-warmed to 37ºC. Two 1.5 mL samples of the neat ejaculate were placed into individual microcentrifuge tubes. One of these samples was stored at -20ºC for later analysis. The other sample was centrifuged at 1000g for 5 minutes to separate the seminal plasma from the sperm cells. The seminal plasma was then placed into a clean microcentrifuge tube and stored at -20ºC for later analysis. Beltsville Thawing Solution (BTS) (37.0g/l anhydrous glucose, 6.0g/l sodium citrate, 1.25 g/l sodium bicarbonate, 1.25 g/l EDTA disodium, 0.75 g/l KCl, pH 7.2, mOsm = 330) was prepared within 24 hours of semen collection and was pre-warmed to 37ºC the morning of collections. The semen samples were then extended in the warmed BTS. Extending the semen was done over a period of about 5 minutes via a slow drip of the warmed BTS into the tube containing the semen. The samples were extended so that there

were 3 billion sperm per 80 ml. This dilution is representative of insemination doses commonly used in commercial production. An example of the calculations for how much extender needed to be added to the neat samples were as follows:

Spermacue Concentration Reading = 250 x 10^6 sperm cells/ml

Volume of ejaculate = 200 ml

$$\frac{3{,}000 \times 10^6 \text{ sperm per dose}}{250 \times 10^6 \text{ sperm cells/ml}} = 12 \text{ ml of semen needed}$$

80 ml total volume – 12ml of semen = 68 ml of extender added

Semen quality evaluations for motility, head and tail morphology, and acrosome morphology were performed once per month beginning when the boars were 210 days of age (approximately 30 weeks of age) until they were 560 days of age (approximately 80 weeks of age). One mL of extended semen was placed into a 3 mL test tube (12 x 75mm; Port City Diagnostics, Inc.; Wilmington, NC). These test tubes were then placed into a pre-warmed incubator (Fisher Isotemp Oven 200 Series; Fisher Scientific; Atlanta, GA) at 37ºC for a minimum of 30 minutes. The samples were then thoroughly mixed by pipetting up and down before a 3 µl sample was removed. This sample was loaded into a pre-warmed Leja slide (Minitube of America, Inc.; Verona, WI). The Leja slides were pre-warmed and maintained at 37ºC on a slide warmer (Minitube of America, Inc.; Verona, WI). The Leja slides were then placed under the microscope (Model BX41; Olympus Optical; Tokyo, Japan) for the determination of motility. Determination of motility was carried out using a computer

assisted semen analysis system called SpermVision® (Minitbe of America; Verona, WI). Five microscopic fields were analyzed for each sample and the average motility, progressive motility and an approximate concentration were recorder for each. The whole procedure was repeated so that each boar's semen was sampled twice and a total of 10 fields (approximately 300-500 cells) were analyzed for motility.

SpermVision® (Minitbe of America; Verona, WI) was also used to analyze the straight line velocity (VSL), curvilinear velocity (VCL) and the straight line distance (DSL). The straight line velocity (µm/second) is a measure of the velocity of the sperm calculated from the straight line distance between the point where the spermatozoa begin to move and the end of the spermatozoa's track. The curvilinear velocity (µm/second) is the velocity calculated over the entire distance that the spermatozoa travel. This includes all small movements made by the head of the spermatozoa while swimming. Finally, the straight line distance (µm) is the distance that the spermatozoa travel calculated from the straight line distance between the point where the spermatozoa begins to move and the end of the spermatozoa's track. These measures are used to determine differences between spermatozoa in the path and distance that they are capable of swimming.

One mL of extended semen was also placed into a 3 mL test tube (12 x 75mm; Port City Diagnostics, Inc.; Wilmington, NC) and 100µl of a 10% Formalin solution was added to the sample as a fixative. The sample was then thoroughly mixed and a 10µl sample was placed onto an ethanol cleaned, glass microscope slide (Fisher Scientific; Atlanta, GA). A coverslip (18 x 18mm; Fisher Scientific; Atlanta, GA) was then placed on top of the sample. The slides were allowed to sit at room temperature for about 1 minute to allow the samples to

settle under the cover slip. The slide was then placed onto the stage of a phase contrast microscope (Zeiss; West Germany). The sample was searched with the 40x objective until spermatozoa were found. Once the spermatozoa were in focus, the objective was moved aside and 1 drop of immersion oil was placed on the slide. The oil immersion objective (100x) was then used to evaluate the sample. The percentage of normal acrosomes was calculated from a random sample of 100 cells. The classification scheme used by Pursel et al. (1972) was used to identify abnormalities in the acrosome.

Another 10µl sample was taken from the test tube containing the extended semen and 10% formalin. This sample was again placed on an ethanol cleaned, glass microscope slide and covered with a coverslip. The sample was again viewed under the 100x oil immersion objective. The percentage of morphologically normal sperm cells was calculated from a random sample of 200 cells. Each sperm cell that was examined was classified into one of the following categories:

1. Morphologically normal
2. Head abnormality: Abnormal size or shape of the head
3. Tail abnormality: Abnormal coiling or attachment of the tail
4. Translocated tail: The tail coils completely around the head of the sperm resulting in a paper clip appearance
5. Presence of a distal cytoplasmic droplet
6. Presence of a proximal cytoplasmic droplet

Testicular Histology

The following reagents and antibodies were used for histological preparation.

Product	**Company**	**Catalog #**
Xylene	Fisher Scientific, (Pittsburg, PA)	X3S-4
Ethanol	Fisher Scientific, (Pittsburg, PA)	AC615090040
Bouin's Fluid	Fisher Scientific, (Pittsburg, PA)	LC 11790-4
Permount Mounting Media	Fisher Scientific, (Pittsburg, PA)	SP15-500
Haematoxylin Stain	Fisher Scientific, (Pittsburg, PA)	SH26-5000
Eosin Stain	Fisher Scientific, (Pittsburg, PA)	E511-100
Paraffin Wax	Fisher Scientific, (Pittsburg, PA)	AC 41677-0020
Alexa Flour 488	Invitrogen Corporation (Carlsbad, CA)	A-11078
Alexa Flour 568	Invitrogen Corporation (Carlsbad, CA)	A-11004
Super Pap Pen	Invitrogen Corporation (Carlsbad, CA)	00-8899
Gata-4 Primary Antibody	Santa Cruz Biotechnology (Santa Cruz, CA)	SC01237
PCNA Primary Anitbody	Santa Cruz Biotechnology (Santa Cruz, CA)	SC56
Ki-67 Primary Anitbody	Santa Cruz Biotechnology (Santa Cruz, CA)	SC23900
Flour Save	EMD-Calbiochem (Temecula, CA)	345789
Normal Goat Serum	Millipore (Billerica, MA)	S26-100ml
Normal Rabbit Serum	Millipore (Billerica, MA)	S20-100ml

Embedding Protocol

At the farm, the right testicle was dissected from the tunica albuginea. It was then punctured with an 18 gauge needle 10-15 times to allow for complete penetration of the bouins fixative. The testicle was then completely submerged into a small glass jar containing Bouins fixative. For the 98 day-old boars and the 330 day-old boars, small sections of the testicle were placed in the jars containing the Bouins instead of the entire testicle. The left testicle was placed in a pre-labeled bag and stored on ice and transported back to the laboratory.

Once back at the laboratory, the left testicle was stored at -22ºC. A gross weight of the testicle and epididymis of the right testicle was taken. The epididymis and excess connective tissue was completely dissected from the testicle and a weight and volume were taken of the testicle and epididymis separately. Each testicle was submerged in a beaker of distilled water. The amount of water displaced by the testicle was recorded as the testicular volume. The testicle was then divided into halves or thirds depending on its size. Testicles from the 14 and 21 day old boars were divided into halves while all others were divided into thirds. A small 1 cm^3 section of tissue was taken from each half or third of the testicle. These small pieces of tissue were placed in a 1.5 ml bullet tube and filled with 1ml of Bouins fixative. A similar sample was also taken from the cauda, corpus and caput epididymis and placed in 1 ml of Bouins fixative. The samples remained in Bouins fixative overnight (approximately 16 hours).

The following morning, the Bouins solution was poured off. The samples were then washed by adding the ethanol solutions of increasing concentrations to the samples in the 1.5 ml bullet tubes. The procedure was as follows:

a. 1 ml 70% ETOH x 1 hr
b. 1 ml 70% ETOH x 1 hr
c. 1 ml 70% ETOH x overnight
d. 1 ml 80% ETOH x 1 hr
e. 1 ml 80 % ETOH x 1 hr
f. 1 ml 95% ETOH x 1 hr
g. 1 ml 95% ETOH x 1hr

h. 1 ml 100% ETOH x 1hr

i. 1 ml 100% ETOH x 1 hr

The tissue was then removed from the bullet tubes and placed into a pre-labeled embedding cassette. All of the cassettes were then placed into a large glass jar containing 100% xylene for 1.5 hours. The cassettes were then placed into pre-warmed paraffin (58ºC) and allowed to incubate overnight (approximately 16 hours). In the morning, the cassettes were removed from the paraffin and the tissue samples were quickly removed. Each sample was placed in a metal embedding mold. The metal mold was covered by a plastic embedding ring. Warm 100% paraffin was then poured into the embedding ring, completely covering the sample. The paraffin was allowed to cool to room temperature before being stored at 4ºC overnight (approximately 12 hours). In the morning, the metal molds were removed from the plastic embedding rings and the samples were checked to make sure that the tissue was completely embedded in the paraffin. Any exposed tissue would be melted and re-embedded in fresh paraffin. All samples were then stored at 4ºC until they were sectioned.

Sectioning

A rotary microtome (Model 820; American Optical Company; Buffalo, NY) was used to slice the paraffin embedded tissue into sections. The microtome was set to create cross sections that were 5µm thick. Ribbons of cross sections of tissue were collected once the full face of the tissue sample was being cut. Every 25th section was mounted cuts through the

middle of the tissue sample. This resulted in at least 9 full-face longitudinal serial sections taken from each testicle.

The ribbons of cross sections were then placed into a pre-warmed water bath. Approximately 1 gram of gelatin was added to the water to assist with the attachment of the tissue section to the slides. The tissue samples were then removed from the water bath with a 25 x 75 mm Superfrost positively charged microscope slide (M110WPL; Port City Diagnostics; Wilmington, NC) and allowed to dry at room temperature for a minimum of 2 hours. Once dry, the slides were placed into an oven at 59ºC overnight (approximately 16-24 hours).

Day 14 samples were not used for histological evaluation during the study. These tissue samples were not processed properly and therefore could not be used for analysis. A small section of the testicle was removed instead of embedding the entire organ. As a result, the sample was extremely small and unstructured. During sectioning, these samples would not adhere to the microscope slides during processing.

Haematoxylin and Eosin Staining and Morphometric Analyses

For each boar, three slides were stained with haematoxylin and counter stained with eosin for visualization of the germ cells. These tissue samples were used to identify spermatogonia that were not undergoing proliferation. Refer to Appendix C for pictures of haematoxylin and eosin stained tissue samples. Slides containing the dried paraffin sections were deparaffinized in xylene and rehydrated through decreasing grades of ethanol. Slides were than rinsed in deionized water before haematoxylin staining. Slides were then

counterstained with eosin and dehydrated through increasing grades of ethanol. A coverslip was then mounted using a xylene based mounting media. Slides were allowed to air dry before morphometric analysis was performed. A detailed description of these procedures is outlined in Appendix H.

The haematoxylin and eosin slides were used to enumerate and generally classify the spermatogonia within the seminiferous tubules. Four slides were prepared for each boar. Each tissue section was divided into four quadrants. One quadrant was randomly selected from each slide. One round seminiferous tubules was photographed from each of the four slides. This resulted in a total of four seminiferous tubules being analyzed per boar. The pictures of these tubules were then used to classify the cells to one of three categories based on the size and shape of the cell as described by Okwun and colleagues in 1996. The first group consisted of all large, round cells lying closest to the basement membrane within the tubule. These would include leptotene, zygotene and pachytene primary spermatocytes. The second grouping consisted of smaller cells that lie closer to the lumen of the tubule. This group was made up of the round and elongating spermatids. The third group consisted of all elongated spermatogonia located closest to the lumen of the seminiferous tubule.

Immunofluorescence Staining and Morphometric Analyses

Gata-4 was used as a marker for sertoli cells within the seminiferous tubule. Proliferating Cell Nuclear Antigen (PCNA) was used as a marker for cells that were undergoing proliferation. The immunohistochemistry procedures were modified slightly from those reported by McCoard et al. (2001) and Tarulli et al. (2006). The use of Ki-67 as a

marker for identification of proliferating sertoli cells was unsuccessful. Details are provided in Appendix B. The slides were removed from the oven and allowed to reach room temperature before beginning the procedure. In general, the procedure consisted of de-paraffinizing and re-hydrating the samples; microwaving the samples for antigen retrieval; immunolocalization of Gata-4 and PCNA antibodies; dehydrating; and drying for histological evaluation. Refer to Appendix C for pictures of fluorescently labeled tissue samples. The detailed procedure is described in Appendix H.

For each boar, three tissue sections from the middle of the embedded tissue section were evaluated for morphometric analysis. The microtome was set to cut 5-µm thick sections. A ribbon of 3-5 slices were taken every 25^{th} slice resulting in each ribbon being approximately a 125µm distance from the previous ribbon. On each slide, one 5-µm section served as a control and was not treated with either Gata-4 or PCNA. The remaining sections were stained with the antibodies and one was used for morphometric analysis.

The sections were examined to identify PCNA-positive and PNCA-negative sertoli cells using fluorescent microscopy. Slides were viewed using a Vanox microscope (Olympus Optical Co. Ltd.; Japan) fitted with an attachment for reflected and transmitted light fluorescence (Model AH2-RFL, Olympus Optical Co. Ltd.; Tokyo, Japan). This attachment allows for fluorescent viewing using blue-violet and green excitation regions (380-490nm and 465-550nm respectively). Digital images of all evaluated tissue sections were taken using a SPOT 2MP 14 bit Insight Color Firewire C-mount Camera (Southern Micro Instruments c/o Nikon Instruments, Inc.; Marietta, GA). Refer to Appendix C for pictures of tissue samples stained for GATA-4 and PCNA.

Microphotographs of tissue samples were divided into four quadrants. Two of the quadrants were randomly selected for histological evaluation. All measurements were made using a computerized software program called Spot Advanced v.4.5 (Diagnostic Instruments; Sterling Heights, MI). The area of the tissue sample representing one quadrant was photographed at a magnification of 200x using blue-violet emission range (Gata-4 emission) for determination of the volume percentage of seminiferous tubules and interstitial tissue. The image was projected onto the computer screen for morphometric measurements. The outline of each seminiferous tubule was circled and their corresponding areas were determined and recorded. Next, the total area of the quadrant was recorded. The volume percentage of seminiferous tubules was determined by calculating a sum of all of the areas of the individual seminiferous tubules and dividing this by the area of the entire quadrant. The volume percentage of interstitial tissue was determined by taking the difference between the volume percentage of the seminiferous tubules and the area of the entire quadrant. These values were then multiplied by the total volume of the testicle in order to estimate the total mass of seminiferous tubules within the testicle.

A second group of pictures that contained at least one round seminiferous tubule were taken of the same quadrant at a magnification of 400x . These pictures were taken using the blue-violet excitation range filter in order to view the cells that were expressing the GATA-4 antibody (cells expressing GATA-4 appear green). These pictures were used to count the number of sertoli cells in each seminiferous tubule. A third group of pictures were taken of the same area using the green excitation range filter in order to view the cells that were expressing the PNCA antibody (cells expressing PCNA appear red). These pictures were

then superimposed onto the second group of pictures (GATA-4 flourescence) to determine the number of sertoli cells expressing PCNA. Using the pictures formed by the merger of the GATA-4 and PCNA staining, cells that appear green inside the seminiferous tubule were considered to be sertoli cells that were not expressing PCNA. Cells in the seminiferous tubule that appeared yellow were considered to be sertoli cells expressing PCNA. Cells that appeared red were considered to be germ cells and spermatogonia because they were not expressing GATA-4 but were located within the seminiferous tubule.

The number of sertoli cells and the number of germ cells located in one round seminiferous tubule per quadrant were determined. The total area of the seminiferous tubule being analyzed was also recorded. The size of the nucleus was also determined for the sertoli and germ cells within the seminiferous tubule. For each cell type, the nucleus was measured using the computer settings for a sphere and the radius was recorded. Due to the irregular shape of the sertoli cell nuclei, two measurements were taken to determine a value for a long and short radius. For germ cells, the radius measurements were taken in duplicate and averaged. Nuclear volume was determined for sertoli cells by using the formula for a prolate sphere ($4/3\pi ab^2$; where 'a' is the long radius and 'b' is the short radius). Abercrombie's formula (Abercrombie, 1946) was used to adjust for capping effects ($4/\pi$). Nuclear volume was determined for the germ cells using the formula for a sphere ($4/3\pi r^3$). No corrections for tissue shrinkage were used because all tissue samples were fixed and processed in the same manner.

The average nuclear volume within each tubule was calculated. This value was then multiplied by the number of sertoli or germ cells in the tubule. The volume density of sertoli

or germ cell nuclei per seminiferous tubule was determined by dividing the mean nuclear volume by the volume of the seminiferous tubule (calculated as the volume of a sphere). This value was then used to calculate the average volume density of sertoli and germ cell nuclei per organ. The average volume density of nuclei per organ equals the average volume density of the nuclei multiplied by the volume density of the seminiferous tubule for that boar. The product of the testis volume and the average volume density of nuclei per organ was divided by the mean nuclear volume in order to determine the number of sertoli and germ cells per organ (Wreford, 1995).

Protein Gel Electrophoresis and Analysis

Two-dimensional gel electrophoresis was performed using a commercially available kit (Invitrogen Corporation, Carlsbad, CA). The following reagents were used:
(all supplies were purchased from Invitrogen Corporation, Carlsbad, CA)

PRODUCT	**CATALOG #**
Isoelecrtic Focusing Gels (IEF)	
Novex® pH 3-10 IEF gel 1.0mm, 10 well	EC6655A
Novex® IEF Cathode Buffer, pH 3-10	LC5310
Novex® IEF Anode Buffer, (50x)	LC5300
Colloidal Blue Staining Kit	LC6025
1-Dimensional Gels	
Novex® Tris-Glycine SDS Running Buffer (10x)	LC2675
See Blue® Plus2 Pre-Stained Standard	LC5925
Gel-Dry™ Drying Solution (1x)	LC4025
Novex® 4-20% Tris-Glycine Gel, 1.5mm x 2D well	EC6029

Two dimensional gel electrophoresis was used to determine the seminal plasma protein profiles. Ejaculates from all boars collected during the week of 10/04/07 (week 77 of the study) were used.

Seminal plasma samples were removed from the freezer and allowed to slowly warm to room temperature. Three boars could be run simultaneously on the same gel. Seminal plasma from a boar not involved in the present study was analyzed with each group of study boars and served as the internal control. Once thawed, 100µl of thoroughly mixed samples were added to 100µl of sample buffer in a clean 1.5ml bullet tube and mixed well. Cathode buffer, anode buffer and the IEF stain were prepared according to the manufacturers instructions.

The IEF gel apparatus (Novex E19001-XCELL II Mini Cell; Invitrogen; Carlsbad, CA) was assembled. Each IEF gel was rinsed with de-ionized water and the comb and tape removed from the gel. The gels were appropriately labeled for the corresponding analysis. The gels were placed in the apparatus and a small amount of cathode buffer was added to the inner chamber to check for leakage between the chambers. If no leakage was present, the inner chamber was filled with cathode buffer and the outer chamber was filled with anode buffer. Samples were run in duplicate by adding 25µl of sample to each corresponding well in the gel. 5µl of the standard were also run in duplicate in the 5^{th} and 10^{th} wells. The IEF markers used as standards were amyloglucosidase (p.I.=3.5), glucose oxidase (p.I.=4.2), trypsin inhibitor (p.I.=4.5), β-lactoglobulin (p.I.=5.3, 5.2), carbonic anhydrase (p.I.=6.0), myoglobin (p.I.=7.4, 6.9) and lectin (p.I.=8.0). Once all samples were loaded the IEF gels

were ran at 100 volts for 1 hour followed by 200 volts for 1 hour followed by 500 volts for 45 minutes.

The gels were then removed from their plastic casing and rinsed for 5 minutes in de-ionized water. The bottom portion of the gel and the "fingers" of the wells was carefully trimmed off the gel. The gels were placed into a fixative (17.3g sulphosalicyclic acid + 57.3g trichloroacetic acid in 500ml of DI water) for 1 hour with gentle agitation. The gels were then rinsed in DI water for an additional 5 minutes before being placed in IEF stain overnight allowing for visualization of the individual lanes of the gel.

The following morning, the gels were rinsed in DI water. Each sample's lanes of the IEF gel were cut down to fit into the well of the 2D gel. Novex pre-cast gels (4-20% Tris-Glycine gel / 1.5mm x 2D well; Invitrogen Corporation; Carlsbad, CA) were used for the 2D analysis. The Tris-Glycine SDS running buffer and staining solution were prepared in advance of the gel run. The gels were prepared in the same manner as the IEF gels.

The cut lanes from the IEF gel were inserted into the well of the 2D gel. The gels were positioned into the gel apparatus (same apparatus as the IEF gel run) and the Tris-Glycine running buffer added to the chambers similar to the IEF gels. 10µof the See Blue Standard (Invitrogen Corporation, Carlsbad, CA) was added to the standard well. The molecular weight markers used as standards were myosin (250 kDa), phosphorylase (148 kDa), BSA (98 kDa), glutamic dehydrogenase (64 kDa), alcohol dehydrogenase (50 kDa), carbonic anhydrase (36 kDa), myoglobin red (22 kDa), lysozyme (16 kDa), aprotinin (6 kDa) and insulin B chain (4 kDa). The gels were run for 1 hour and 45 minutes at 125 volts.

The gels were then removed from their plastic casing and rinsed for 5 minutes in de-ionized water. The gels were then placed in the tris-glycine stain and allowed to stain overnight (length of staining depends on age of stain where older stain may require additional staining time) with gentle agitation.

Once the gels were stained so that all of the protein bands were visible, the gels were allowed to rinse in DI water until the background became clear. This time varied from 1 hour to 5 hours. Gels were then allowed to soak in the Gel Dry™ Solution (Invitrogen Corporation; Carlsbad, CA) for at least 10 minutes. The Gel Dry™ Solution is designed to control the rate at which the gels dry to prevent the gels from cracking. The soaked gels were carefully placed in between two pieces of cellophane (also pre-soaked in Gel Dry™ Solution) on a drying rack. The gels were placed in a cool, dry place for 48 hours to dry. Once dry, the cellophane was trimmed from the edges of the gel and the gels were placed in individual pieced of blotting paper. The gels were appropriately labeled for identification and stored beneath weighted objects to minimize curling of the gels until analysis.

For analysis, the IEF standards p.I. range needed to be marked on each gel to allow for easy reference between the p.I.s and molecular weights. There were 9 standard p.I.'s marked; 3.5, 4.2, 4.5, 5.2, 5.3, 6.0, 6.9, 7.4 and 8.0.

Each gel was then scanned into the computer to create a digital image using a Hewlett Packard hp Scanjet 8200 scanner. Refer to Appendix E for pictures of the scanned gel images. A horizontal and a vertical image were created for analysis of the molecular weight and p.I.s, respectively. A computer software program, Image Quant TL (Amersham Biosciences; Piscataway, NJ), was used for protein detection. In general, all of the

identifiable proteins on the gel were marked and the volume normalized to myosin (250 kDa). All data was printed and saved for comparison between boars.

The boar that was used as an internal control had the greatest number of different proteins present in his seminal plasma. Therefore, the image of his gel was used to make a template by which to compare all of the other boar's seminal plasma protein profiles to. Each protein on the template was given an identification letter based on the location in the gel vertically. Then each protein on the template was given a number based on the location in the gel horizontally. This resulted in each protein having a letter and a number identification. Images of gels from all other boars were compared to the template and all proteins were assigned a letter and number identification. The molecular weight, isoelectric point, volume and normalized volume were recorded for each protein from each boar. The normalized volume was then compared to the total protein content of the ejaculate. This compared value was then analyzed statistically for differences between boars.

Radioimmunoassays

Concentrations of estradiol-17β, testosterone and insulin-like growth factor (Siemens Medical Solutions Diagnostics; Los Angeles, CA and Diagnostic Systems Laboratories; Webster, TX) were determined with commercially available radioimmunoassay kits that were validated previously for porcine serum. All samples were assayed according to the manufacturer's instructions provided with the kits. The inter and intra-assay coefficients of variation for estradiol and testosterone were: 2.17, 0.62; and 2.03, 4.98. All assays for IGF were run on one day, therefore the inter-assay coefficient of variation was 4.59.

Serum FSH was analyzed using a double antibody radioimmunoassay. All reagents were purchased from Dr. A. F. Parlow of the National Hormone and Pituitary Program (Harbor-UCLA Medical Center, Los Angeles, CA). Purified pFSH used for iodination and standards was lot # AFP10640B and the pFSH antisera was lot # AFP 2062096RB. Iodination was performed according to protocol from a personal communication from Dr. J. J. Ford (USDA, Clay Center, NE). Briefly pFSH was diluted in 0.01 M sodium bicarbonate and 2.5 ug of FSH was then aliquotted into vials for iodination. Ten uL of 0.5 M PO_4 was added for volume and then 0.5 mCi of I^{125} (Perkin-Elmer,) in 25 uL 0.5 M PO_4 pH 7.5 was added. Then 2.2 uL of chloramine-T (12.5 mg per 20 mL in 0.01 M PO_4 pH 7.5) was added for 45 seconds. The reaction was stopped by addition of 50 uL sodium metabisulfite (12 mg per 10 mL of 0.01 M PO_4 pH 7.5). Then 200 uL of KI was added to the tube and the entire contents then transferred to the column. The reaction tube was then rinsed with 100 uL of KI and this was added to the column. The column was prepared according to the iodination method described by Kraeling et al., (1982) and the fraction with the highest counts was then further purified using the method described by Rayford et al., (1974). The iodination column used AG 1-X8 anion exchange (Bio-Rad,) 0.5 cc in a 3 cc syringe which was prepared using two mL of 0.5 M PO_4 , one mL of 1% BSA in 0.1M PO_4, and two mL of 0.05 M PO_4. Elution was done using 2 mL of 0.05M PO_4. Purification of the fraction was done using a disposable Pasteur pipette packed loosely with 4 cm of AG 1-X8 anion exchange resin. The column was pre-washed with 5 mL of PBS, 500 uL of porcine serum, and then again 5 mL of PBS. Then 100 uL of the peak fraction was added to the column and eluted with 800 uL of PBS.

On the first day of the assay 400 uL of serum was added to the tubes. Following this step 100 uL of the I^{125} pFSH containing approximately 50,000 cpm was added to all tubes. The FSH antibody (200 uL) was added to all tubes except the total counts and non-specific binding tubes at a dilution of 1:40,000. Tubes were vortexed briefly in a multi-tube vortexer and were left to incubate for 48 h at room temperature. The second antibody was obtained from Dr. Parlow (goat anti-rabbit gamma globulin) and was diluted 1:20 with PBS. Two hundred uL was added to all tubes except total counts, tubes were vortexed briefly with a multi-tube vortexer and left to incubate overnight at room temperature. The next day 2 mL of cold PBS was added to all tubes except total counts and the tubes were then centrifuged for 30 minutes at 4ºC and then decanted and left to dry. Dried tubes were counted for one minute using a COBRA gamma counter as described above. Serum concentrations were determined by an automatic program by comparison with the standard points.

Standards and pooled aliquots of porcine sera were parallel over a serum volume ranging from 50 to 400 μL. Recovery of known amounts of pFSH added to serum pools averaged 93.8%. Sensitivity of the assay was 0.3 ng/mL. The inter and intra-assay coefficients of variation for testosterone, estrogen and LH were: 2.03, 4.98; 2.17, 0.62; and 2.75, 5.2, respectively. The assays for FSH and IGF were all performed on one day. The inter-assay coefficients of variation for FSH and IGF were 2.59 and 4.59, respectively.

Enzyme-Linked Immunosorbent Assays

Commercially available enzyme-linked immunosorbent assays were used to analyze tumor necrosis factor alpha (Porcine TNFα ELISA Kit; Pierce Biotechnology, Rockford,

IL,), transforming growth factor beta (TGFβ_1 E_{MAX} Immunoassay System; Promega Corporation; Madison, WI.) and heat shock protein 70 (Hsp 70 ELISA Kit; Assay Designs; Inc., Ann Arbor, MI) in the seminal plasma samples. These kits were validated using boar seminal plasma (Appendix G). Each seminal plasma sample was centrifuged (10,000g for 10 minutes) to remove cellular debris and 100 µl was analyzed in duplicate. Assays were run following manufacturers instructions with the exception of the TNFα, where the seminal plasma samples were not diluted prior to analysis. The inter- and intra-assay coefficients of variation were as follows: TNFα: 9.84, 10.45, TGFβ: 8.9, 11.6, HSP70: 0.339, 5.73.

Statistics

All statistical analyses were performed using Statistical Analysis System® (SAS) version 9.1 (Cary, NC). Specific details of each program are included in Appendix D.

Body Weight, Testicular Measures and Hormone Concentrations

Body weight, testicular measures and serum concentrations of hormones were analyzed using analysis of variance procedures for repeated measures (Gill and Hafs, 1971). The statistical model included treatment (FSH-early; FSH-late; or Control), boar age (weeks 2 through 77) and the treatment by boar age interaction. Boar nested within treatment group was used as an error term to test for treatment effects. When main effects were significant, Student-Newman-Keuls Multiple Range Test (SNK) was used to determine differences among treatments or boar age.

Morphometric Analyses

The number of sertoli cell per organ; the number of germ cells per organ; the number of sertoli cells per gram of testis; the number of germ cells per gram of testis; percentage of sertoli expressing PCNA; the percentage of germ cells expressing PCNA; the mass and percentages of seminiferous tubules and interstitial space occupied in the testis were all analyzed using analysis of variance procedures for general linear models (Snedecor and Cochran, 1989). The model included treatment (FSH-early, FSH-late and Control), boar age (21, 42, 98 and 330 days) and a treatment by boar age interaction. All percentage data were square root transformed prior to statistical analysis. When main effects were significant, Student-Newman-Keuls Multiple Range Test (SNK) was used to determine differences among treatments or boar age.

Using the haematoxylin and eosin stained slides, germ cells were counted and classified into one of three categories based on the shape and size of the nucleus. These data were analyzed using analysis of variance for general linear models (Snedecor and Cochran, 1989). The model included treatment (FSH-early, FSH-late and Control), boar age (21, 42, 98 and 330 days) and a treatment by boar age interaction. When main effects were significant, Student-Newman-Keuls Multiple Range Test (SNK) was used to determine differences among treatments or boar age.

Semen Evaluation

The total number of sperm in the ejaculate was analyzed using proc Mixed in SAS. The model included the fixed effects of week of age and treatment and the random effects of individual boars.

All measures of semen quality were arcsine transformed prior to statistical analysis. They were then analyzed using analysis of variance for repeated measures (Gill and Hafs, 1971). The model included treatment (FSH-early, FSH-late and Control), boar age (30 through 77 weeks) and the treatment by boar age interaction. Boar nested within treatment group was used as an error term to test for treatment effects. When main effects were significant, Student-Newman-Keuls Multiple Range Test (SNK) was used to determine differences among treatments or boar age.

Training to Mount and Collect

A boar was considered successfully trained once they had successfully collected for three consecutive days and were placed on a weekly collection regimen. The length of time from the start of training until the boar was successfully collected was calculated and used to analyze differences between FSH treatment groups as well as differences between the various training regimens. Length of time to train was analyzed using analysis of variance procedures for general linear models (Snedecor and Cochran, 1989). The model included FSH treatment group (FSH-early, FSH-late and Control), training treatment group (2 days a week or 5 days a week) and a FSH treatment group by training treatment group interaction.

When main effects were significant, Student-Newman-Keuls Multiple Range Test (SNK) was used to determine differences among treatments or training treatment group.

Seminal Plasma Proteins, TNFα, TGFβ and HSP70

The normalized volume of the seminal plasma proteins were analyzed two ways. First, analysis of variance for general linear models was used to determine differences between boars in the concentrations of the proteins. The model included treatment (FSH-early, FSH-late and Control). When main effects were significant, Student-Newman-Keuls Multiple Range Test (SNK) was used to determine differences among treatments.

Results

Body Weight and Testicular Size

There was not a significant effect of treatment on body weight of the boars ($p=0.763$). There was a significant effect of age on body weight of the boars ($p<0.0001$) where body weight increased linearly with age (Figure 2). The treatment by week of age interaction was also significant ($p<0.0001$). At 46 weeks of age, the control boars weighed significantly more than the FSH treated boars (Figure 2).

The area of the testicles did not differ among treatment groups ($p=0.4731$, Figure 3). Testicular area increased linearly with age ($p<0.0001$, Figure 3). There was also a treatment by age interaction ($p<0.001$). Prior to 19 weeks of age, testicular area did not increase rapidly. However after 19 weeks of age, testicular area increased linearly.

Testis area to body weight ratio was used to adjust for any effects of body weight on testicular growth. No differences were seen between treatments ($p=0.6034$, Figure 4) in the testis size to body weight ratio. However, there were significant differences in testis size to body weight ratio due to boar age ($p<0.0001$, Figure 4). Prior to 19 weeks of age, the testis area to body weight ratio decreased. After 19 weeks of age, the testis area to body weight ratio increased linearly.

At castration testicular and epididymal weights and volumes were measured. There were no differences among treatments in testicular or epididymal weights and volumes ($p=0.7710$; $p=0.4053$ and $p=0.0.7735$; $p=0.4875$, respectively, Figures 5 and 6). There were differences among castration ages on the weight and volume of the testicle ($p<0.0001$, Figures 5 and 6) where the testicles of the 330 day old boars weighed significantly more than

the other treatment groups and also had a greater volume. There were significant differences among castration ages in the weight and volume of the epididymis ($p<0.0001$, Figures 7 and 8) where the 98 day-old and 330 day-old boars had increased weights and volumes of the epididymis compared to other castration ages.

Radioimmunoassays for Serum Hormone Concentrations

Estimation of serum hormone concentrations for individual boars before 5 weeks of age were not possible due to the small volume of blood collected. There was a treatment by week interaction for serum FSH concentrations ($p=0.016$, Figure 9). At 6 weeks of age, the late FSH-treated boars had significantly higher FSH concentrations. At 7 weeks of age, the early FSH-treated boars had increased FSH concentrations. In general, FSH concentrations were greatest at 5 and 6 weeks of age and decreased to concentrations below the sensitivity of the assay after 9 weeks of age.

There was a significant interaction between treatment and week of age for testosterone concentrations ($p=0.0001$, Figure 10). At 77 weeks of age, the early FSH treated boars had an increased testosterone concentration compared with the control boars and the late FSH-treated boars ($p<0.0001$). Testosterone concentrations remained low from 5 weeks of age through 9 weeks of age. At 16 weeks of age, testosterone concentrations began to increase.

During week 77, the average estrogen concentration for all boars was beyond the highest value for the sensitivity of the assay (>500pg/ml), therefore these values could not be analyzed. There were no differences among treatment groups ($p=0.5890$, Figure 11) for

serum estrogen concentrations. There was a significant effect of time ($p<0.0001$, Figure 11) on serum estrogen concentrations as they were highest on weeks 59 and 69 weeks of age.

The average concentrations of LH and IGF were not analyzed statistically for individual boars because of insufficient volumes of serum for analysis. However, samples were pooled within treatment group and mean values for serum LH and IGF are presented in Figure 12.

Histological Examination of the Testicles

There were no differences among treatments for seminiferous tubule mass ($p=0.406$, Figure 13), proportion of the testicle occupied by the seminiferous tubule ($p=0.621$, Figure, 14), the mass of the interstitial space ($p=0.406$, Figure 15) or proportion of the testicle occupied by the interstitial space ($p=0.405$, Figure 16). However, each of these increased with boar age ($p<0.0001$, Figures 13, 14, 15 and 16).

Sertoli cells were identified by the uptake of GATA-4. There were no significant differences in the number of sertoli cells per organ among treatments ($p=0.7010$, Table 1). There were differences ($p<0.0001$, Table 1) among ages in the number of sertoli cells per organ. The 98 day-old boars had a significantly higher number of sertoli cells per organ compared to all other castration ages. There were no differences between the number of sertoli cells per gram of testis between treatment groups ($p=0.2050$, Table 2). However, there was a significant effect of time on the number of sertoli cells per gram of testis ($p<0.0001$, Table 2) where boars that were 21 days old had an increased number of sertoli cells per gram of testis compared to all other castration ages. There were no differences among treatments

in the average nuclear volume of the sertoli cells (p=0.8413, Figure 17). There were also no effects of age on the average nuclear volumes of the sertoli cells (p=0.173, Figure 17).

Germ cells were enumerated using PCNA staining. There were no significant differences among treatment groups in the number of germ cells expressing PCNA (p=0.1545, Table 3). There were differences among ages in the number of germ cells expressing PCNA ($p<0.0001$, Table 3) where the 330 day old boars had a significant increase in the number of germ cells expressing the PCNA antibody (Figure 18). It should be noted that the PCNA only stained the primary spermatocytes and not other sperm cells. There were no differences among treatment groups in the number of germ cells expressing PCNA per gram of testis (p=0.1325, Table 4). There was an effect of age on the number of germ cells expressing PCNA per gram of testis ($p\leq0.0001$, Table 4) where the 21 day-old boars had significantly more germ cells expressing PCNA compared to all other castration ages. There were no differences among treatments or castration ages for germ cell nuclear volume (p=0.3137 and p=0.077, respectively, Figure 19).

Germ cells were also enumerated using slides stained with haematoxylin and eosin. The average number of germ cells located within a cross section of a round seminiferous tubule did not differ among treatments (p=0.7844, Figure 20). There was a significant effect of age on the number of germ cells in the cross section of a seminiferous tubule ($p<0.0001$, Figures 20 and 21). The 330 day old boars had significantly higher numbers of spermatozoa present in the tubules compared to all other age groups.

There was an age by treatment interaction for the number of germ cells classified as belonging to group 1 (primary spermatocytes) (p=0.0005, Figure 22). In the 330 day-old

group, animals receiving the early FSH treatment had a significantly higher number of primary spermatocytes per cross section of a single seminiferous tubule compared to the control boars or the boars ($p<0.0001$, Figure 22). There were no treatment differences among the numbers of spermatozoa classified as group 2 or group 3 cells ($p=0.1681$ and 0.5265, respectively, Figure 23). There was an effect of age ($p<0.0001$, Figure 23). This was due to the observation that the 330 day-old boars were the only animals that had any group 2 and group 3 spermatozoa located within the tubule.

The percent of the total number of sertoli cells undergoing proliferation, as indicated by PCNA uptake by the nuclei, were not different among treatments ($p=0.1480$, Figure 24). There was a significant effect of time on the number of sertoli cells undergoing proliferation ($p<0.0001$, Figure 25) where the 98 and 330 day old boars had significantly less proliferating sertoli cells. At 98 days, only the FSH-treated boars have any sertoli cells undergoing proliferation.

Semen Analysis

There was a tendency for the total number of sperm per ejaculate to be different among treatment groups ($p=0.06$, Figure 26) where the average number of sperm cells per ejaculate was higher for the boars in the early FSH treatment group compared to the control boars or the boars in the late FSH treatment group. There was also a significant effect of time on the number of sperm cells per ejaculate ($p<0.0001$, Figures 27 and 28) where a linear increase in sperm production occurred as boars aged irrespective of treatment group.

There were no differences among treatments in the percentage of motile ($p=0.3630$) or progressively motile ($p=0.3589$) spermatozoa in the ejaculate (Figure 29). There was no effect of time on the percentage of motile spermatozoa in the ejaculate ($p=0.0913$, Figure 28). There was an effect of time on the percent of progressively motile spermatozoa present in the ejaculate ($p\leq0.0001$, Figure 30) where a significant decrease in progressively motile sperm were seen at 48 weeks of age and a significant increase in progressively motile sperm occurred during weeks 69 and 77.

Three additional measures of sperm motility were examined: curvilinear velocity (VCL), straight line distance (DSL) and straight line velocity (VSL). No significant differences among treatments were found ($p=0.9538$; $p=0.4001$; and $p=0.4014$, respectively, Figure 31). There were differences among weeks of age for each mobility measure ($p\leq0.0001$, Figure 31) where mean values increased at 64 weeks of age and remained elevated through 77 weeks of age.

There were no differences among treatments in the percentage of normal acrosomes ($p=0.3601$), percentage of morphologically normal spermatozoa ($p=0.4414$), percentage of spermatozoa with distal cytoplasmic droplets ($p=0.3857$), percentage of spermatozoa with proximal cytoplasmic droplets ($p=0.4161$), percentage spermatozoa with translocated tails ($p=0.3714$), percentage of spermatozoa with tail abnormalities ($p=0.4316$), or percentage of spermatozoa with abnormal heads ($p=0.4165$) (Table 4). There were differences among weeks of age for percentage of spermatozoa with normal acrosomes ($p\leq0.0001$) and percentage of spermatozoa with abnormal tails ($p=0.0352$) where random variation occurred from week to week (Figure 32).

Training to Mount and Collect on a Dummy Sow

All boars that were trained to mount and collect on a dummy sow were randomly assigned to be trained either 2 days per week or 5 days per week. The effects of FSH treatment as well as the effects of a 2 versus 5 day a week training regimen were examined. No differences between FSH treatment groups ($p=0.9592$) or training 2 days a week compared to 5 days a week ($p=0.2061$) were found (Figure 33).

Seminal Plasma Protein Concentrations

Fifty-four proteins were identified in the seminal plasma for evaluation. There were no differences among treatment groups in the relative concentrations of the individual proteins per mg of total protein present in the seminal plasma (Table 5). There were also no significant differences among treatment groups in the amount of total protein present in the seminal plasma ($p=0.8734$).

Heat Shock Protein 70, TNFα and TGFβ were also examined in the seminal plasma in the adult boars. The amount of HSP70, TNFα and TGFβ were analyzed as a concentration per milligram of total protein in the seminal plasma. There were no differences among treatments on HSP70, TNFα or TGFβ concentrations ($p=0.3593$, $p=0.4740$, and $p=0.6980$, respectively, Table 6).

Discussion

The objectives of this study were to determine the effects of FSH administration during the neonatal period on sertoli cell mitotic activity and adult sperm production. It was found that adult sperm production was increased with administration of FSH from 4-22 days of age. This is in agreement with studies performed in rats (Orth,1984; Meachem et al., 1996) and contrary to studies performed in pigs (Wagner and Claus, 2008). The sperm concentration in the ejaculates of the early treated boars was 116% of control boars. This increase in sperm number is most likely correlated to an increase in testosterone production in the adult boars treated with FSH from day 4-22 of age. The boars that received FSH injections from day 4-22 had increased testosterone concentrations as adults. These results are similar to data reported by Swanlund and colleagues (1995) where there was a tendency for boars receiving FSH injections from 8-40 days of age to have higher testosterone concentrations at 100 days of age. If this study had continued past 100 days of age and into pubertal development, a significant difference in testosterone concentrations may have been evident. The present study did not see significant differences until 77 weeks of age. Studies in rats have found that FSH can stimulate leydig cell function by increasing the number of LH receptors on the leydig cell (Orth et al., 1977, Chen et al., 1976). Injections of FSH during the neonatal period could be increasing the number of LH receptors on the leydig cells. Leydig cells produce testosterone in response to LH. Therefore if there are more LH receptors present on the leydig cells, then a greater amount of testosterone would be produced.

Another possible mechanism for an increase in testosterone in these boars would be that the sertoli cell produces a steroidogenic factor in response to FSH. This factor has been identified in *in vitro* studies in the rat and pig where stimulation of sertoli cells with FSH has increased testosterone production from leydig cells in co-culture (Verhoeven and Cailleau, 1985, Benahmed et al., 1982). The boars that received FSH injections from day 22-40 did not show an increase in testosterone concentrations as adults. A possible explanation for this could be because negative feedback has begun after three weeks of age. Therefore increasing peripheral FSH concentrations may provide negative feedback on the hypothalamus to decrease the amount of GnRH secretion, therefore decreasing FSH and LH production in these boars. Prior to three weeks of age, the boars' hypothalamo-pituitary-gonadal axis is functioning without negative feedback mechanisms. Therefore, the increase in peripheral FSH concentrations can act as a stimulatory factor on the gonad.

Neonatal FSH administration resulted in an increase of roughly 9 billion sperm per ejaculate. The industry standard for insemination doses in swine is 3 billion sperm per dose. Therefore, this increase in concentration would correlate to three additional semen doses produced from a single ejaculate. Theoretically, three additional sows could be mated from the ejaculate of an early FSH-treated boar compared to a control boar. This could have a great economic impact for the producer because three additional matings could potentially result in the production of roughly 30 additional piglets born. When considered over the average productive life of commercial boars (78 weeks @ 1.5 collections per week), this would translate into an advantage of 1,053 billion total spermatozoa or 351 insemination doses.

Most of the research in rodents evaluates germ cell numbers histologically and not in the ejaculate of the adult rat. Germ cell numbers were also examined histologically in the present study. Using the slides fluorescently labeled with PCNA allowed only the identification of germ cells that were proliferating at the various ages. While this may be an adequate measure of the small number of cells in the younger boars, it did not provide a good representation of the numbers of spermatogonia in the adult boar. Germ cell counts were then performed on the haematoxylin and eosin stained slides. These are reported as the total number of germ cells present within a single cross section of the seminiferous tubule. These do not represent the numbers of cells within the entire organ. These suggest that the increase in the numbers of sperm cells seen in the ejaculates of early treated boars could be due to an increase in the number of primary spermatocytes located within the seminiferous tubule. Testosterone has been shown to be necessary for the maintenance of spermatogenesis. The androgen dependant phases of spermatogenesis occur during stages of the cycle of the seminiferous epithelium when the B spermatogonia are entering mitosis. The boars that were treated with FSH from days 4-22 also had significantly higher testosterone concentrations as adults. This increase in testosterone production could account for an increase in the numbers of primary spermatocytes located within a cross section of the seminiferous tubule.

There was also an effect of time on sperm production in the boars. At puberty, germ cells in the testicle will undergo mitotic events in the process of spermatogenesis. An individual boar's sperm production will initially increase linearly as the boar ages until it plateaus when the boar reaches its adult sperm production. Griffith (2006) compared timing of the initiation of collection regimens (160 days vs 190 days) on the age at which adult

sperm production was achieved. They found that beginning collections at 190 days of age resulted in adult sperm production being achieved between 10-11 months of age compared to 13-14 months of age for boars collected at 160 days of age. The boars in the present study began training regimens at roughly 180 days of age. It appears as though they reached a plateau in sperm production at about 43 weeks (11 months) of age. There were additional fluctuations in sperm production after this age. There may be a slight increase in sperm production around 57 weeks of age (14 months) as well as 72 weeks of age (18 months). This increase at 57 weeks could actually be when the boars are reaching their adult sperm production. After that, the fluctuations in sperm production could be due to seasonal differences in sperm production. The boars were 18 months of age (72 weeks) during the month of October. This coincides with the time of year when sperm concentrations have been shown to be higher as compared to the summer months.

No negative effects on semen quality were associated with the FSH-induced increases in spermatozoa production. For most of the estimates of semen quality, FSH-treated boars had a numerically greater numbers of morphologically abnormal spermatozoa in their ejaculates compared to control boars, however these differences were not significant. Flowers (1997) found no differences in fertilizing capabilities between ejaculates that had >60% motility, >70% normal acrosome morphology and >70% normal sperm morphology. Even if all of the morphological abnormalities were summed together in the present study, they would not total greater than 30% of the sperm cells in the ejaculate. Therefore, from a biological perspective, the negative effects on estimates of semen quality probably would not cause a reduction in the number of live offspring produced from that boar. However, this

study did not go as far as to inseminate multiple sows to determine the number of live offspring produced by each boar.

It is normal for the semen quality estimates to vary from week to week. In the present study, there were significant differences between the semen quality estimates from week to week without an identifiable pattern to the variation. Griffith (2006) analyzed semen from maturing boars of similar genetic make up to the present study. They found that motility fluctuated around 80%. They also reported normal acrosome morphology to fluctuate between 70 and 90%. This study summed all of the morphological abnormalities together and reported variation from 5 -25 % of the sperm in the ejaculates as being characterized as abnormal. Results of the present study are similar to these findings.

Neonatal FSH treatment did not influence the measures of velocity and distance that the spermatozoa traveled. However there were some differences in these measures over time, similar to what was seen for all semen quality estimates. The use of computer-assisted methods for evaluating motility, velocity and distance have been studied in humans, rats and pigs. In humans and rats, correlations between the velocity of spermatozoa and *in vitro* measures of fertility have been found (Holt et al., 1985, 1989, Moore and Akhondi, 1996). However, few studies have examined the relationship between the measures of distance and velocity of the spermatozoa and *in vivo* measures of fertility. Holt and colleagues (1997) examined the relationship between these motility parameters and number of pigs born alive. They found that the curvilinear velocity was a significantly correlated with litter size, however these estimates are dependant on the amount of time that the spermatozoa are incubated in media prior to insemination. The estimates of VCL and VSL in the present

study are slightly higher after 30 minutes of incubation in semen extender compared to fresh and 2-hour incubated samples in the study performed by Holt and colleagues (1997). However, these differences could be due to the different semen extenders that were used. Holt and colleagues (1997) used a Tris-buffers media for extending semen where as the present study used Beltsville Thawing Solution (BTS) containing supplemental glucose. This additional energy in the BTS could increase the velocity of the spermatozoa. The present study does not have any data to report on litter sizes for the boars, so a correlation between these estimates of semen quality and fertility cannot be made.

It has been well established in rodents that sertoli cell proliferation during the fetal and neonatal periods is representative of the adult sertoli cell population (Orth, 1988). Further, supplemental FSH during this neonatal period can increase the rate of sertoli cell mitosis resulting in an increase in the population of sertoli cells (Meachem et al., 1996, Sharpe, 1994). Similar results have been suggested in pigs where neonatal FSH administration resulted in an increase in the length of the seminiferous tubule (Swanlund et al., 1995). This increase in the population of sertoli cells is thought to be responsible for the increase in spermatogonial numbers. Contrary to this research, the increase in adult sperm production in the present study was not associated with an increase in sertoli cell number or mitotic activity as indicated by the uptake of PCNA. The effects of neonatal FSH treatment were assessed by measuring the mass of the seminiferous tubules and the relative numbers of sertoli cells per organ and per gram of tissue. The results of the present study suggest that neonatal treatment with FSH does not increase the number of sertoli cells in the adult boar (330 days of age). Although not significant, there does appear to be a tendency for the

treated boars to have an increased number of sertoli cells per organ at day 98 compared to the treated boars. At 21 and 42 days after birth there appears to be a tendency for the FSH treated boars to have a lower number of sertoli cells compared to the control boars. The lack of differences in the number of sertoli cells undergoing proliferation during the treatment period has also been found in similar studies in rats using BrdU labeling as a measure of mitotic activity (Meachem et al., 1996). However, in the present study, the sertoli cells of the FSH treated boars tend to proliferate over a longer period of time. At 98 days of age, the FSH treated boars still have some sertoli cells that are taking up the PCNA stain. It is a small percentage of the total number of cells, however this could explain why there is a tendency for the treated boars to have higher numbers of sertoli cells at day 98 compared to control boars. So taken together, one explanation for these results would be that neonatal FSH treatment does not increase the rate of proliferation of sertoli cells but increases the length of time over which proliferation occurs, or delays maturation of the sertoli cells resulting in an increase in the length of time over which proliferation can occur. These results would be different from studies in rats where FSH treatment increased the rate of mitosis whereas neonatal hypothyroidism delayed maturation of the sertoli cells (Sharpe, 1994).

Another possible explanation for a lack of an increase in sertoli cell number could be due to length of time that FSH was administered or the amount of FSH. Swanlund and colleagues (1995) administered FSH at a concentration of 100µg/kg body weight daily from 8-40 days of age. The present study administered 100µg/kg body weight every other day from either day 4-22 or day 22-40. Collectively, the present study only administered about 1/3 of the total amount of FSH as Swanlund and colleagues did. Due to a limited number of

animals available for the present study, a combined treatment group (pigs receiving FSH from day 4-40) was not able to be added to the study. Meachem and colleagues (1996) administered FSH to neonatal rats and found an increase in sertoli cell numbers in the testicle. This study administered FSH daily from birth until day 5, 10, 15 or 20 after birth. They found that sertoli cell number was increased the greatest with 15 days of FSH administration. In the rat, sertoli cell mitosis ends between 15 and 17 days of age. Therefore, FSH administration over the entire length of time for which mitosis is occurring was the most effective at increasing sertoli cell numbers in the testicle. The present study may have not administered enough total FSH or over a long enough period of time to see an increase in sertoli cell numbers in the testicle.

Interpretation of the numbers of sertoli cells present at 330 days of age may need to be examined carefully. In some boars, Gata-4 staining in the 330 day old boars was of a lesser visual intensity compared to staining at younger ages. The intensity of the Gata-4 was not measured in this study. This made counting the nuclei of the sertoli cells more difficult at this age than at the younger ages. In addition, some of the tissue samples collected at 330 days of age may not have been fully engorged with Bouin's fixative immediately following castration. This could result in a loss of sensitivity to the Gata-4 staining. Gata-4 has been identified as a marker for sertoli cells within the seminiferous tubule at postnatal, prepubertal and postpubertal ages (McCoard et al., 2001). This paper did not make mention of the intensity of Gata-4 staining at different ages, just that it was present in the sertoli cell nuclei. In 2003, McCoard and colleagues examined the uptake of Gata-4 in testicular samples taken from white cross boars during the fetal and neonatal periods. They found that Gata-4

staining increased in intensity from birth until 14 days of age and then decreased until 25 days after birth. This study did not follow the boars until maturity, so it is not know whether the intensity of Gata-4 staining continued to decrease as the boar matured. In the hamster and rat, Gata-4 staining has been shown to be intense in the immature and mature animals (Tarulli et al., 2006). This study by Tarulli and colleagues (2006) also showed non-specific staining for Gata-4 in the acrosomes of elongating spermatids. Similar results were seen in the present study in the boar. However, other studies have reported that Gata-4 is always specific to the sertoli cells within the seminiferous tubules at all ages using a Vectastain Elite ABC kit (McCoard et al., 2001, 2003).

Previous studies have determined that proliferation of sertoli cells is greatest from birth until 1 month of age with an additional slight increase in mitotic activity and hypertrophy at about 3 months of age in the pig (Franca et al., 2000). The present study confirms that the greatest amount of mitotic activity is occurring during the early neonatal period. However, only the FSH treated boars were still undergoing sertoli cell hyperplasia at 98 days of age. Control boars did not have any cells still undergoing mitotic activity at 98 days of age. These results are different from the previous reports (Franca et al., 2000). Assuming that the cells proliferating at 98 days of age in the FSH treated boars is due to FSH administration, then there is no discernible increase in mitotic activity occurring between 3 and 4 months of age as reported in the Piau pig (Franca et al., 2000).

There is debate as to what histological method is the best for identifying proliferating cells. Some research uses a halogenated form of thymidine, 5-bromodeoxyuridine (BrdU), which is capable of incorporation into DNA as it is being synthesized *in vivo*. In this case,

the BrdU would have to be injected into live animals. Therefore, *in vitro* experiments have been designed to use BrdU labeling on paraffin-embedded tissue sections (Meachem et al., 1996). The BrdU expression is evident during the S phase of the cell cycle. Other nuclear proteins have been used to identify proliferating cells as well. Ki-67 is a nuclear protein that is present in the G1, S and G2 phases of the cell cycle and is thought to be necessary for the maintenance of cell proliferation in humans. Therefore Ki-67 is a specific marker for proliferating cells. However, this protein has a short half life and there is some debate as to how well it is expressed in cells from other species besides humans. Therefore antibodies known as MIB-1 and MIB-5 have been generated from the human Ki-67 cDNA which are capable of reacting with the proliferation protein found in rodents. This may be a better alternative to Ki-67 for use in other species besides humans. Proliferating Cell Nuclear Antigen (PCNA) is another nuclear protein expressed in the G1 and S phase of the cell cycle. This protein is thought to be necessary for DNA synthesis and repair. Therefore some cells could be expressing PCNA during the repair of DNA and not the synthesis of DNA leading to false positives. In the present study, Ki-67 was attempted for use in histological evaluation of the testicles due to its high specificity for identifying proliferating cells. Use of Ki-67 was unsuccessful at producing consistent, reproducible results. Therefore PCNA was selected as an alternative to Ki-67. PCNA has less specificity and could have produced some false positives. However, this protein was easy to work with during the processing of the tissue samples and produced repeatable results.

Follicle stimulating hormone injections during the neonatal period have not been shown to increase body weight in pigs or rodents (Meachem et al., 1996, Swanlund et al.,

1995). The present study also did not show an increase in body weight associated with FSH administration with the exception of week 46 of age. Roughly 12 boars were removed from the study at each castration age. By week 46, 36 boars had been removed from the study after castration. Therefore, there is a chance that the heaviest boar from one treatment and the lightest boar from another treatment could have been removed at the same time. This may account for some of the variation in body weights seen this week. However, there were differences in body weight over time as the boars matured. Changes in body weight were similar to those previously reported (Allrich et al., 1983). Boars increased in body weight from an average of 1.61 kg at birth to 245 kg at roughly 530 day of age. Body weight began to significantly increase around 14 weeks (98 days, 3.5 months) of age. This is prior to the onset of puberty coincident with an increase in testosterone concentrations.

Studies in rats have demonstrated an increase in testis size in response to neonatal FSH treatment (Meachem et al., 1996). However similar results have not been shown in pigs treated with FSH (Swanlund et al., 1995). The present study also did not see any effects of FSH treatment on the area or weight of the testis. At 330 days of age, the weights of the removed testis were numerically higher in the FSH treated boars than the control boars, however these differences were not significant.

There was an increase in testis size as the boars matured. The area (cm^2) of the testicles followed a similar pattern to body weight. The rate of increase in the area of the testicles was slow from birth until between 14 and 19 weeks of age when a rapid increase in the area of the testicles took place. This coincides with the period of pubertal development and the re-initiation of spermatogenesis. It has been previously discussed that the periods of

rapid sertoli cell mitotic activity occurs in the first month after birth. Between three and four months of age, there is an additional period where the sertoli cells undergo some mitotic activity as well as mature and begin to produce a multitude of proteins and other biological products needed to support germ cells throughout development (Franca et al., 2000). Therefore this rapid increase in the size of the testicle between three and four months of age is most likely not due to an increase in the number of sertoli cells in the testicle.

In the pig, the leydig cells undergo three distinct phases of mitotic activity: the first occurs during early fetal development; the second during the perinatal period; and the third from 3 months through adulthood (Franca et al., 2005). Therefore, the increase in the area of the testicle during pubertal development can be due, at least in part, to an increase in leydig cell number. The present study did not enumerate leydig cells. However the percent of the testicle occupied by the interstitial space and the mass of the interstitial space were measured. As the boars aged, the percent of the testicle occupied by the interstitial space decreased while the mass of the interstitial space increased. The increase in the mass of the interstitial space would account for part of the increase in the weight and area of the testicle as the boar ages. However, because the percent of the testicle occupied by the interstitial space decreases with age, it can be assumed that the seminiferous tubules are increasing at a rapid pace during this time.

Another factor contributing to the increase in the size of the testicle prior to puberty is an increase in the seminiferous tubule by the formation of the tubule lumen and the increase in mitotic activity of the developing germ cells. In the present study, the volume percentage of the seminiferous tubules increased as the boar aged, similar to results previously reported

(Allrich et al., 1983, Franca et al., 2000). From evaluation of the 98 day histological samples of the testis, this period of time also coincided with the appearance of the lumen within the seminiferous tubules and a significant increase in the number of germ cells present in the tubule.

Follicle stimulating hormone administration should result in an increase in circulating concentrations of FSH in the animal (Meachem et al., 1996; Wagner and Claus, 2008; Swanlund et al., 1995). In the present study, circulating FSH concentrations in the boars were only different at 6 weeks of age, being higher in the boars treated with FSH from day 22-40. This does coincide with the period of time when these boars were receiving their FSH injections. However, the boars were also receiving FSH injections in weeks 5 and 7. The lack of a difference in FSH concentrations can be accounted for by the timing of the blood sample relative to the timing of the FSH injection. The lapse in time between injection and sampling varied from 24-48 hours. Boars were only receiving FSH injections every 48 hours from the 4^{th} day after birth. The pigs were born over the course of a week. Boars within a treatment were not receiving their FSH injections all on the same days since day 4 of life would have come over the course of a week as well. Blood samples were taken at weekly intervals on the Monday morning of each week, prior to the FSH injections for that day. So, if a boar received his FSH injection on Sunday morning, and a blood sample was taken Monday morning, then 24 hours had lapsed between the injection and blood sample. Other boars within the same treatment group may have received their FSH injection on Saturday, 48 hours prior to the blood sampling. Swanlund and colleagues (1995) administered FSH of similar concentration to boars. At 39 days of age, they took hourly blood samples for 24

hours after injection to determine the clearance of FSH from the treated boars. The FSH treated boars had a peak in serum FSH concentrations within 1 hour of injection and this concentration was still slightly elevated at 24 hours after injection. FSH concentrations are known to be elevated at birth in the pig and declining during the neonatal period (McCoard et al., 2003). At 25 days of age, McCoard and colleagues (2003) reported that Yorkshire x Landrace boars had a serum FSH concentration of roughly 0.75 ng/ml. Swanlund and colleagues reported similar concentrations in Duroc x Landrace-Yorkshire crossbred boars at 39 days of age (0.61-0.89 ng/ml). In the present study, boars that were 5 weeks of age (35 days of age) had FSH concentrations of 1.5 ng/ml. This is a higher concentration of FSH compared to the previous studies. This could be due to the crossbreeding scheme used in the present study where Yorkshire x Landrace x Large White sows were bred to Hampshire x Duroc x Pietran boars to produce the boars for this study. Also, there could also be a tendency for FSH concentrations to vary between seasons (Borg et al., 1993) which could also account for the differences seen in basal FSH concentrations. Accurate measures of FSH concentrations were difficult in the older animals as the secretion of FSH during these times would be pulsitile. A more accurate measure of FSH concentration could be determined with more frequent blood sampling.

Follicle stimulating hormone administration during the neonatal period has not been shown to effect circulating LH concentrations in pigs (Wagner and Claus, 2008). In the present study, LH concentrations were not analyzed statistically due to a lack of available serum for evaluation. Luteinizing hormone concentrations have been shown to be high just after birth reaching maximal concentrations between 2 and 3 weeks after birth

(Colenbrander, 1982a). The present study shows similar results where LH concentrations appear to peak at 3 weeks of age and decrease after that. Similar to FSH concentrations in the adult, LH becomes pulsitile and therefore can be hard to detect depending on the time between sampling and each pulse of LH. A more precise measure of LH could be obtained with more frequent blood sampling.

Sertoli cells of the testicle also produce IGF-1 in response to FSH stimulation. This IGF-1 is thought to play an important role in development of the germ cells within the seminiferous tubule. Some studies have also suggested a role for IGF-1 in leydig cell proliferation and differentiation in the rat (Mendis-Handagama and Ariyaratne, 2001). The present study shows an increase in IFG-1 production increasing from 2-3 weeks, then declining until about 8 weeks of age. This initial rise in IGF-1 correlates with the period of leydig cell proliferation in the boar (Franca et al., 2005). It has been demonstrated in rats, Meishan boars and crossbred domestic boars that serum IGF-1 increases prior to puberty (Zamaratskaia et al., 2004, Kanematsu et al., 2006, Handelsman et al., 1987). This increase is associated with an increase in growth hormone concentrations thought to account for increases in body weight as puberty approaches. The increase in IGF-1 occurs earlier in the Meishan boar (3-5 weeks of age) as compared to domestic crosses (8 weeks) (Zamaratskaia et al., 2004, Kanematsu et al., 2006). The present study shows similar results where serum concentrations of IGF-1 begin to rise around 7-8 weeks of age and remain high throughout pubertal development.

Conclusions

Overall, it appears as though FSH administration can increase the numbers of spermatozoa present in the ejaculate in adult boars. This is most likely the result of an increase in testosterone production. This could also be the result of an increase in the length of time over which sertoli cell mitosis takes place or a delay in the maturation of sertoli cells allowing for additional mitotic activity to take place. Further research into this hypothesis would be necessary to determine the exact role that FSH plays in regulating mitotic activity in the boar. First, examination of the effects of FSH treatment for the entire duration of mitotic activity on sertoli cell numbers in the adult would need to be examined. In addition, some measures of sertoli cell maturation could also be investigated to determine if FSH treatment extends the period of mitotic activity by delaying maturation of the sertoli cells. However, it is doubtful that FSH acts alone in regulating mitotic activity in the boar. Further investigation into the roles of thyroid hormone, inhibin and testosterone on sertoli cell mitotic activity during the neonatal period may be warranted.

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Figures and Tables

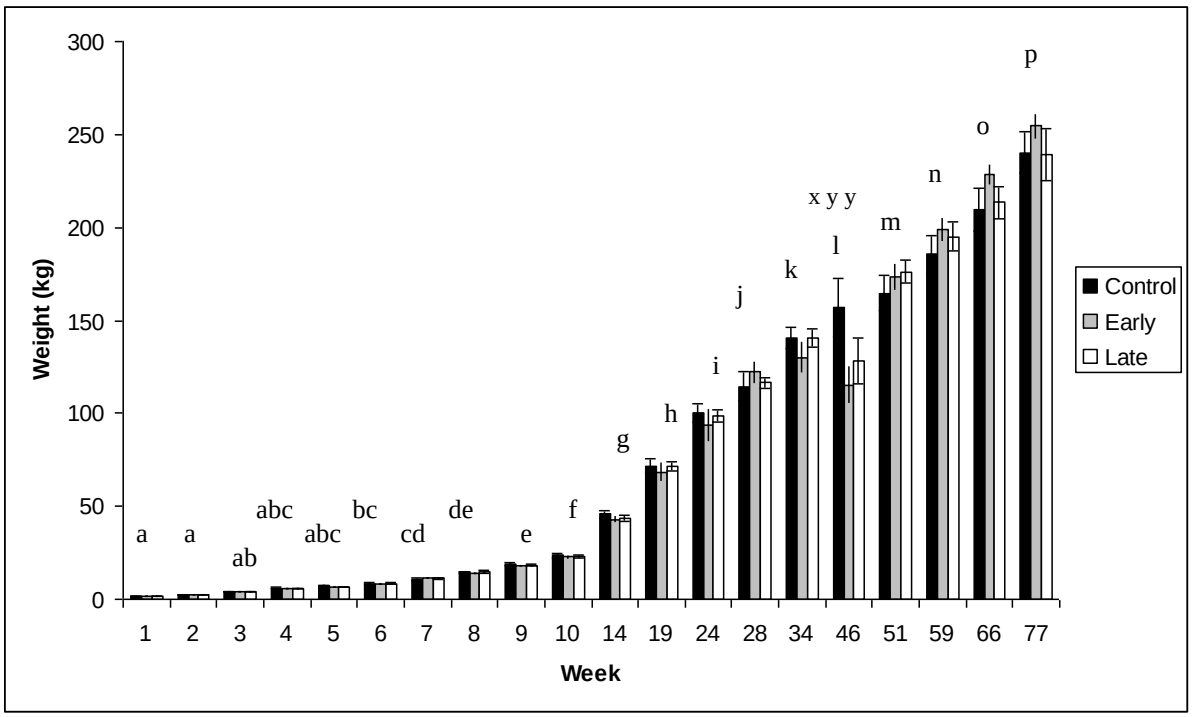


Figure 2. Effects of FSH and age on body weight. Data is presented as means ± S.E. There was a linear increase in body weight over time. At week 46, control boars weighed significantly more than FSH treated boars.

^{xy}: Differences in superscripts indicate a difference at p≤0.05 among treatment groups at a specific week.

$^{a-p}$: Differences in superscripts indicate a difference at p≤0.05 among weeks of age.

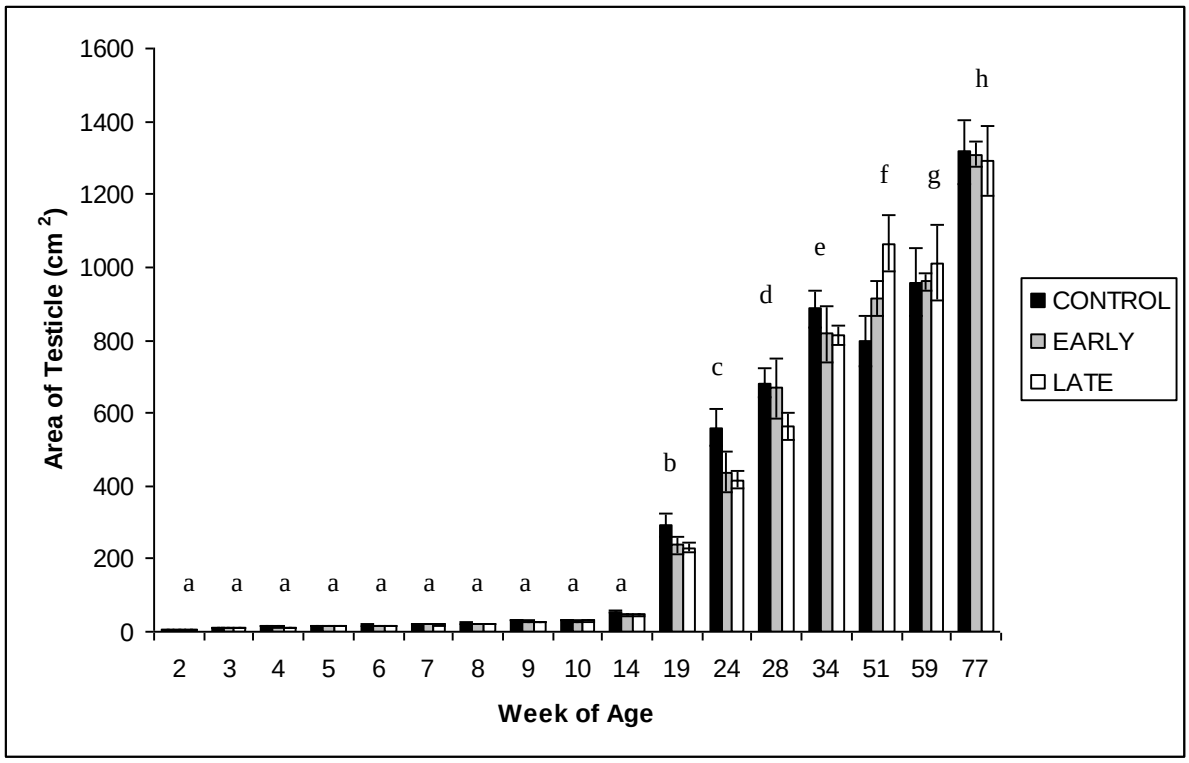


Figure 3. Effects of age on area of the testicle (mean ± S.E.) over time.

[a-h]: Differences in superscripts indicate a difference at p≤0.05 among weeks of age.

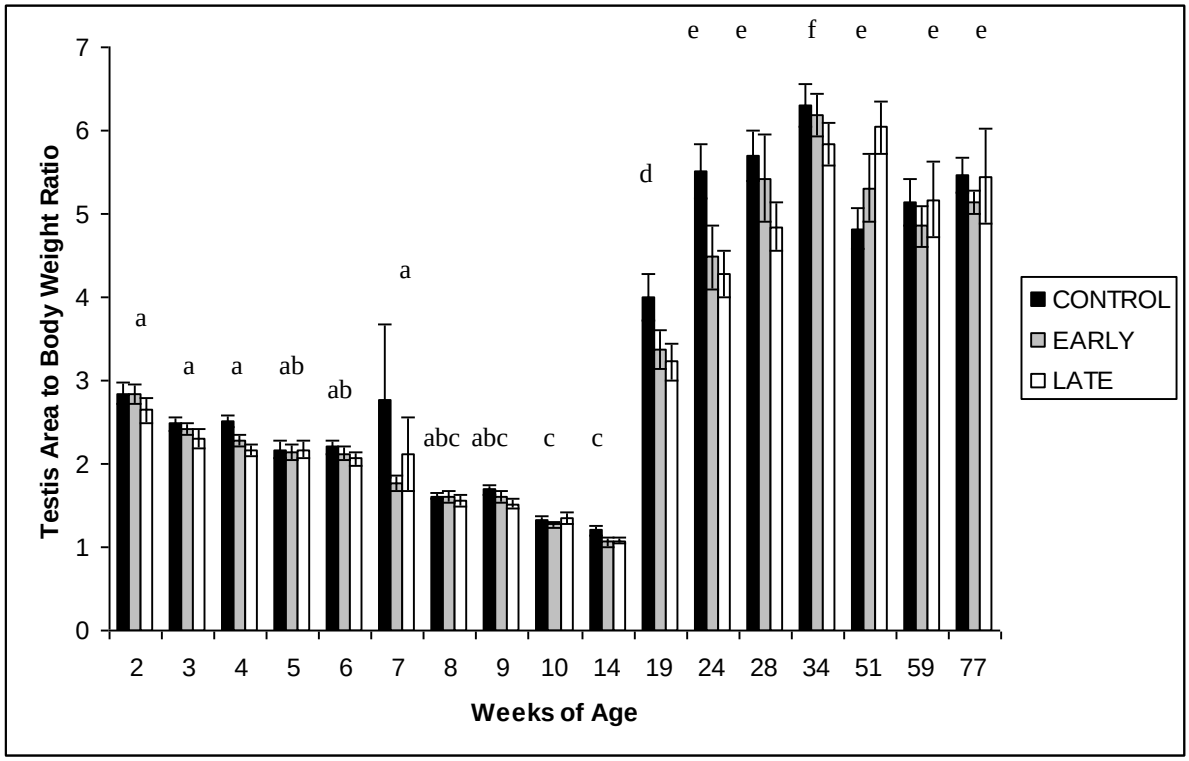


Figure 4. Effects of age on testicular area to body weight ratio (mean ± S.E.) over time.

[a-e]: Differences in superscripts indicate a difference at p≤0.05 among weeks of age.

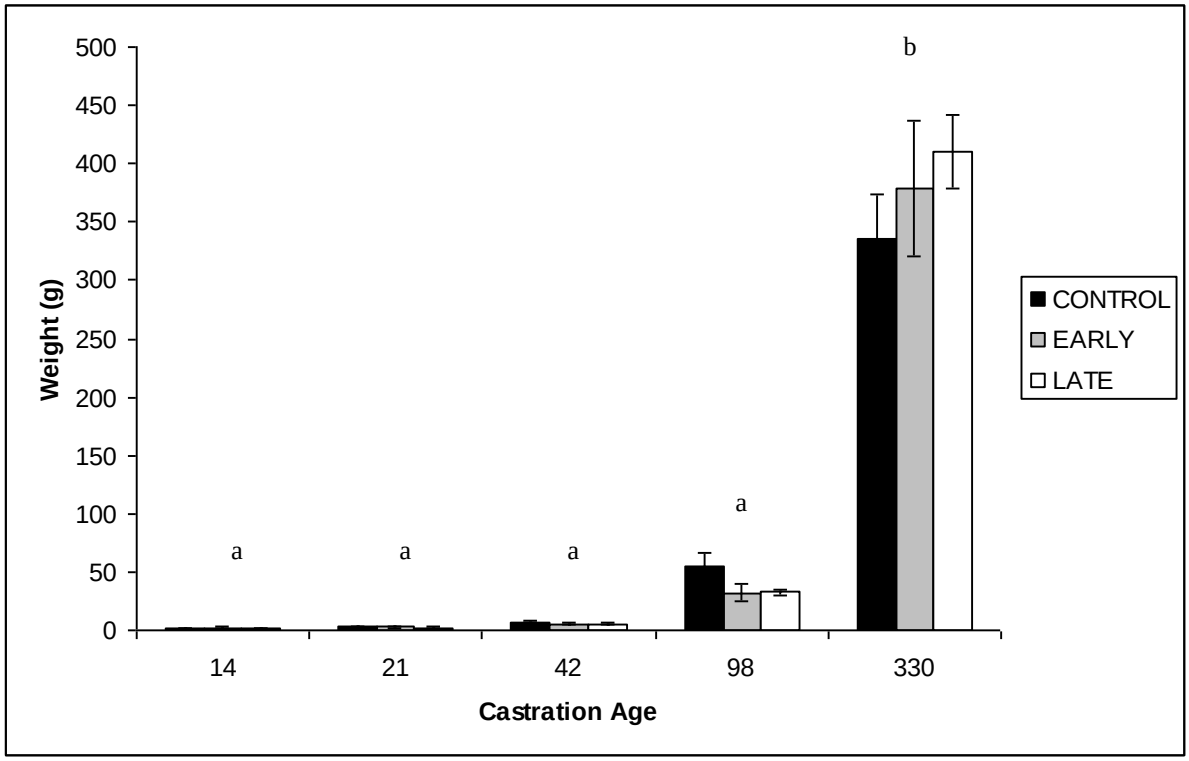


Figure 5. Effect of age at castration on the weight of the testis (means ± S.E.) for each treatment group. This data represents the weight of the testicle after removal of the epididymis and surrounding connective tissue.

[ab]: Differences in superscripts indicate a difference at p≤0.05 among castration ages in the weight of the testicle.

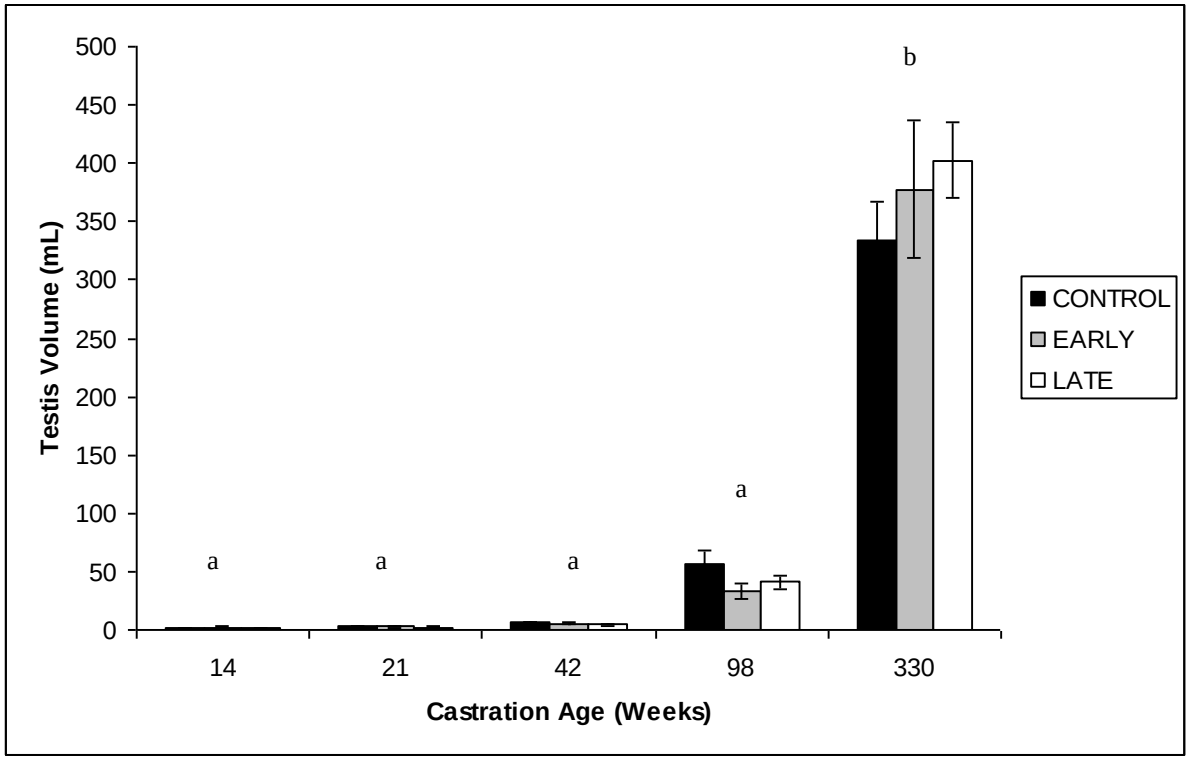


Figure 6. Effect of age at castration on the volume of the testis (means ± S.E.) for each treatment group. This data represents the weight of the testicle after removal of the epididymis and surrounding connective tissue.

[ab]: Differences in superscripts indicate a difference at p≤0.05 among castration ages in the volume of the testicle.

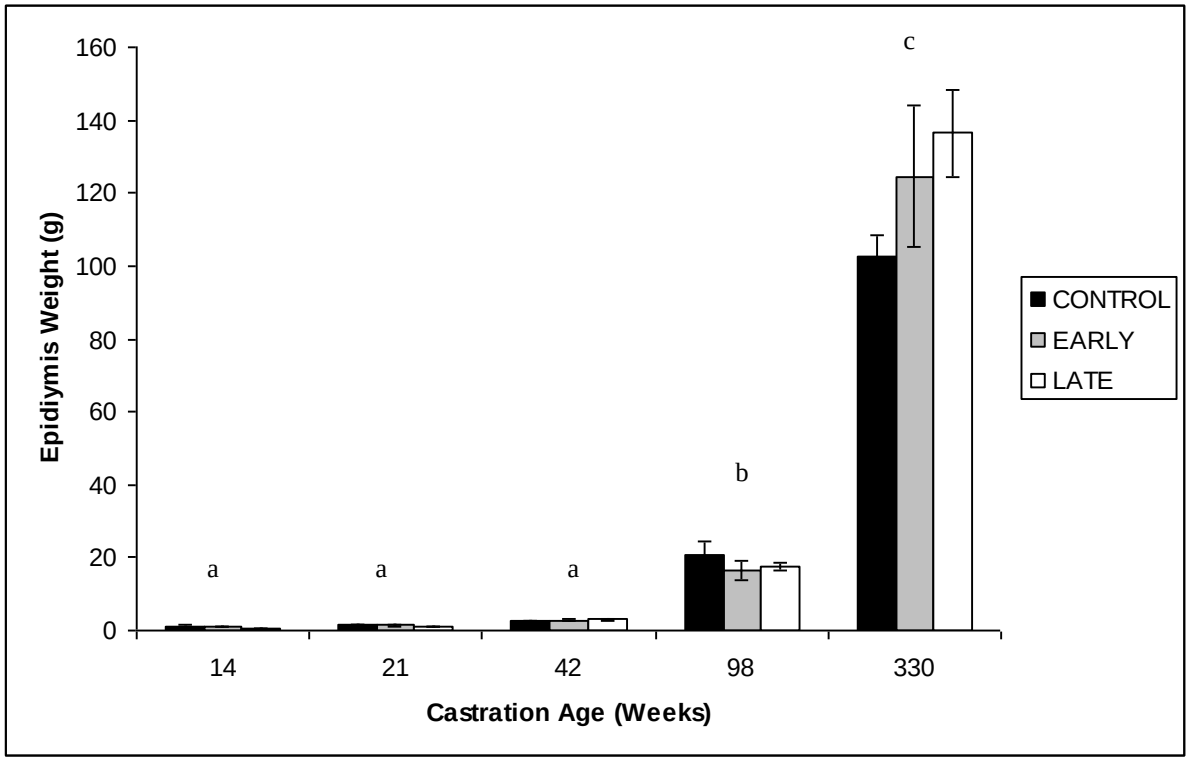


Figure 7. Effect of age at castration on the weight of the epididymis (means ± S.E.) for each treatment group.

[abc]: Differences in superscripts indicate a difference at p≤0.05 among castration ages in the weight of the epididymis.

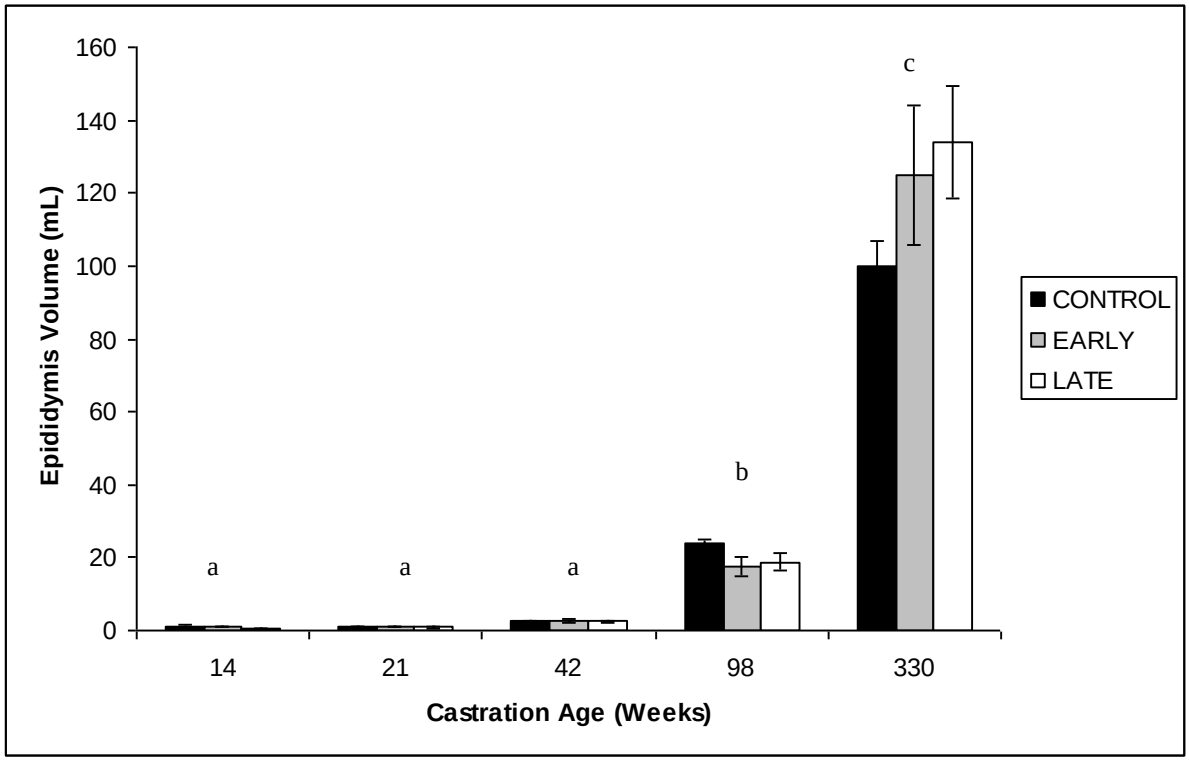


Figure 8. Effect of age at castration on the volume of the epididymis (means ± S.E.) for each treatment group.

[abc]: Differences in superscripts indicate a difference at p≤0.05 among castration ages in the volume of the epididymis.

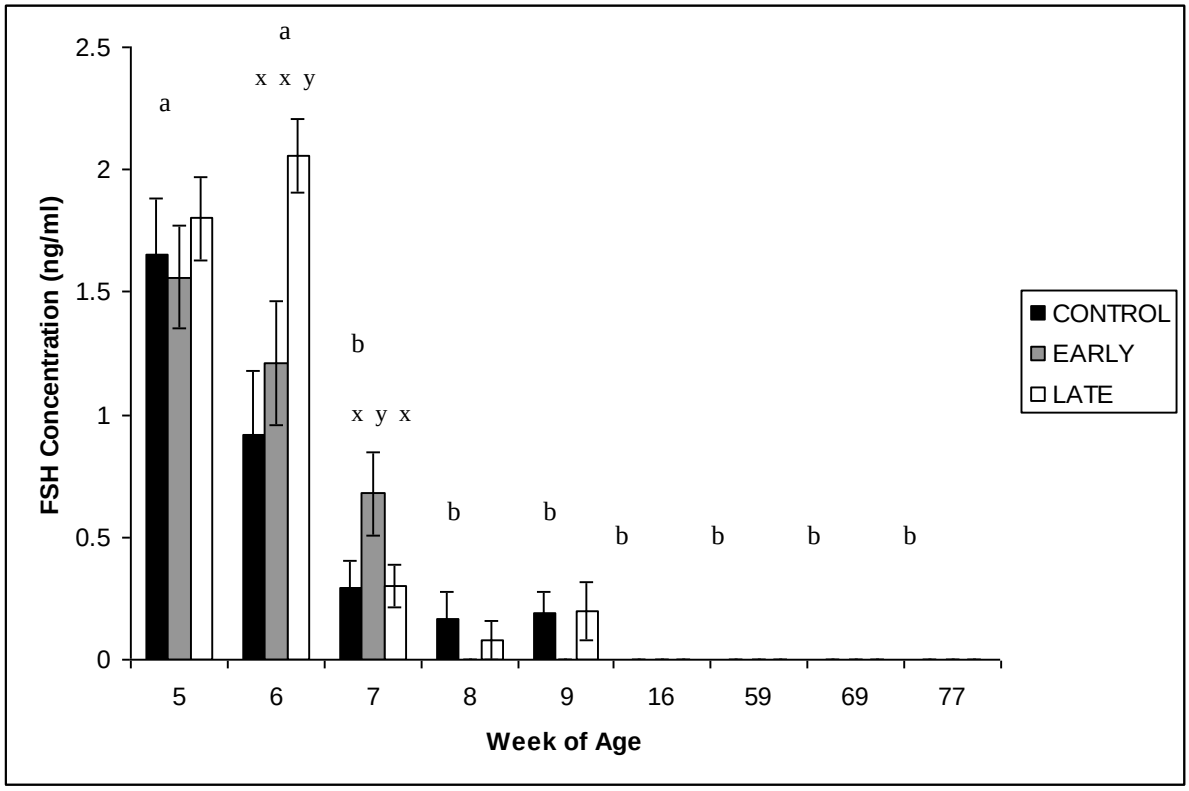


Figure 9. Effects of FSH treatment on average FSH concentrations (means ± S.E.) over time.

[xy]: Differences in superscript represent differences among treatments at p≤0.05.

[ab]: Differences in superscript represent differences among weeks of age at p≤0.05.

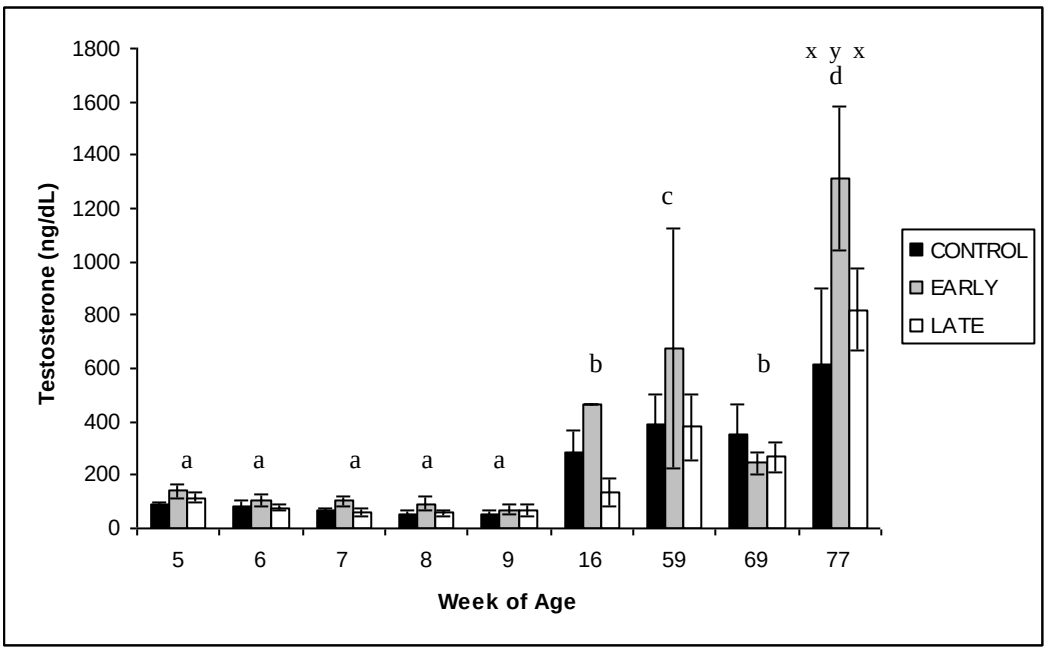


Figure 10. Effects of FSH treatment on average testosterone concentrations (means ± S.E.) over time.

[xy]: Differences in superscript represent differences among treatments at p≤0.05.

[a-d]: Differences in superscript represent differences among weeks of age at p≤0.05.

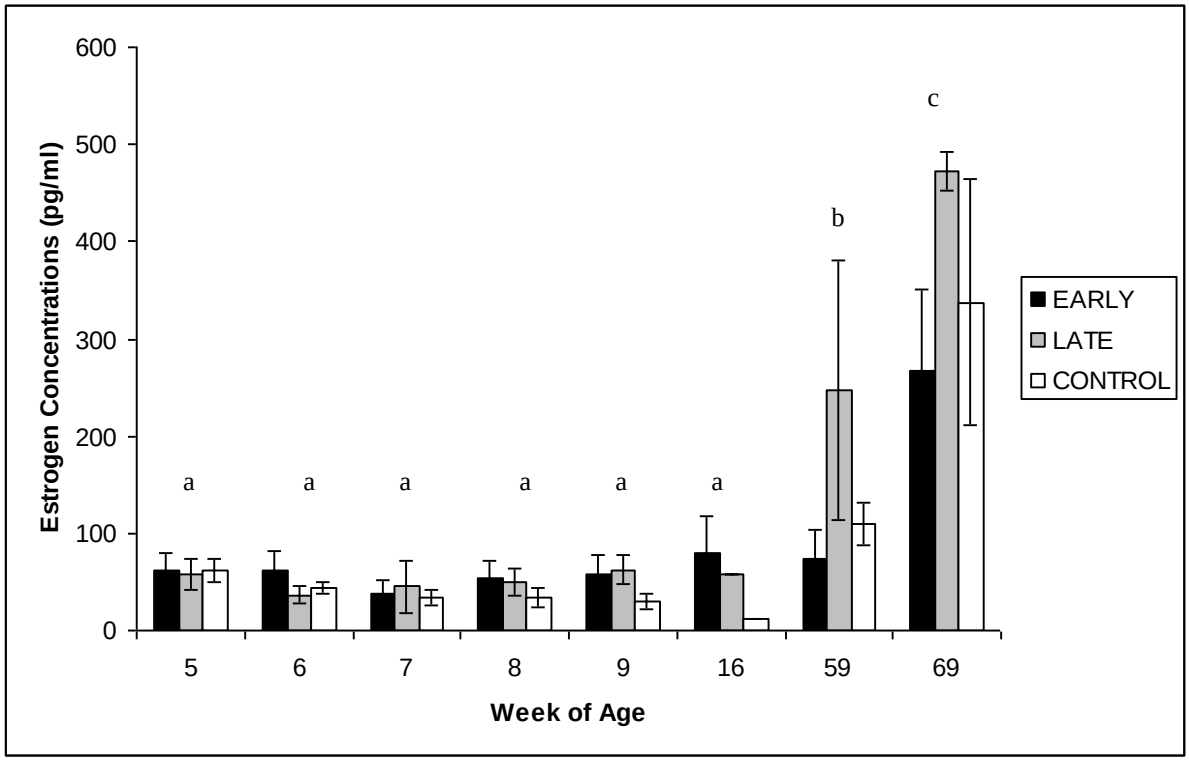


Figure 11. Effects of FSH treatment on average estrogen concentrations (means ± S.E.) over time. Week 77 is not shown because all boars were above the maximum value on the standard curve of the assay.

[abc]: Differences in letter represent differences among ages at p≤0.05.

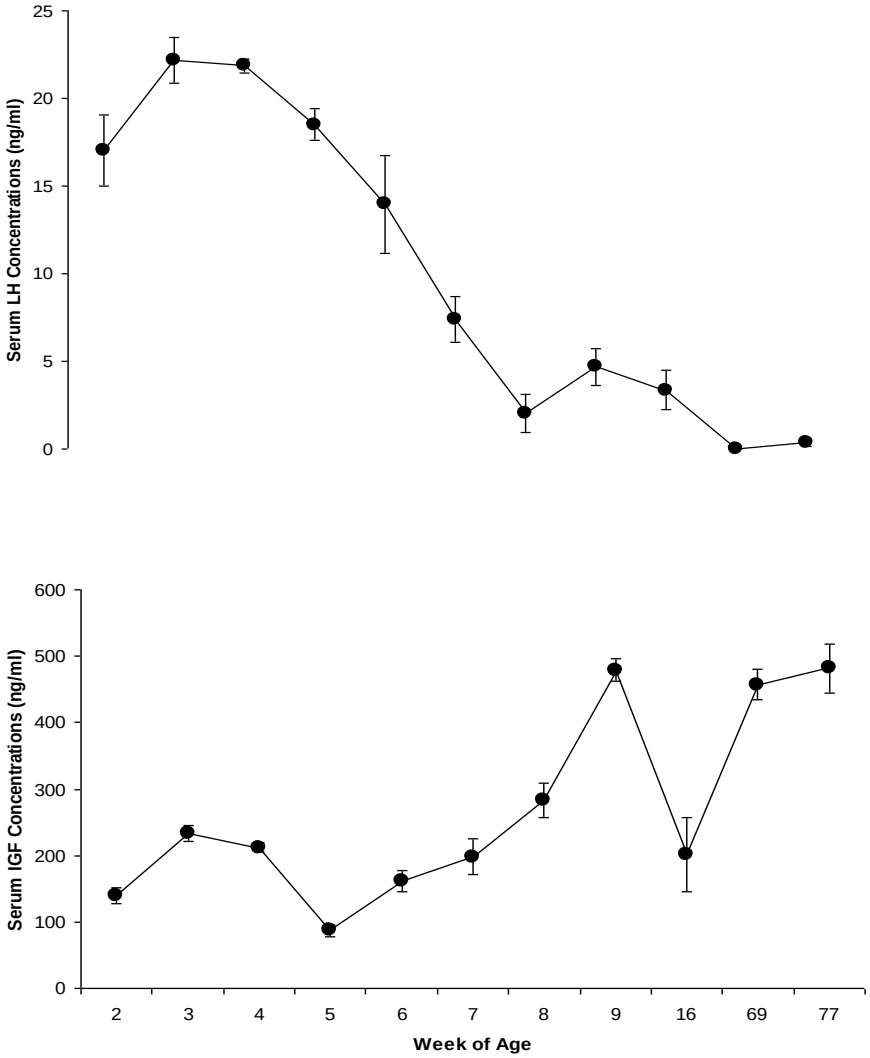


Figure 12. Average serum LH and IGF (means ± S.E.) concentrations over time. These concentrations were determined from pooled blood samples from all boars within each treatment group. Treatment groups were analyzed individually for treatment differences and then averaged for presentation.

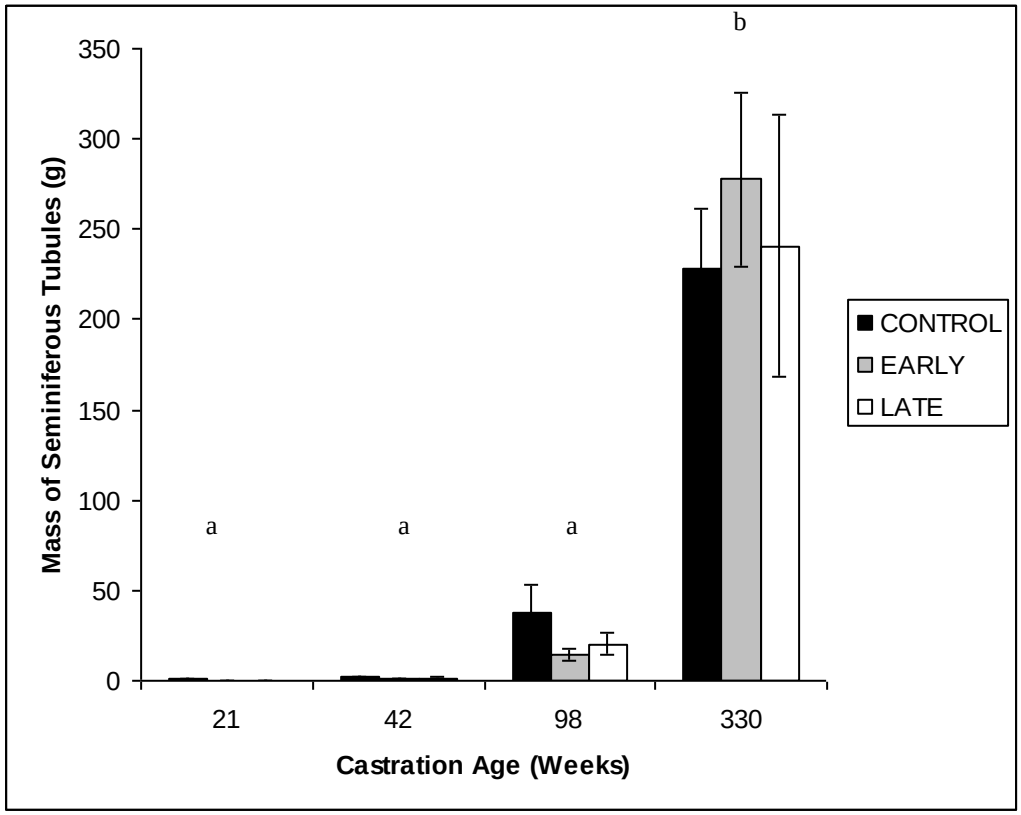


Figure 13. Effects of FSH treatment on the mass of the seminiferous tubule (mean ± S.E.) at the different castration ages.

[ab]: Differences in letter represent differences among castration ages at p≤0.05.

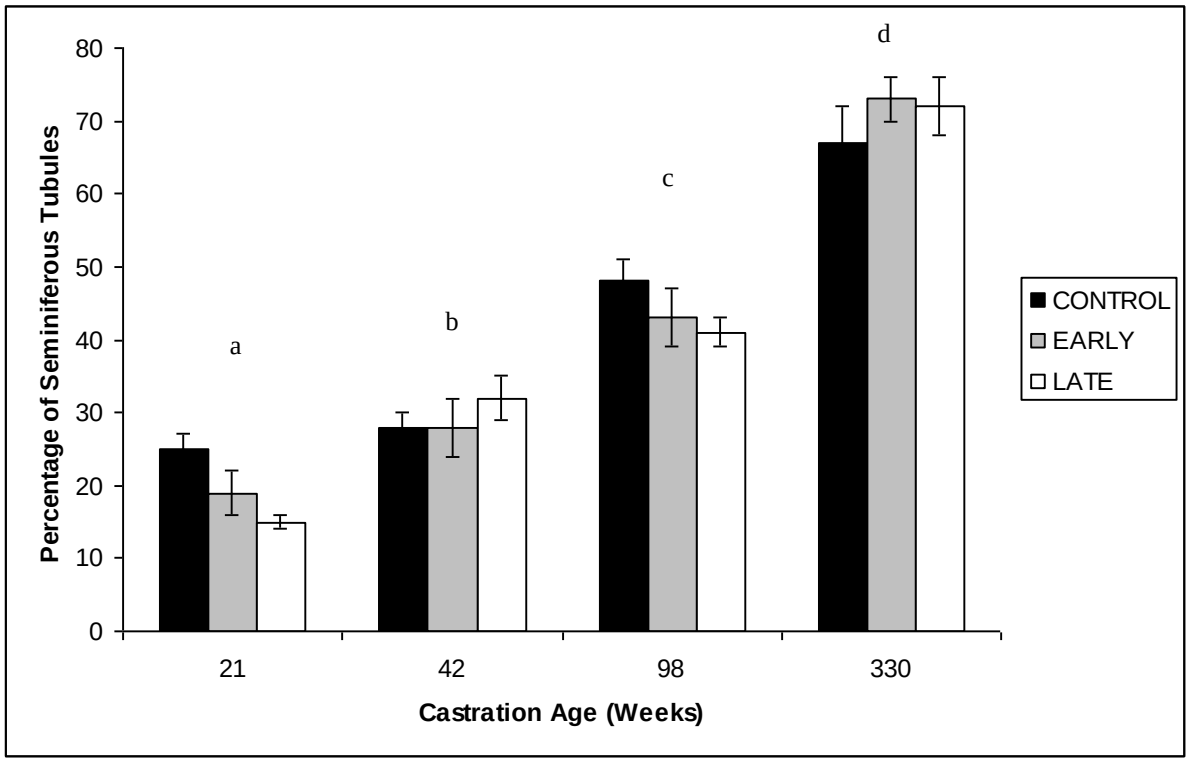


Figure 14. Effects of FSH treatment on the percentage of testicle occupied by the seminiferous tubule (mean ± S.E.).

[a-d] : Differences in letter represent differences among castration ages at p≤0.05.

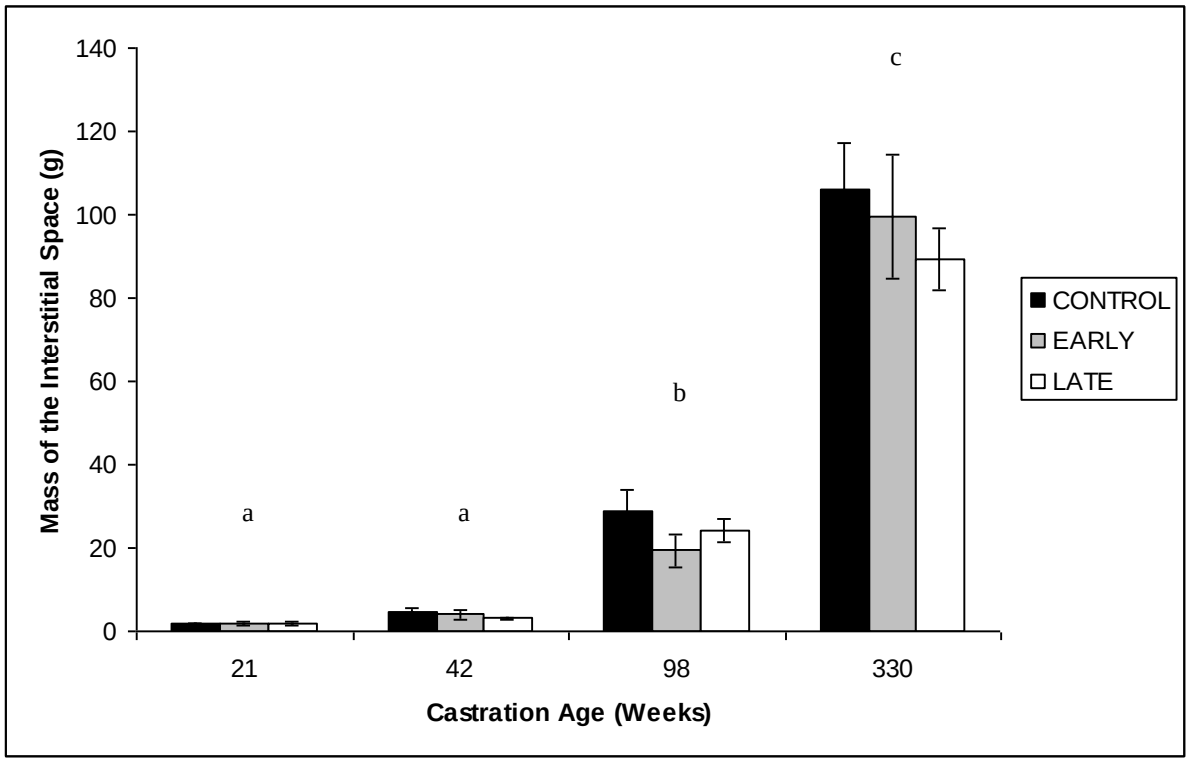


Figure 15. Effects of FSH treatment on the mass of the interstitial space in the testicle (means ± S.E.) across the different castration ages.

[abc] : Differences in letter represent differences among castration ages at p≤0.05.

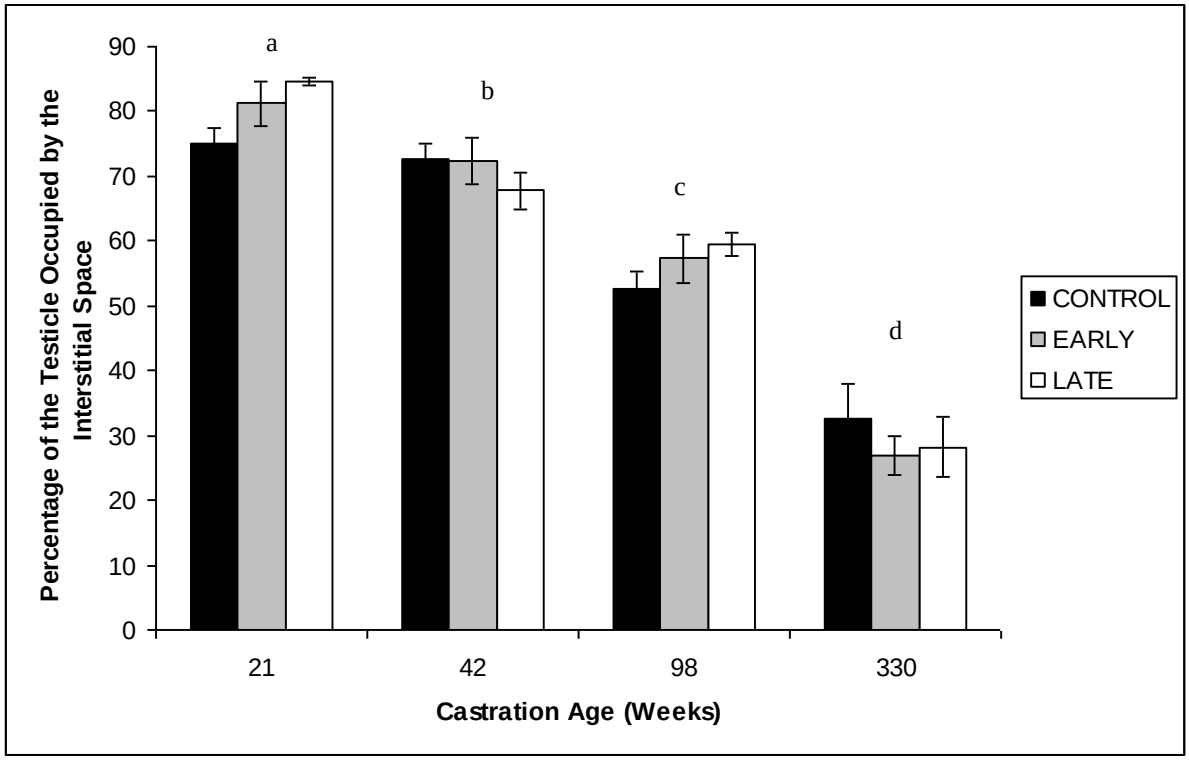


Figure 16. Effects of FSH treatment on the percentage of the testicle occupied by the interstitial space (means ± S.E.) across the different castration ages.

[abc] : Differences in letter represent differences among castration ages at p≤0.05.

Table 1. Effects of FSH treatment on the number of sertoli cells per organ ($x10^6$) (mean ± S.E.). No differences were seen among treatments (p=0.701).

Castration Age	Control (n)	Early (n)	Late (n)	Castration Age Mean
21	588.1.1 ± 30.3 (5)	686.8 ± 129.3 (4)	499.8 ± 167 (4)	**591.3 ± 63.2** [a] **(13)**
42	1363.5 ± 168.0 (4)	1042.2 ± 240.4 (4)	862.3 ± 99.6 (4)	**1089.3 ± 112.4** [a] **(12)**
98	2146.5 ± 380 (4)	3484.1 ±653.5 (4)	3914.1 ± 1241 (4)	**3181.6 ± 493.4** [b] **(12)**
330	1577.5 ± 351.7 (4)	922.3 ± 106.4 (4)	1553.9 ± 686.4 (4)	**1310.6 ± 199.1** [a] **(12)**
Treatment Mean	**1369.9 ± 184.1 (17)**	**1533.8 ± 333.6 (16)**	**1729.4 ± 514.0 (16)**	--

[ab] : Differences in superscript represent differences among castration ages at p≤0.05.

Table 2. Effects of FSH treatment on the number of sertoli cells per gram of testis ($x10^6$) (mean ± S.E.). No differences were seen among treatments (p=0.205).

Castration Age	Control (n)	Early (n)	Late (n)	Castration Age Mean
21	422.1 ± 81.7 (5)	738.1 ± 196.7 (4)	946.8 ± 283 (4)	**680.8 ± 118.5 [a] (13)**
42	116.6 ± 9.4 (4)	150.6 ± 31.2 (4)	146.8 ± 35.9 (4)	**138.0 ± 15.3 [b] (12)**
98	2.5 ± 1.4 (4)	10.7 ± 3.9 (4)	5.7 ± 1.8 (4)	**6.3 ± 1.7 [b] (12)**
330	0.03 ± 0.02 (4)	0.01 ± 0.004 (4)	0.03 ± 0.02 (4)	**0.02 ± 0.007 [b] (12)**
Treatment Mean	**152.2 ± 50.2 (17)**	**224.9 ± 89.8 (16)**	**314.1 ± 134 (16)**	

[ab] : Differences in superscript represent differences among castration ages at p≤0.05.

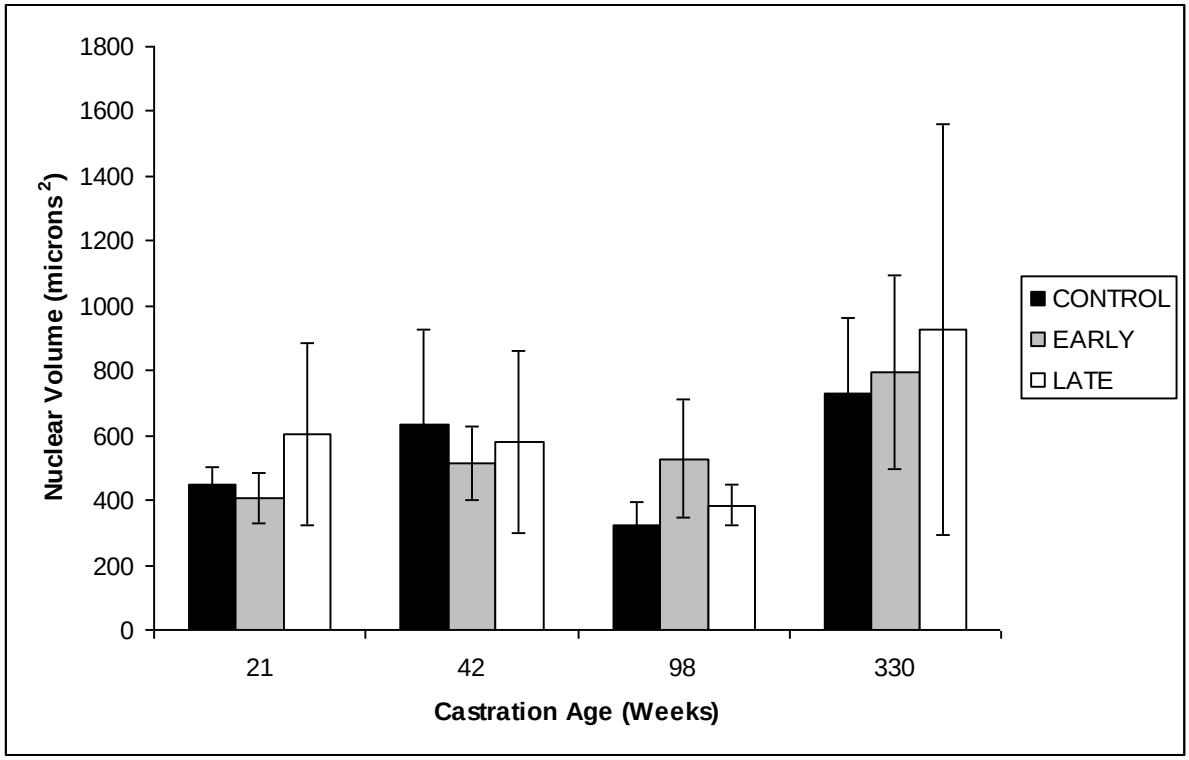
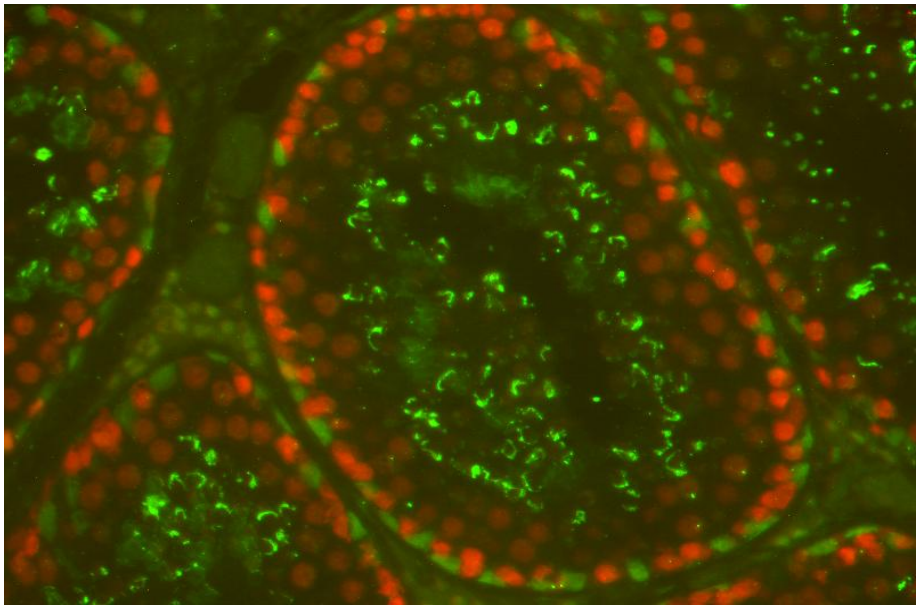


Figure 17. Effects of FSH treatment on sertoli cell nuclear volume (means ± S.E.) for the different castration ages. There were no differences among treatments (p=0.8413) or across time (p=0.1730).

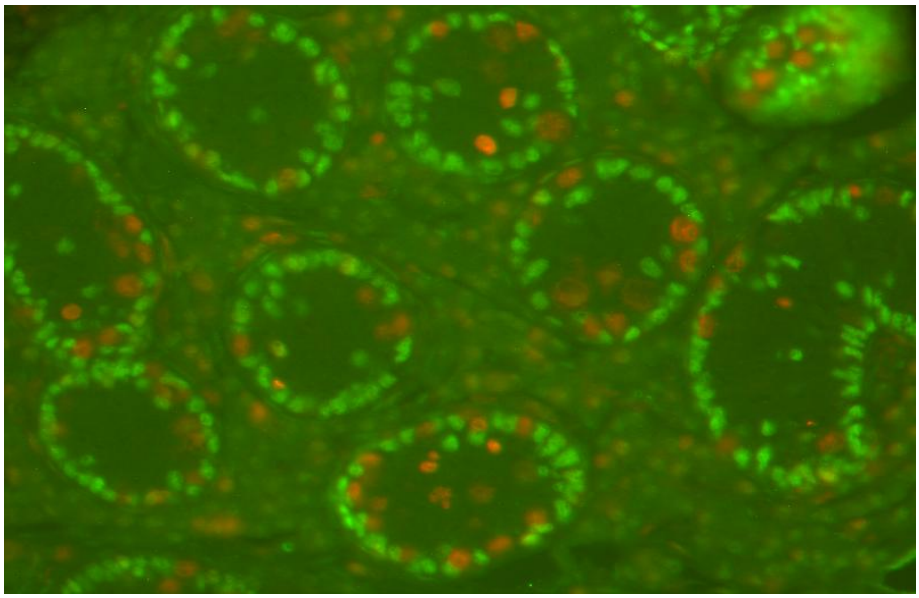
Table 3. Effects of FSH treatment on the number of proliferating germ cells per testis ($x10^6$) (mean ± S.E.). No differences were seen among treatments (p=0.1545).

Castration Age	Control (n)	Early (n)	Late (n)	Castration Age Mean
21	47 ± 5.9 (5)	97 ± 27.4 (4)	63 ± 6.6 (4)	**67.3 ± 10.1 [a] (13)**
42	126 ± 33.4 (4)	83 ± 26.7 (4)	87 ± 27.7 (4)	**98.8 ± 16.4 [a] (12)**
98	1250 ± 322.9 (4)	482 ± 141.9 (4)	959 ± 180.2 (4)	**897 ± 152.9 [a] (12)**
330	5361 ± 1707.5 (4)	2963 ± 384.8 (4)	5443 ± 2784.2 (4)	**4417.9 ± 858.9 [b] (12)**
Treatment Mean	**1599 ± 647.5 (17)**	**906.1 ± 322.8 (16)**	**1094.5 ± 584.2 (16)**	**--**

[ab] : Differences in superscript represent differences among castration ages at p≤0.05.



A



B

Figure 18. Effects of FSH treatment on the number of germ cells expressing PCNA.

Panel A: Immunflourescent picture of a crossection from a boar castrated at 330 days of age (Boar #13806). Green cells are sertoli cells expressing Gata-4. Red cells are germ cells expressing PCNA. Panel B: Immunflourescent picture of a crossection from a boar castrated at 98 days of age (Boar #13712). Green cells are sertoli cells expressing Gata-4. Red cells are germ cells expressing PCNA.

Table 4. Effects of FSH treatment on the number of proliferating germ cells per gram of testis ($x10^6$) (mean ± S.E.). No differences were seen among treatments (p=0.1325).

Castration Age	Control (n)	Early (n)	Late (n)	Castration Age Mean
21	34.9 ± 10.6 (5)	88.3 ± 27 (4)	198.1 ± 90.3 (4)	**101.5 ± 32.9** [a] **(13)**
42	9.8 ± 1.8 (4)	11.6 ± 4 (4)	16.55 ± 7.3 (4)	**12.6 ± 2.7** [b] **(12)**
98	0.84 ± 0.3 (4)	1.19 ± 0.4 (4)	1.4 ± 0.3 (4)	**1.1 ± 0.2** [b] **(12)**
330	0.09 ± 0.03 (4)	0.03 ± 0.005 (4)	0.11 ± 0.09 (4)	**0.07 ± 0.02** [b] **(12)**
Treatment Mean	**12.8 ± 4.7 (17)**	**25.3 ± 11.3 (16)**	**61.7 ± 33.4 (16)**	**--**

[ab] : Differences in superscript represent differences among castration ages at p≤0.05.

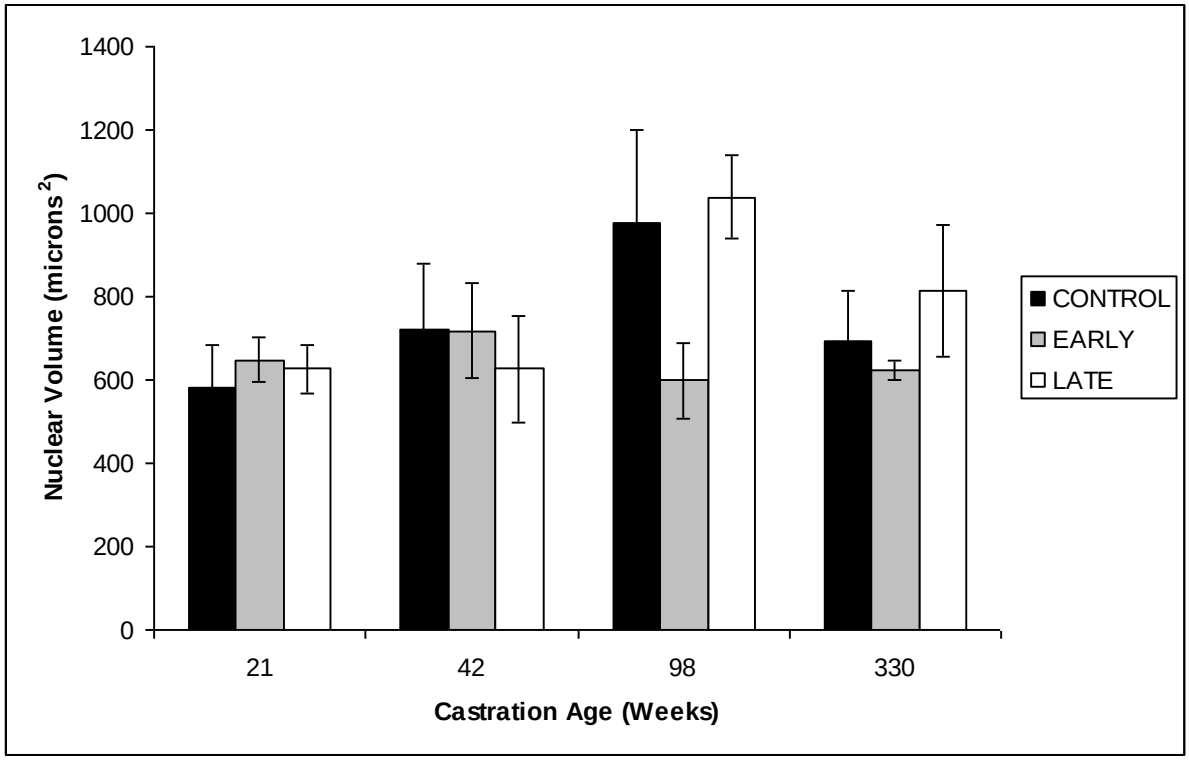


Figure 19. Effects of FSH treatment on germ cell nuclear volume (means ± S.E.) for the different castration ages. There were no differences among treatments (p=0.3137) or across time (p=0.077).

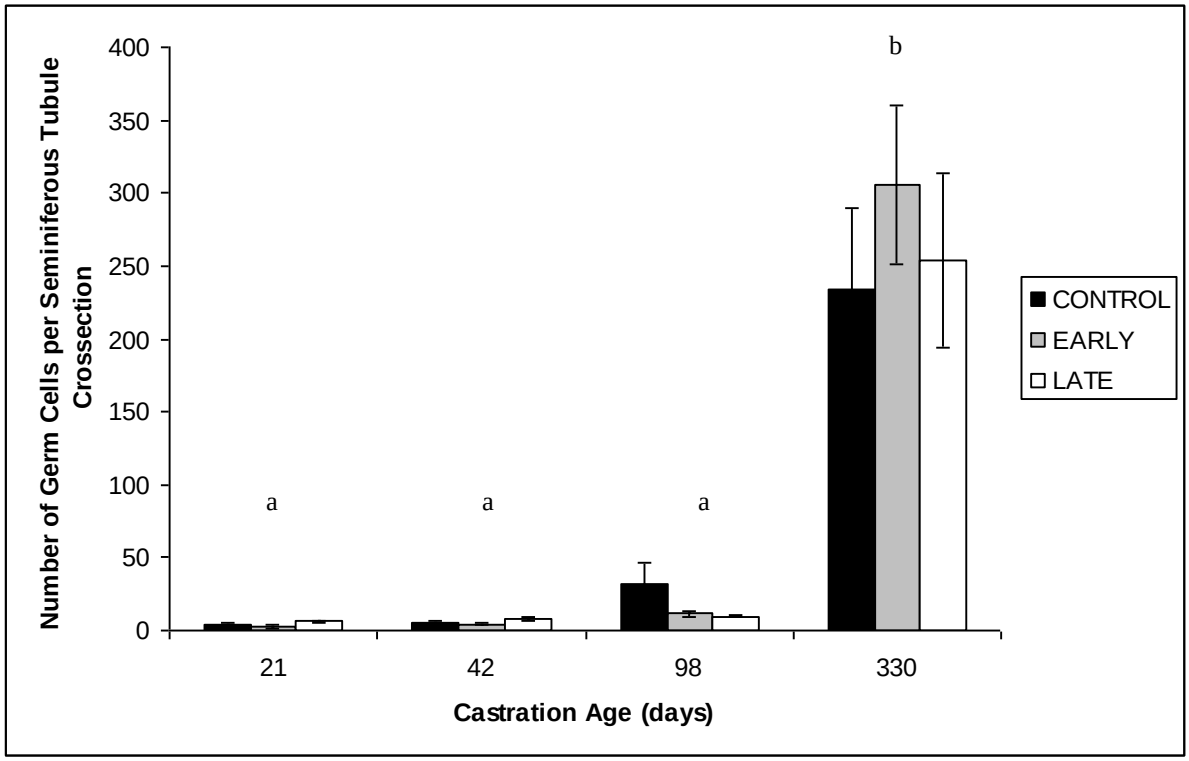
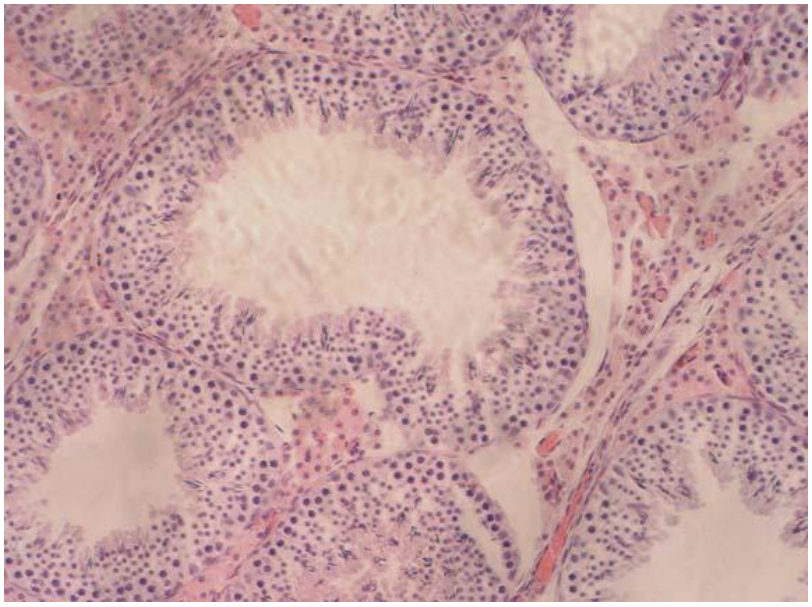
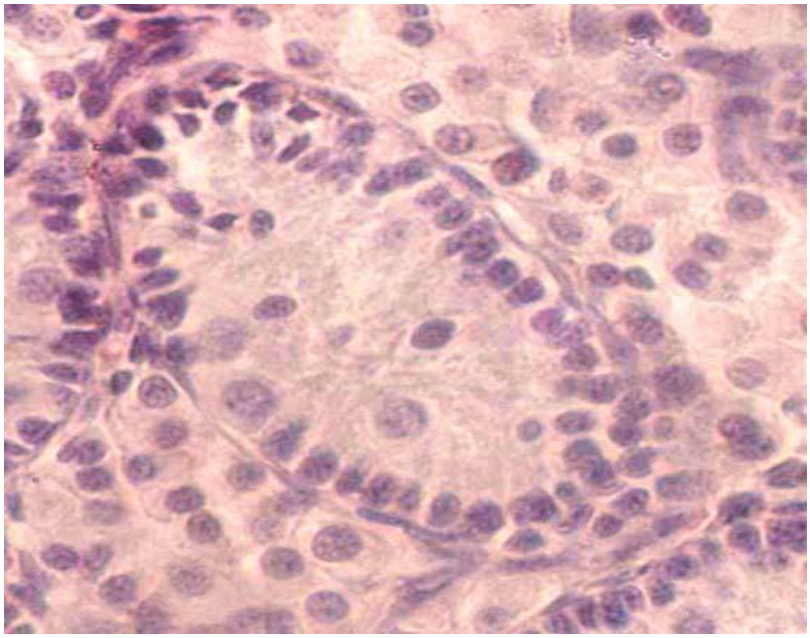


Figure 20. Effect of FSH treatment on the number of germ cells per seminiferous tubule crossection using haematoxylin and eosin staining (means ± S.E.). There were no differences among treatment groups (p=0.7844).

[ab] : Differences in superscript represent differences among castration ages at p≤0.05.



A



B

Figure 21. Effects of FSH treatment on the number of germ cells present in the seminiferous tubule using tissue samples stained with haematoxylin and eosin.

Panel A: Haematoxylin and eosin stained testicular tissue from a 330 day old boar (Boar # 13804). Germ cells are identified by the purple staining and are classified by the shape of the nucleus (details in Materials and Methods). Panel B: Haematoxylin and eosin stained testicular tissue from a 42 day old boar (Boar # 13014). Germ cells and sertoli cells appear purple in this picture. Germ cells are identified by their round shaped nucleus compared to the irregular shape of the sertoli cell nucleus.

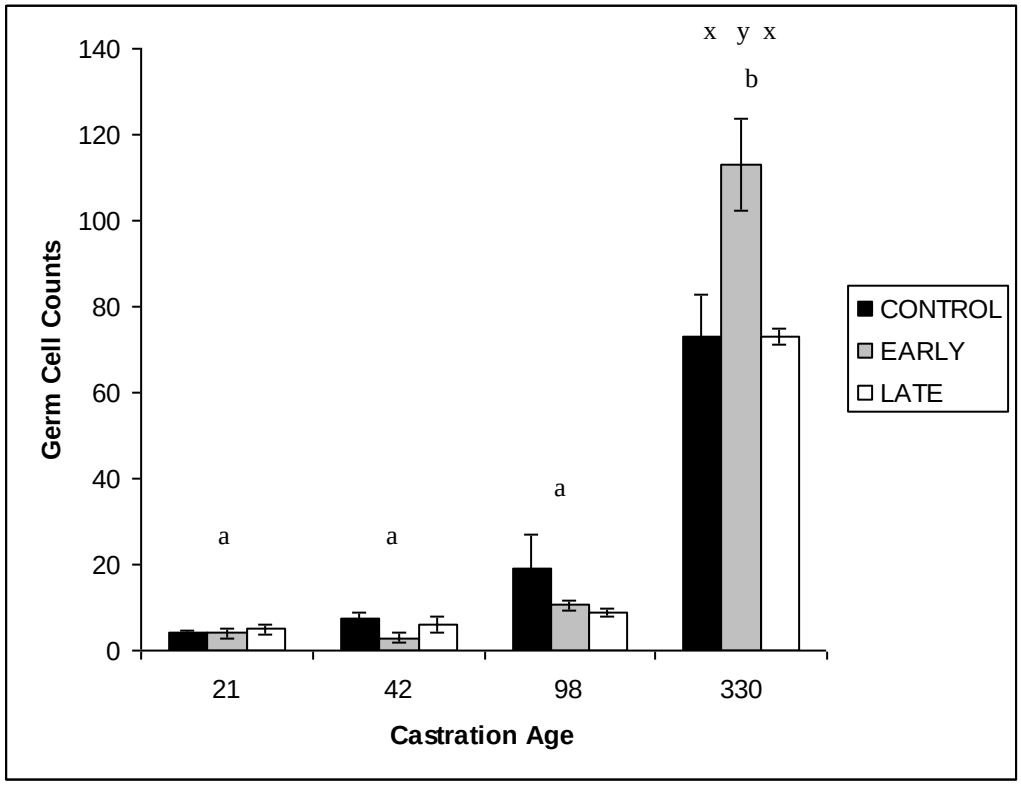


Figure 22. Effects of FSH treatment on the number of primary spermatocytes per seminiferous tubule cross section (mean ± S.E.) at all castration ages.

^{xy} : Differences in superscript represent differences among treatment groups at p≤0.05.

^{ab}: Differences in superscript represent differences among castration ages at p≤0.05.

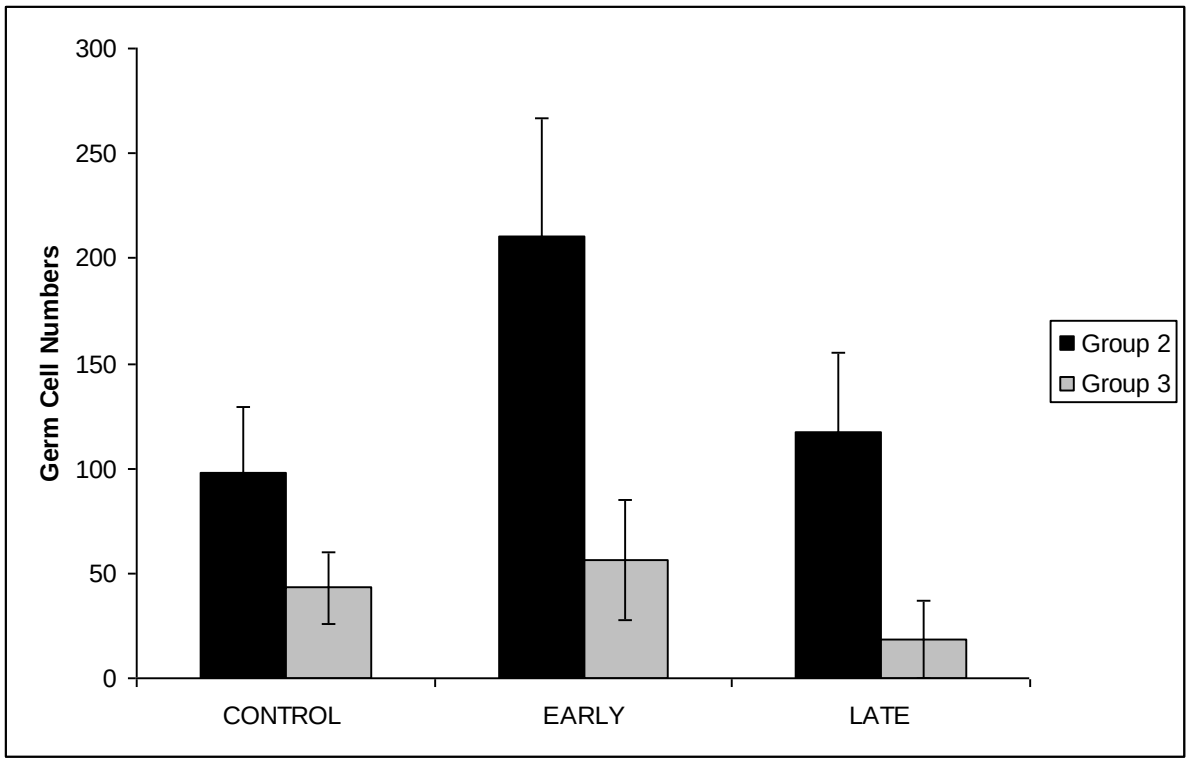


Figure 23. Effects of treatment on the number of germ cells (mean ± S.E.) classified as group 2 or 3 in the boars that were castrated at 330 days of age. The boars castrated at 330 days of age were the only boars that had germ cells classified as group 2 or 3 in the seminiferous tubule. Counts were averaged from 3 cross sections from each boar stained with haematoxylin and eosin. There were no differences among treatments for group 2 or 3 germ cells (p=0.1681 and p=0.5265, respectively).

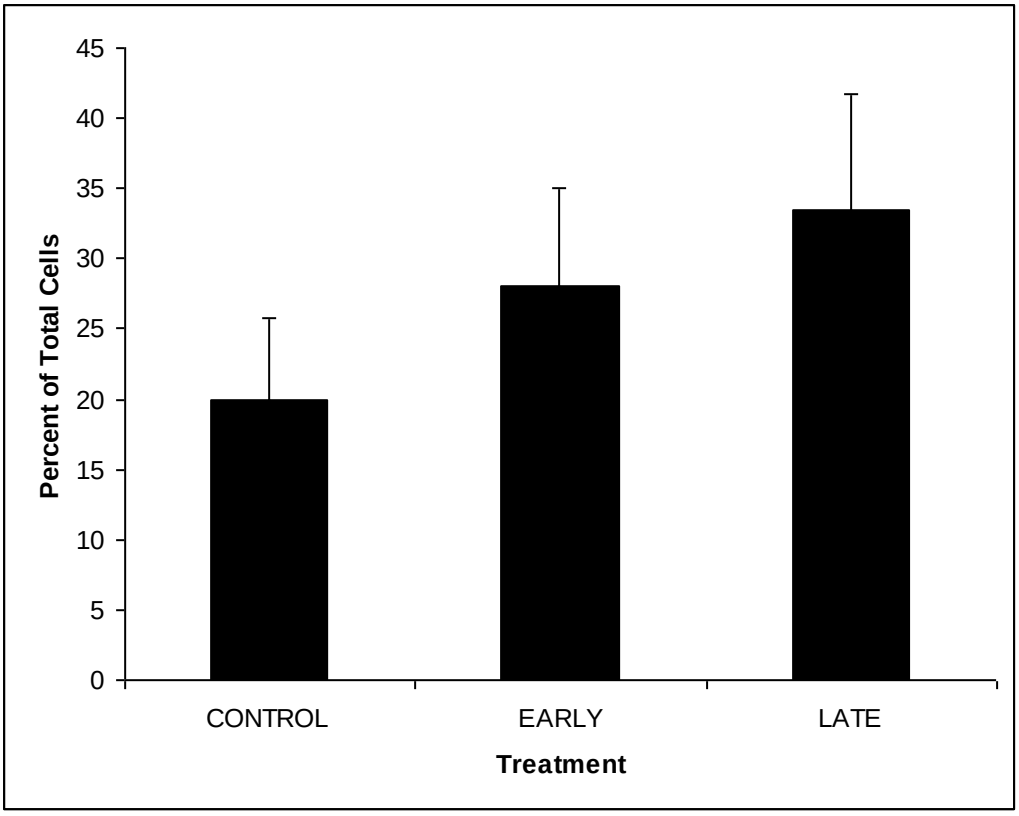


Figure 24. Effects of FSH treatment on the percent of sertoli cells undergoing proliferation (±S.E.) in the different treatment groups. Data is presented as the average percentage of cells undergoing proliferation. Data was square root transformed for statistical analysis. There were no differences among treatments in the percent of sertoli cells undergoing proliferation (p=0.1480).

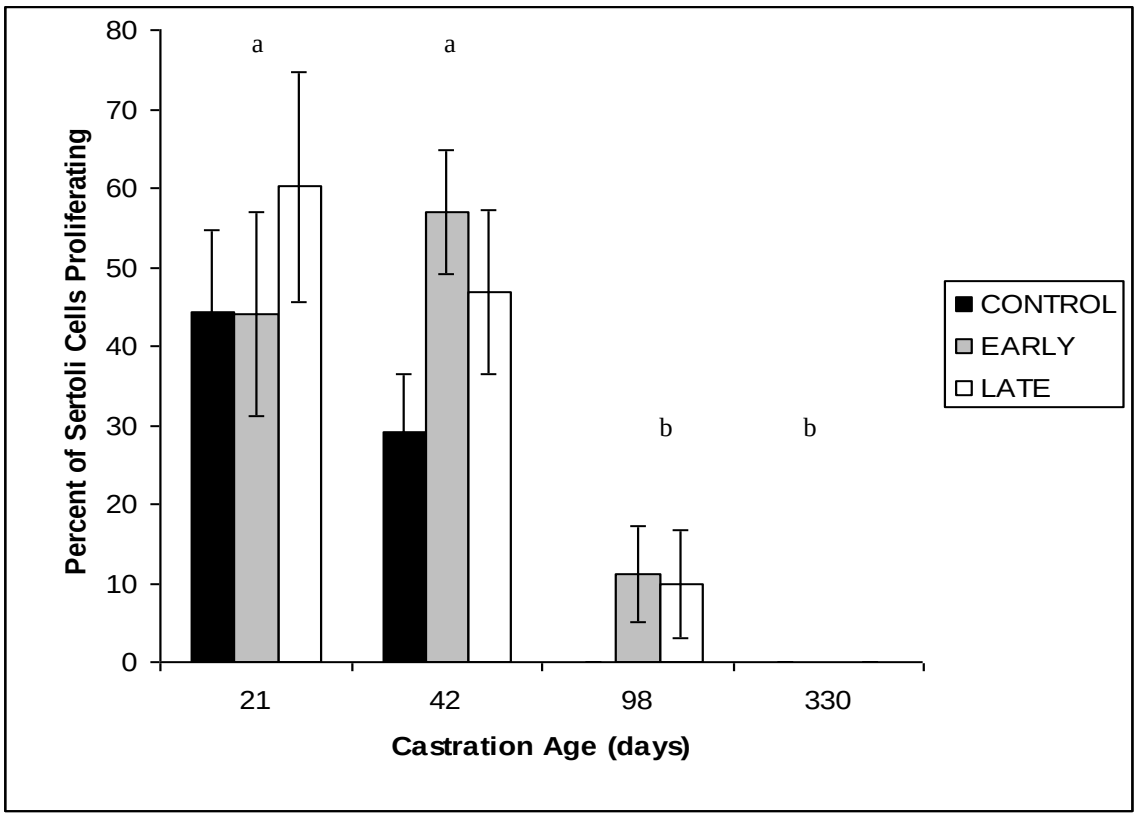


Figure 25. Effects of treatment on the percent of sertoli cells undergoing proliferation (±S.E.) at the various castration ages. Data is presented as the average percentage of cells undergoing proliferation. Data was square root transformed prior for statistical analysis.

[ab]: Differences in superscript represent differences among castration ages at p≤0.05.

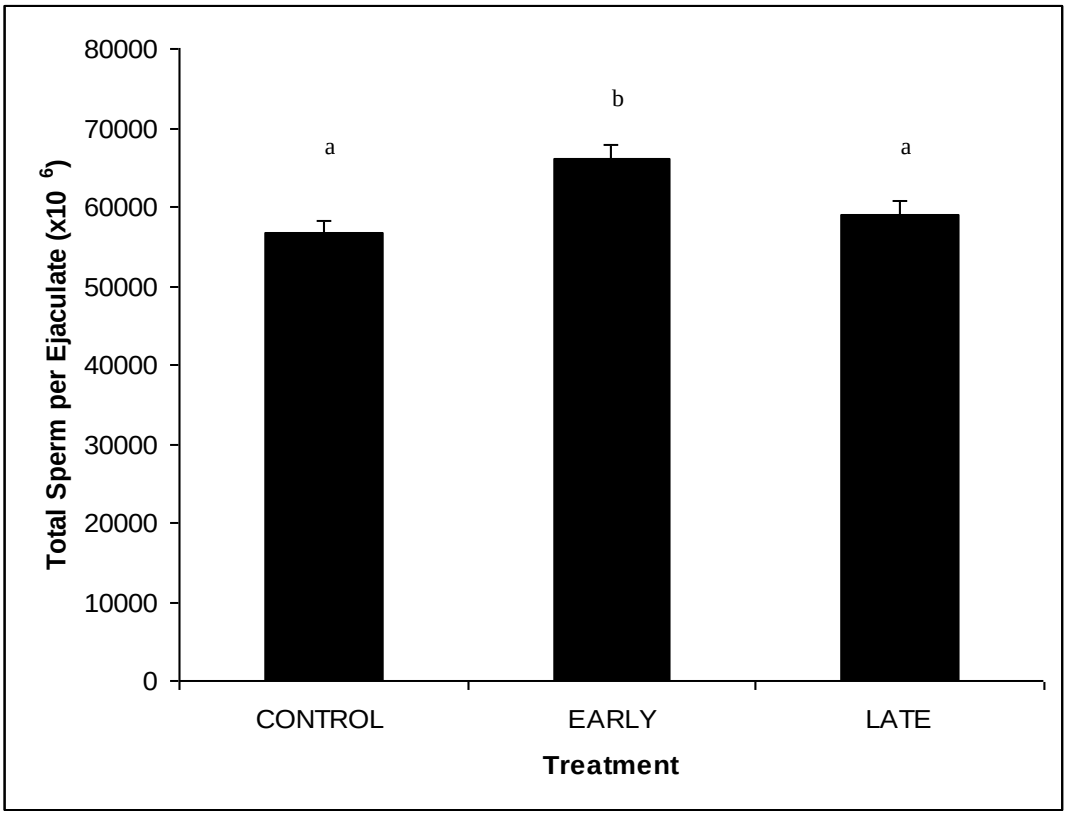


Figure 26. Effects of FSH treatment on the total number of sperm cells per ejaculate (mean ± S.E.).

[ab]: Differences in superscript represent differences between treatments at p≤0.06.

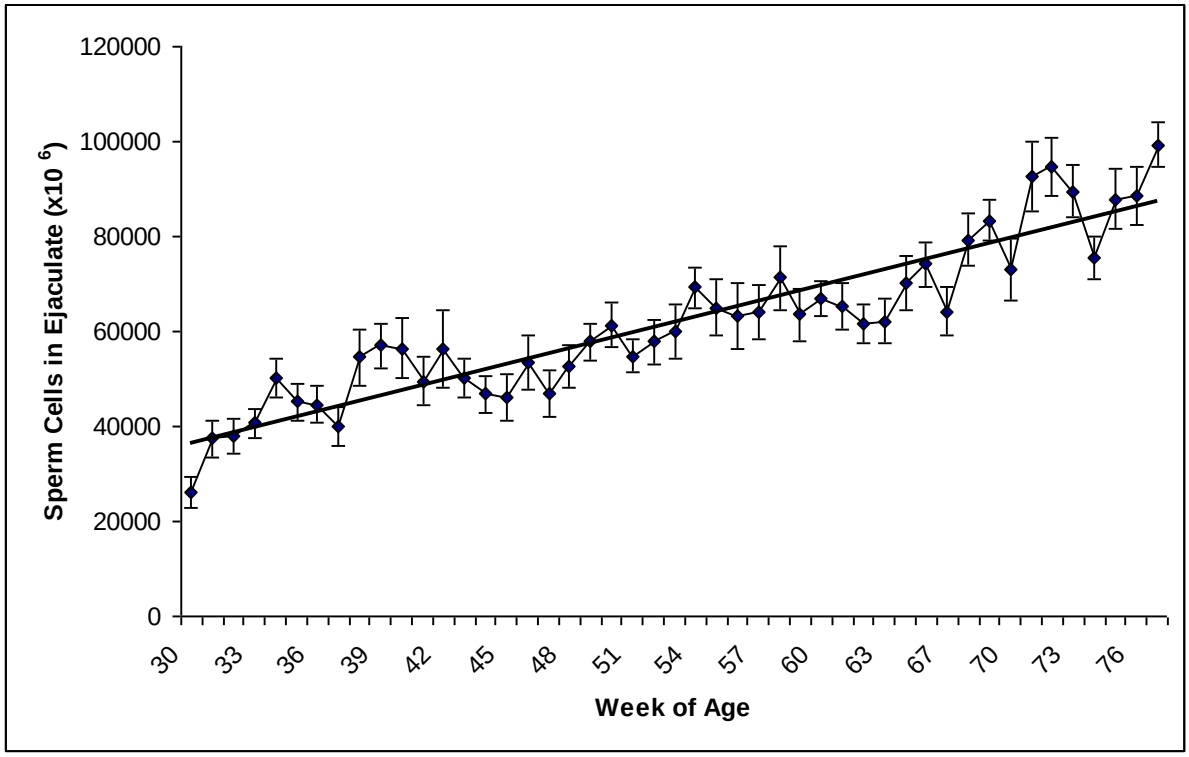


Figure 27. Average number of sperm cells per ejaculate (mean ± S.E.) over time for all boars on study. There was a linear increase in sperm production as the boars aged ($p \leq 0.0001$).

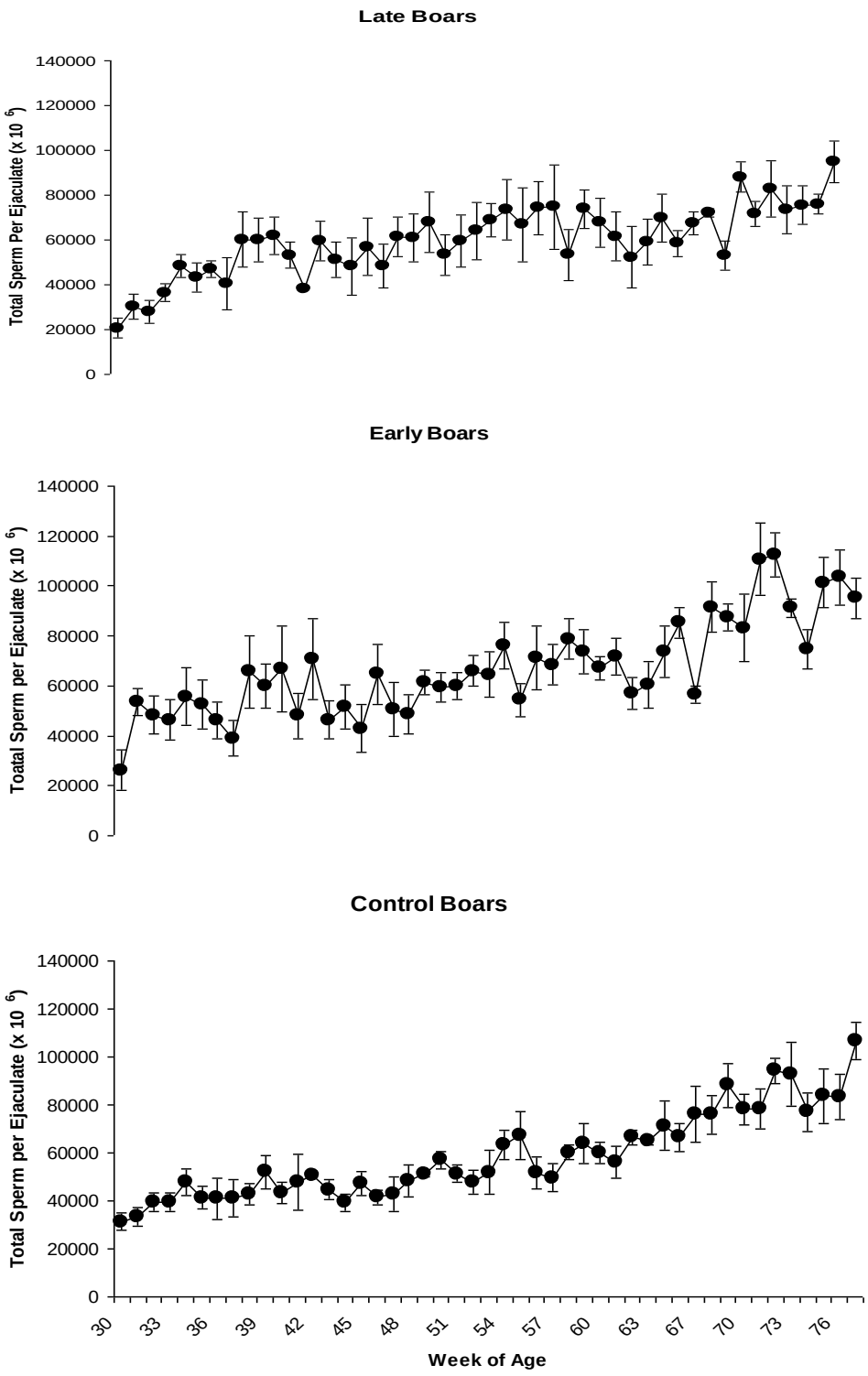


Figure 28. Effects of FSH treatment on the total number of sperm cells per ejaculate (mean ± S.E.) over time.

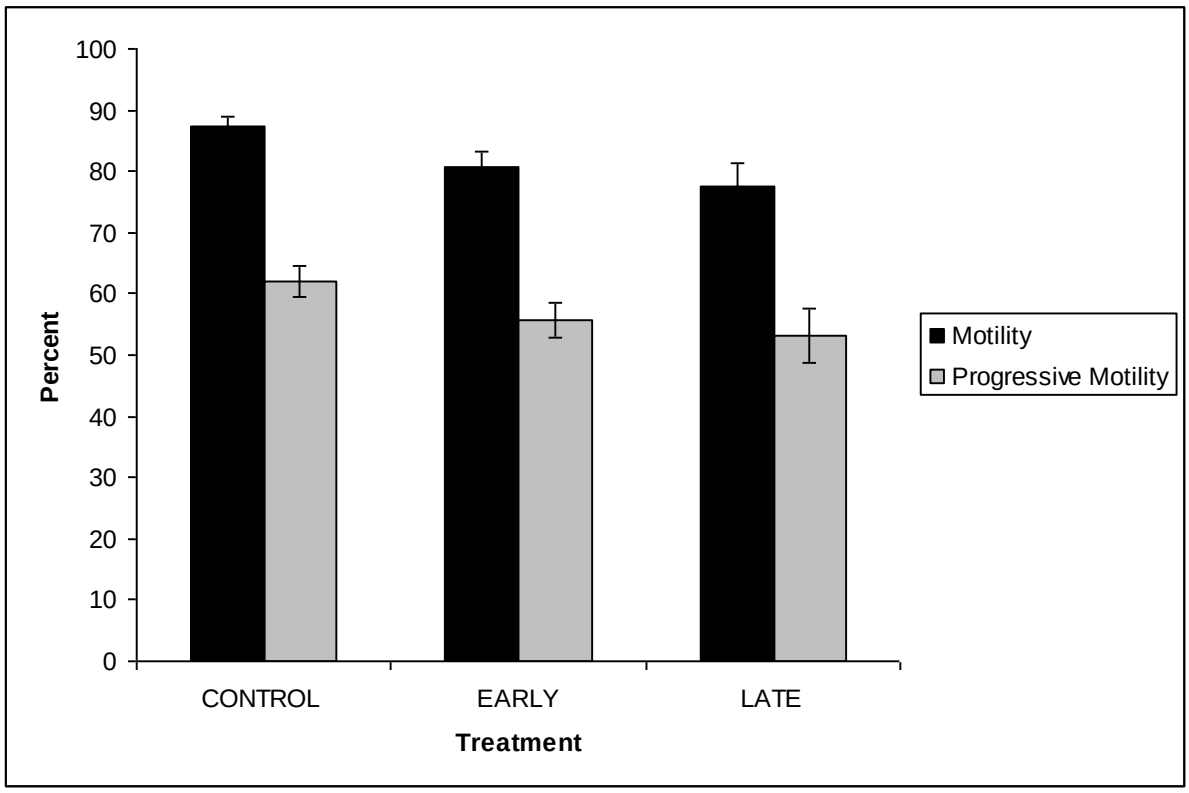


Figure 29. Percent of motile and progressively motile sperm (±S.E.) in the ejaculates across treatment groups. The data are presented as the mean percentage of motile and progressively motile sperm cells. The data was arcsine transformed prior to statistical analysis. There were no differences among treatments in the percent of motile spermatozoa (p=0.3630) or progressively motile spermatozoa (p=0.3589) in the ejaculates.

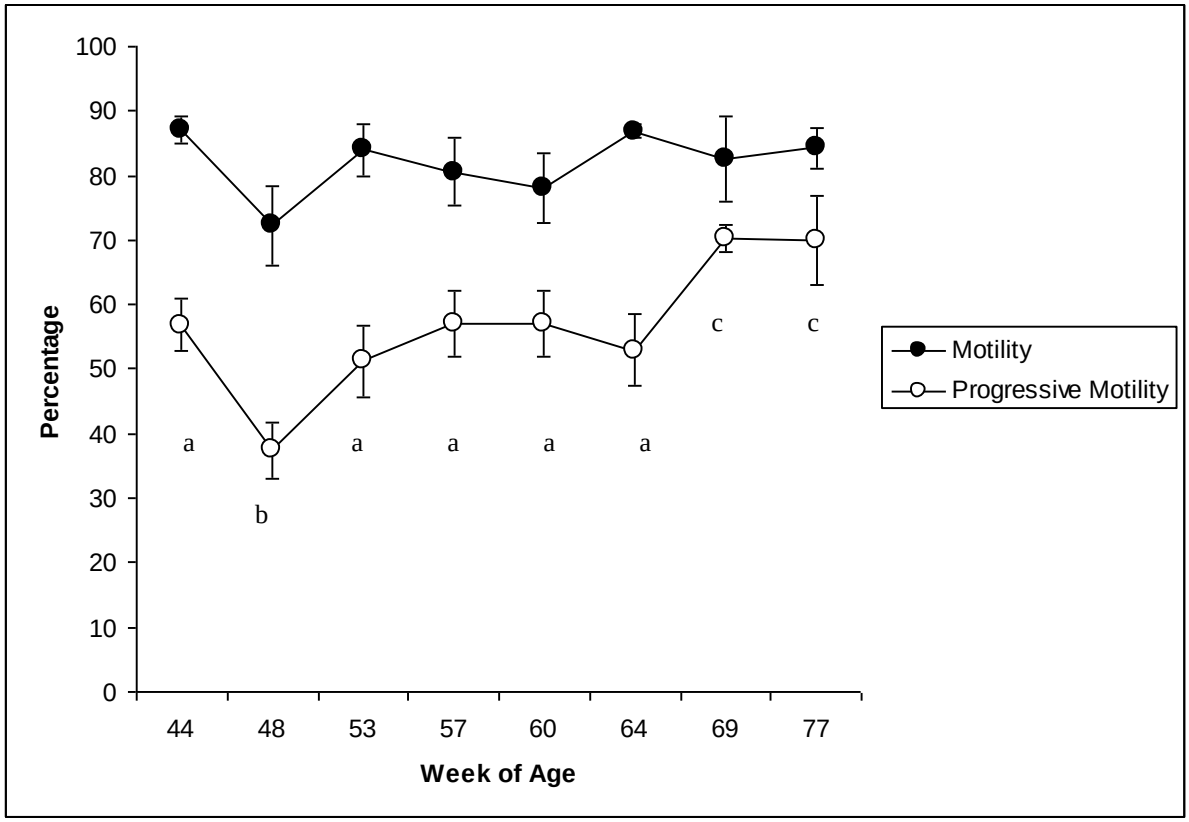


Figure 30. Effects of FSH treatment on the percentage of motile and progressively motile spermatozoa in the ejaculate. Data is presented as means ± S.E., however all data was transformed for statistical analysis. No differences over time were seen for the percentage of motile spermatozoa in the ejaculate (p=0.0913).

[abc]: Differences in superscript represent differences among weeks in progressive motility at p≤0.05.

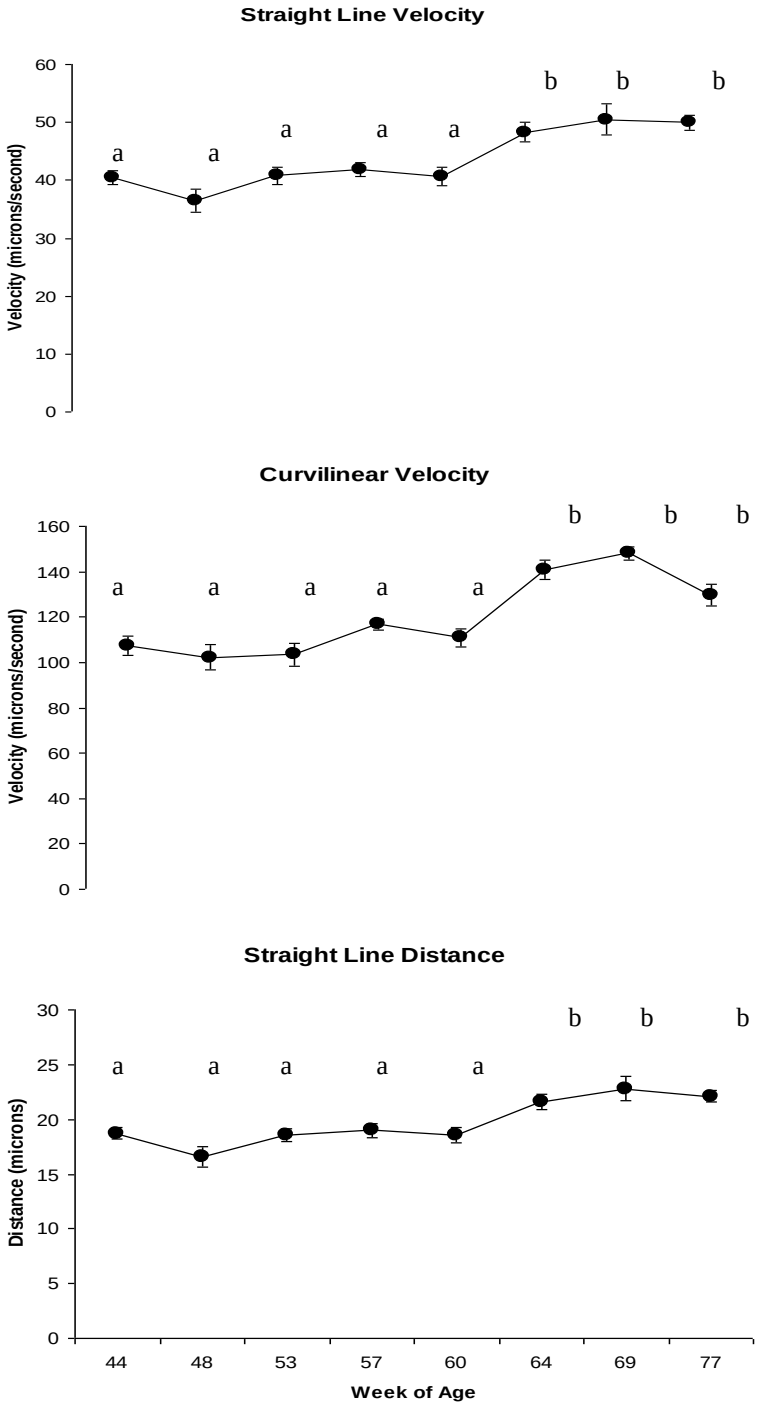


Figure 31. Effects of FSH treatment on average straight line velocity, curvilinear velocity and straight line distance of spermatozoa over time (mean ± S.E.).

^{abc}: Differences in superscript represent differences among weeks of age ($p \leq 0.05$).

Table 5. Effects of FSH treatment on measures of semen quality. Data are presented as means ± S.E. however all data was arcsine transformed prior to analysis. There were no differences among treatment groups for any of these measures of semen quality.

Measure	Treatment p-value	Control (%) (n)	Early (%) (n)	Late (%) (n)
Acrosome	0.3601	81.8 ± 1.7 (34)	81.4 ± 2.3 (33)	80.2 ± 2.7 (30)
Normal Sperm	0.4414	92.6 ± 1.5 (34)	83.7 ± 7.7 (33)	84.9 ± 6.7 (30)
Distal Droplets	0.3857	5.4 ± 0.9 (34)	11.5 ± 2.9 (33)	10.7 ± 2.4 (30)
Proximal Droplets	0.4161	5.0 ± 0.8 (34)	7.5 ± 1.8 (33)	7.3 ± 1.3 (30)
Translocated Tail	0.3714	2.2 ± 0.5 (34)	7.3 ± 3.0 (33)	8.2 ± 4.0 (30)
Tail Abnormalities	0.4316	0.8 ± 0.2 (34)	1.5 ± 0.3 (33)	2.5 ± 0.9 (30)
Head	0.4165	0.9 ± 0.2 (34)	0.7 ± 0.2 (33)	1.6 ± 0.3 (30)

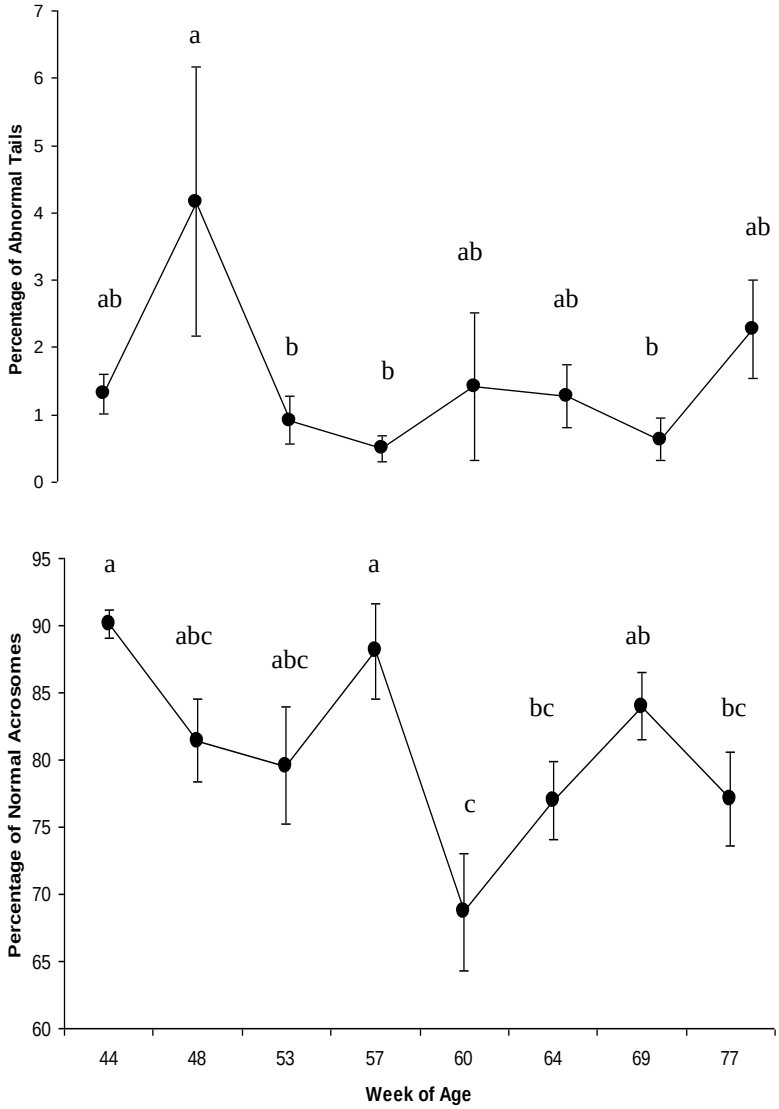


Figure 32. Effects of FSH treatment on the percentage of normal acrosomes and percentage of abnormal tails over time. Data is presented as percentages ± S.E. All data was arcsine transformed prior to statistical analysis.

^{abc}: Differences in superscript indicate differences between weeks of age at $p \leq 0.05$.

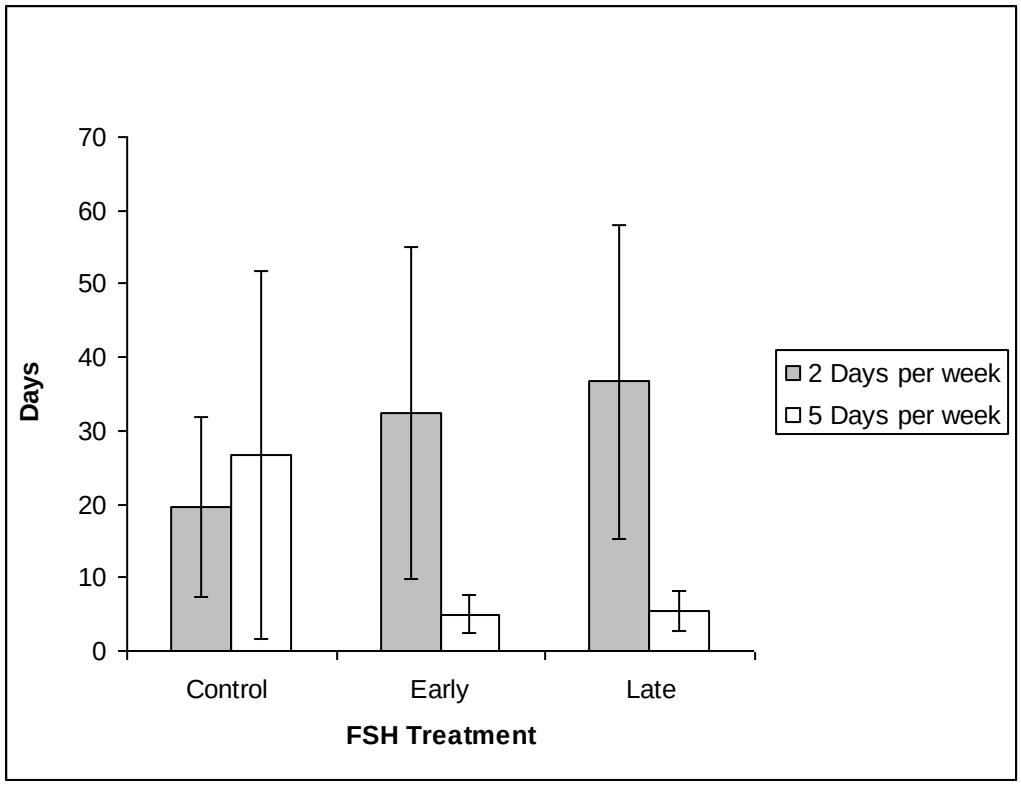


Figure 33. Effects of FSH treatment and number of days per week of training on number of days of training to learn to mount a dummy sow for semen collection (means ± S.E.).

Table 6. Effects of FSH treatment on µg of 54 different seminal plasma proteins per mg of total protein in the seminal plasma. There were no differences among treatment groups for any of the seminal plasma proteins (p-values in table).

Protein Code	Molecular Weight	Isoelectric Point	FSH Treatment Effects P-value
A1	16.6	9.36	0.3780
A2	14.51	8.59	0.6482
A3	16.15	7.94	0.4544
A4	16.6	7.15	0.6512
A5	17.46	6.79	0.1733
A6	16.89	6.38	0.2830
A7	17.3	5.27	0.2057
A8	16.15	4.87	0.2981
A9	16.6	4.73	0.3956
A10	15.54	4.63	0.6002
A11	15.66	4.39	0.2548
B1	21.62	5.19	0.4633
B2	21.62	4.72	--
B3	22.18	4.56	--
C1	34.27	8.61	0.8805
C2	32.24	7.81	0.8432
C3	30.31	6.9	0.3855
C4	28.115	6.50	0.7270
C5	27.14	5.56	0.0757
C6	26.86	5.14	0.4707
C7	30.31	4.97	--
C8	31.31	4.65	--
C9	28.4	4.58	--
D1	43.82	5.74	0.3312
D2	43.82	5.11	0.7794
D3	43.82	4.75	0.3945
D4	43.82	4.63	0.2557
E1	54.8	4.58	--

Table 6 (continued). Effects of FSH treatment on µg of 54 different seminal plasma proteins per mg of total protein in the seminal plasma. There were no differences among treatment groups for any of the seminal plasma proteins (p-values in table).

Protein Code	Molecular Weight	Isoelectric Point	FSH Treatment Effects P-value
F1	82.57	7.82	0.4640
F2	82.57	7.34	0.5470
F3	82.57	6.96	0.4875
F4	82.57	6.65	0.5241
F5	82.57	6.19	0.8425
F6	82.57	5.34	0.5315
F7	79.29	4.81	0.3668
F8	82.57	4.61	0.6226
F9	76.05	4.49	0.2824
F10	69.93	4.36	0.8147
G1	110.03	4.51	0.1692
G2	110.03	4.42	0.9969
G3	110.03	4.36	0.1407
H1	142.33	8.2	0.2873
H2	136.94	7.59	0.4180
H3	136.94	6.88	0.1580
H4	131.87	6.41	0.1250
H5	136.94	5.69	0.2948
H6	136.94	4.96	0.8862
H7	136.94	4.58	--
I1	239.53	7.67	0.2892
I2	229.12	6.93	0.5786
I3	218.84	6.47	0.2943
I4	229.12	5.8	0.3127
I5	229.12	5.0	0.9420
I6	208.76	4.59	--

Table 7. Effects of FSH treatment on total protein, heat shock protein 70, TGFβ and TNFα concentrations in seminal plasma (means ± S.E.). There were no differences among treatments for total protein (p=0.8734), heat shock protein (p=0.3593), TGFβ (p=0.6980) or TNFα (p=0.4740).

	CONTROL (n)	**EARLY (n)**	**LATE (n)**
Total Protein (µg)	5822 ± 1166 (4)	6128 ± 341 (4)	5533 ± 143 (3)
Heat Shock Protein	15.4 ± 2.3 (4)	16.4 ± 3.8 (4)	23.3 ± 5.5 (3)
TGFβ	0.22 ± 0.14 (4)	0.12 ± 0.08 (3)	0.09 ± 0.03 (3)
TNFα	0.26 ± 0.05 (4)	0.20 ± 0.01 (4)	0.24 ± 0.03 (3)

Appendices

Appendix A

Appendix A.1. All 85 boars that were born during the week of April 15th, 2006 were placed on trial. Each of the boars was assigned to a treatment group. Their fate during the course of the study is presented here.

Sow Nursed	Pig ID	Date of Birth	TRT	Fate During Study
03235405	12808	4/15/06	2	Collected for Semen Evaluation
0414008	12809	4/15/06	3	Castrated Day 21
03235405	12810	4/15/06	3	Collected for Semen Evaluation
03235405	12811	4/15/06	1	Castrated Day 98
0414008	12812	4/15/06	2	Castrated Day 21
0414008	12813	4/15/06	1	Cull
03235405	12904	4/15/06	1	Castrated Day 42
0414008	12905	4/15/06	1	Castrated Day 330
0414008	12906	4/15/06	2	Castrated Day 42
03235405	12907	4/15/06	3	Castrated Day 21
0414008	12908	4/15/06	3	Died
03235405	12909	4/15/06	2	Castrated Day 21
0414004	13010	4/19/06	2	Died
0414004	13011	4/19/06	3	Castrated Day 14
0414004	13012	4/19/06	1	Castrated Day 330
0414004	13013	4/19/06	2	Collected for Semen Evaluation
0414004	13014	4/19/06	3	Castrated Day 42
9932101	13103	4/20/06	2	Collected for Semen Evaluation
0414004	13104	4/19/06	1	Castrated Day 21
0519504	13105	4/20/06	1	Castrated Day 98
9932101	13106	4/20/06	3	Castrated Day 42
0519504	13107	4/20/06	2	Cull
0519504	13108	4/20/06	3	Collected for Semen Evaluation
9932101	13109	4/20/06	1	Collected for Semen Evaluation
0519504	13110	4/20/06	1	Castrated Day 42
0519504	13209	4/20/06	2	Castrated Day 14
0519504	13210	4/20/06	3	Castrated Day 330
9932101	13211	4/20/06	1	Castrated Day 14
9932101	13212	4/20/06	3	Castrated Day 42
0323905	13308	4/20/06	2	Castrated Day 42
0323905	13309	4/20/06	1	Castrated Day 21
04151008	13310	4/20/06	1	Castrated Day 21
04151008	13311	4/20/06	2	Castrated Day 14
0323905	13312	4/20/06	3	Cull
0520902	13313	4/20/06	1	Castrated Day 98
04151008	13314	4/20/06	3	Castrated Day 21
0521002	13406	4/20/06	1	Collected for Semen Evaluation
0521002	13407	4/20/06	3	Castrated Day 98
0520902	13408	4/20/06	3	Collected for Semen Evaluation

Appendix A.1, Continued

0521002	13409	4/20/06	2	Castrated Day 42
0520902	13410	4/20/06	2	Died
0520902	13507	4/20/06	3	Castrated Day 14
0521002	13508	4/20/06	1	Castrated Day 21
0521002	13509	4/20/06	3	Castrated Day 98
0520902	13510	4/20/06	2	Castrated Day 21
0520902	13511	4/20/06	1	Castrated Day 42
0520902	13512	4/20/06	3	Collected for Semen Evaluation
0520902	13513	4/20/06	2	Castrated Day 330
0404102	13606	4/20/06	1	Castrated Day 21
0404102	13607	4/20/06	2	Castrated Day 98
0404102	13608	4/20/06	3	Castrated Day 98
0404102	13609	4/20/06	1	Collected for Semen Evaluation
0404102	13610	4/20/06	2	Castrated Day 330
0324901	13707	4/20/06	2	Runt Removed from Study
0414506	13708	4/20/06	1	Castrated Day 14
0414506	13709	4/20/06	2	Castrated Day 21
0324901	13710	4/20/06	3	Runt Removed from Study
0414506	13711	4/20/06	3	Cull
0414506	13712	4/20/06	1	Castrated Day 98
0414506	13713	4/20/06	2	Castrated Day 98
0414506	13714	4/20/06	3	Castrated Day 98
0414506	13715	4/20/06	2	Castrated Day 98
0429702	13804	4/20/06	3	Castrated Day 330
0429702	13805	4/20/06	2	Castrated Day 42
0429702	13806	4/20/06	1	Castrated Day 330
0429702	13807	4/20/06	3	Castrated Day 14
0404102	13811	4/20/06	3	Castrated Day 42
0324901	13907	4/21/06	2	Castrated Day 330
0324901	13908	4/21/06	1	Runt Removed from Study
0429702	13909	4/21/06	2	Collected for Semen Evaluation
0521002	13910	4/20/06	2	Died
0429702	13911	4/21/06	1	Castrated Day 42
04151008	13911	4/20/06	3	Cull
0324901	14003	4/20/06	1	Runt Removed from Study
0324901	14004	4/20/06	3	Runt Removed from Study
04151008	14005	4/20/06	3	Castrated Day 21
04151008	14006	4/20/06	1	Cull
04151008	14007	4/20/06	2	Died
0323905	14008	4/20/06	1	Castrated Day 330
27604	14107	4/24/06	2	Castrated Day 14
27604	14108	4/24/06	1	Collected for Semen Evaluation
0520106	14203	4/24/06	2	Castrated Day 330
0520106	14204	4/24/06	3	Castrated Day 330
0520106	14205	4/24/06	2	Castrated Day 98

Appendix B

Ki-67 Immunohistochemistry

In the present study, it was imperative that cells undergoing mitotic divisions were identified. Prior to the use of the fluorescence protocol with the PCNA antibody, other markers for identifying proliferating cells were tried. One of these was Ki-67. Ki-67 is a protein expressed in the nucleus of proliferating cells (Key et al., 1993). The Ki-67 antibody can be purchased commercially for use in immunohistochemistry in pigs (McCoard et al., 2001). Other antibodies have been identified that can bind to and identify the Ki-67 antigen. An example is the MIB 1 antibody that is used extensively in human research to identify the Ki-67 antigen from paraffin embedded testicular samples (Key et al., 1993). It is unclear whether the Ki-67 or the MIB 1 antibody identify the Ki-67 antigen more readily in pigs.

It was attempted to identify proliferating sertoli cells using a Ki-67 antibody (Santa Cruz Biotechnology, SC-23900, Santa Cruz, CA) and a commercially available immunoperoxidase kit, Vectastain Elite ABC kit (Vector Laboratories, pk-6105, Burlingame, CA). A chromagen called Novared (Vector Laboratories, sk-4800, Burlingame, CA) was used to identify cells that were positive for the Ki-67 antigen.

The procedure was as follows:

1. xylene 3 x 3 min.
2. 100% ETOH 3 x 3 min.
3. 95% ETOH 1 x 3 min.
4. 80% ETOH 1 x 3 min.
5. Tap water 1 x 5 min.

6. Microwave slides submerged in 0.1M Sodium Citrate (pH=6.0) for 10 minutes

7. Allow slides to cool to room temperature

8. 3% H2O2 for 10 minutes (to quench peroxidase activity)

9. Tris Buffer 3 x 5 min.

10. 1% normal blocking serum for 20 minutes (Vectastain Elite ABC Kit)

11. Ki-67 Primary Antibody for 1 hour (Vector Laboratories) diluted 1:100 in blocking solution

12. Tris Buffer 3 x 5 min.

13. Biotintylated secondary antibody for 40 minutes (Vectastain Elite ABC Kit)

14. Tris Buffer 3 x 5 min.

15. ABC Reagent (Vectastain Elite ABC Kit) for 40 minutes

16. Tris Buffer 1 x 5 min

17. Novared for 15 minutes protected from light (Vector Laboratories)

18. Rinse in water

19. Hematoxylin for 30-45 seconds depending on age of stain

20. 95% ETOH 1 x 5 min.

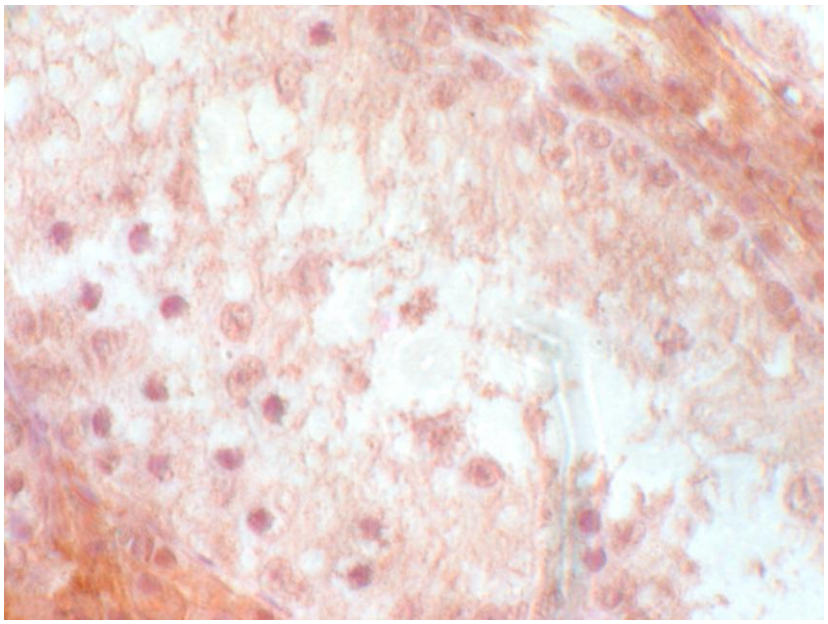
21. 100% ETOH 1 x 5 min.

22. xylene 3 x 15 min. and allow to soak overnight

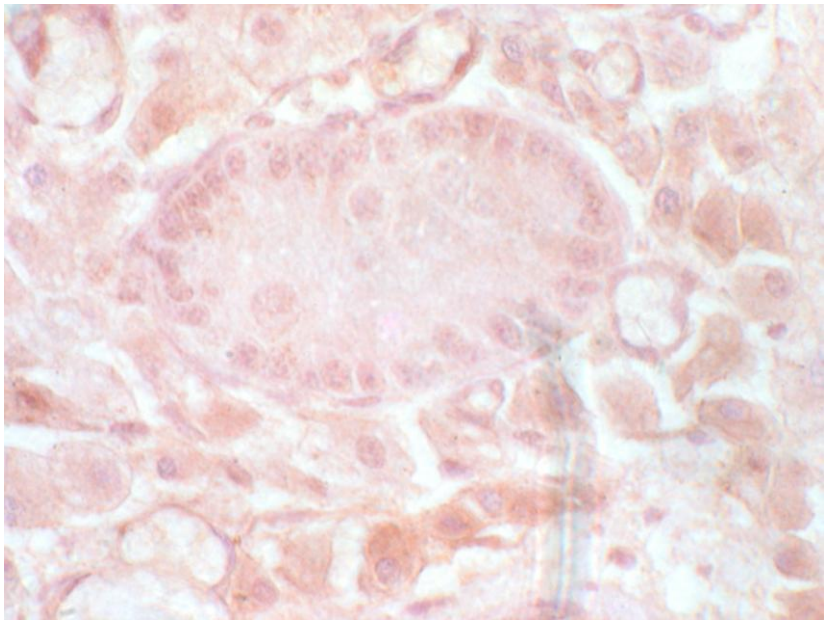
23. Place 1 small drop of permount on the slide and cover with a coverglass.

Many aspects of this procedure were modified in an attempt to improve the results. The length of time the samples were microwaved, the concentration of the primary antibody, the length of incubation in the blocking solution and incubation at 4ºC in the primary

antibody versus room temperature are examples. Consistent, reproducible results were not obtained. Below are example pictures of the tissue samples after staining for Ki-67.



A

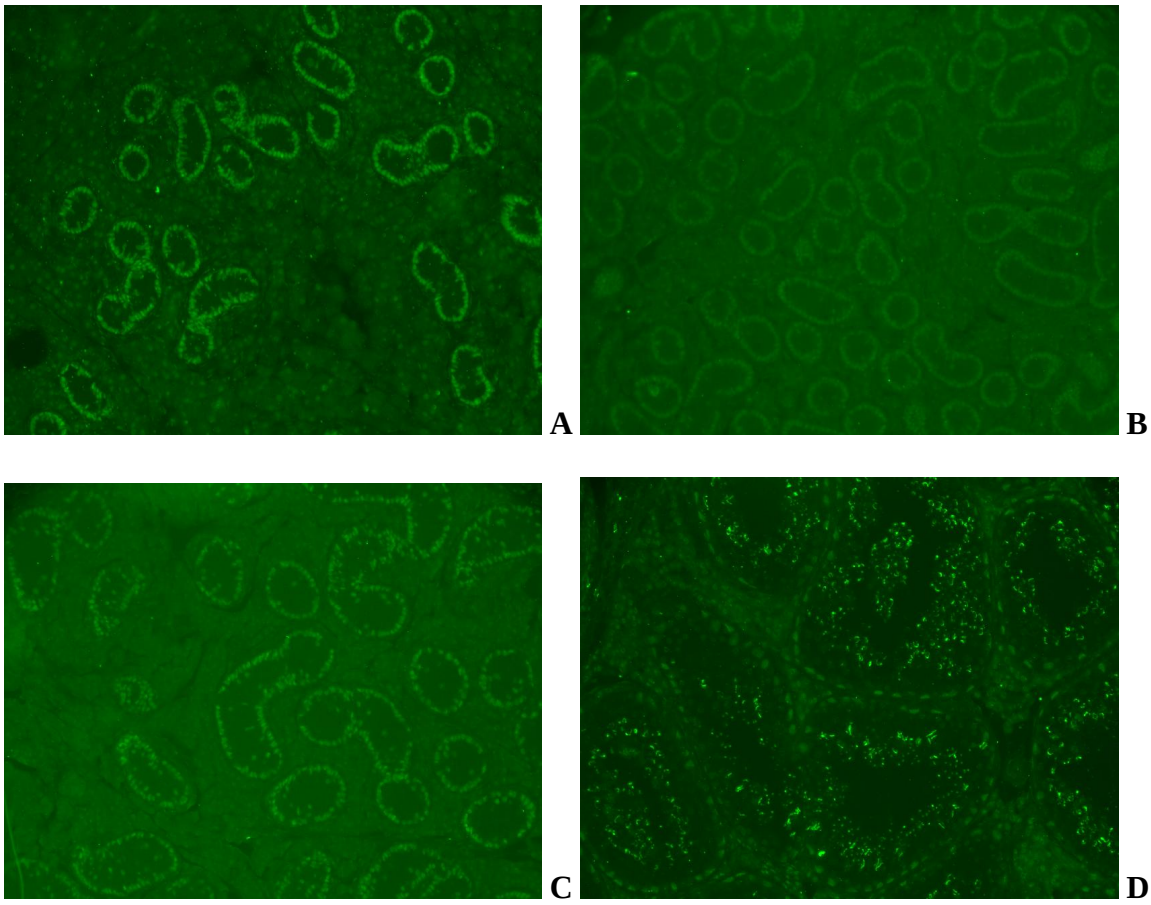


B

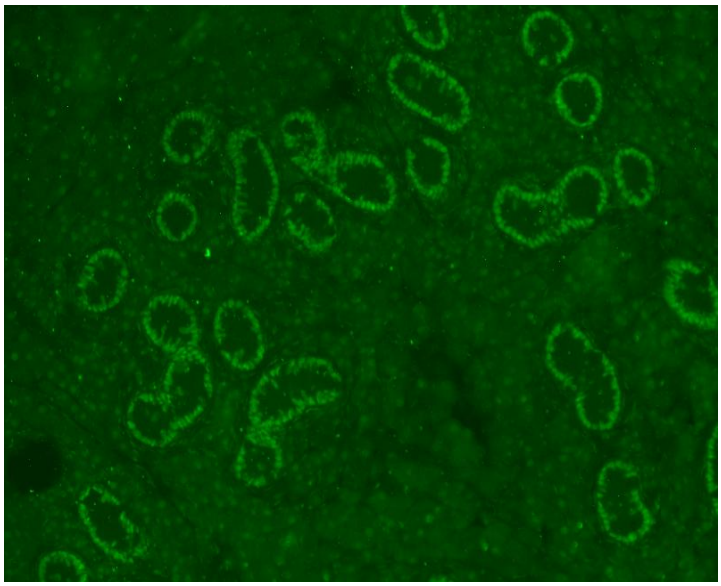
Appendix B.1. Pictures of 98 day (A) and 14day (B) old boar's testicles stained with Ki-67 antibody using a Vectastain Elite ABC kit. Novared was used as the chromagen.

Appendix C

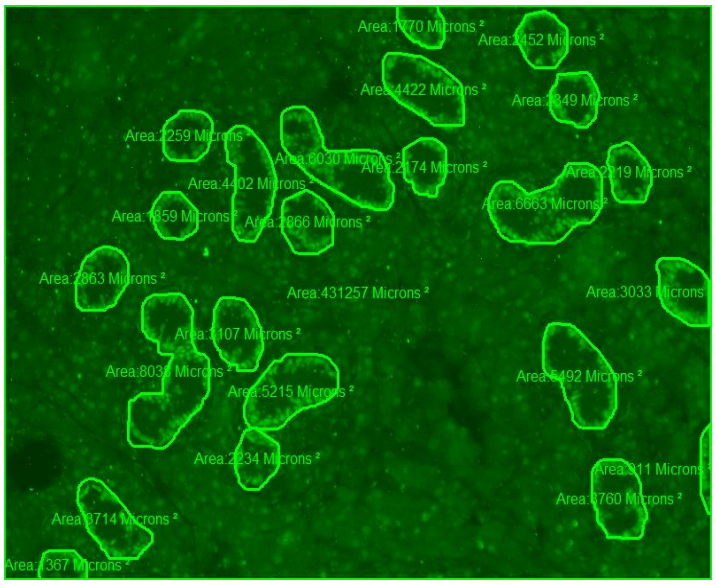
Immunofluorescence Pictures



Appendix C.1. These pictures are taken at a 20x objective of testicular tissue from 21 (A), 42 (B), 98 (C) and 330 (D) day old boars. The tissue has been fluorescently stained for the GATA-4 antibody. This antibody has been shown to stain only the sertoli cells within the seminiferous tubule as well as the leydig cells. There was also the uptake of stain in the 330 day old boar (D) by the acrosomal membranes of the elongated spermatozoa. These pictures were used to estimate the volume percentage of the testicle comprised of the seminiferous tubules and interstitial space.

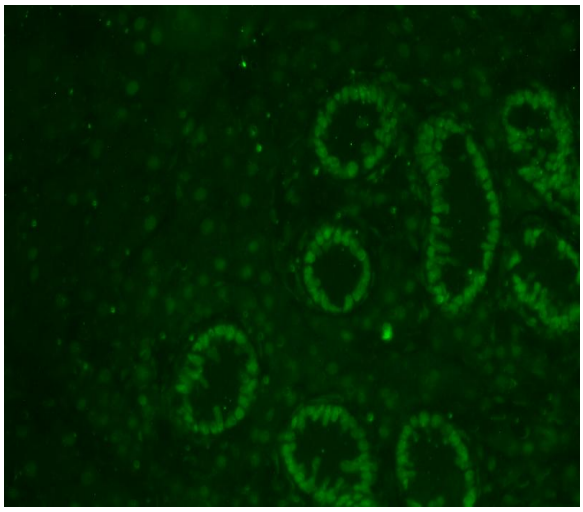


A

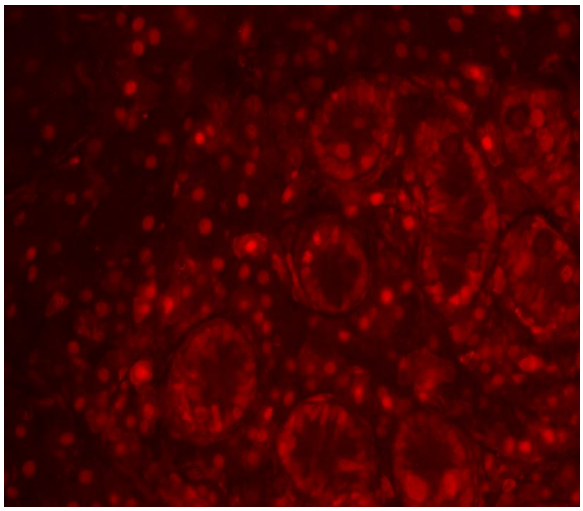


B

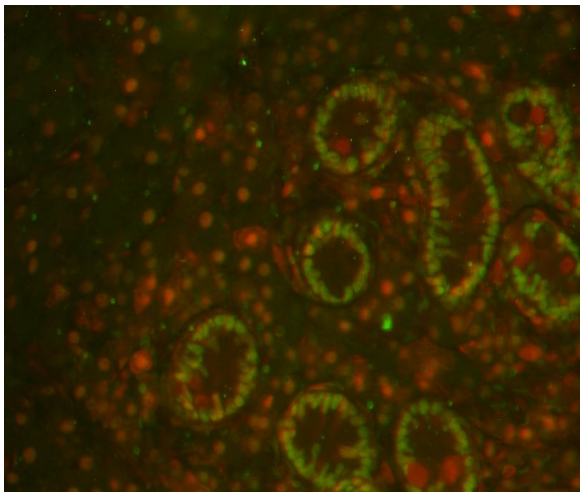
Appendix C.2. This is an example of how the measurements were taken to calculate the volume percentage of seminiferous tubules and interstitial space. These tissue samples are from a 21 day old boar stained for GATA-4 (A). The volume percentage of the testicle comprised of the seminiferous tubules were calculated by summing all of the areas of the seminiferous tubules within a field and dividing that number by the total area of the view (B).



A

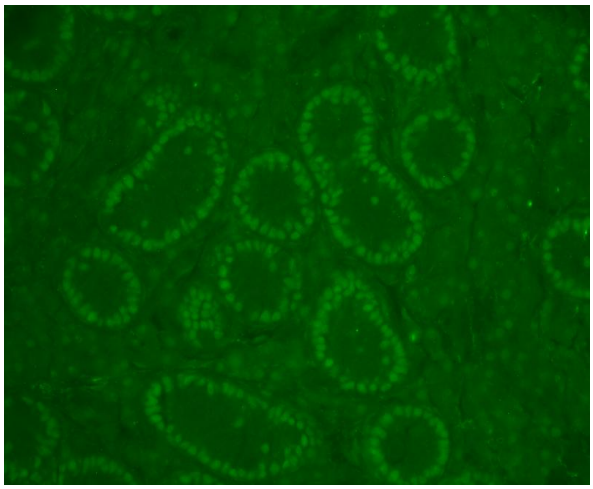


B

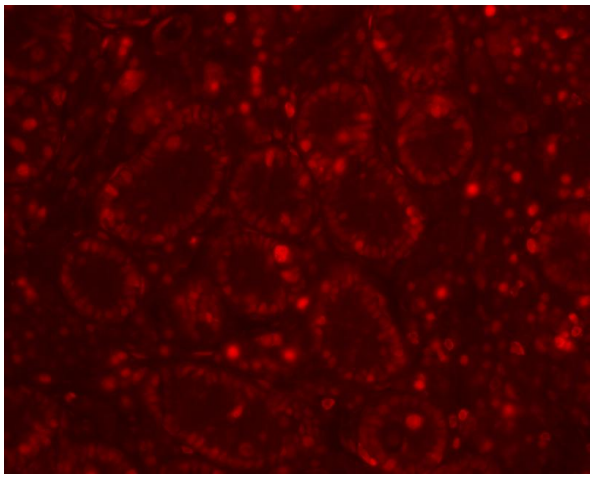


C

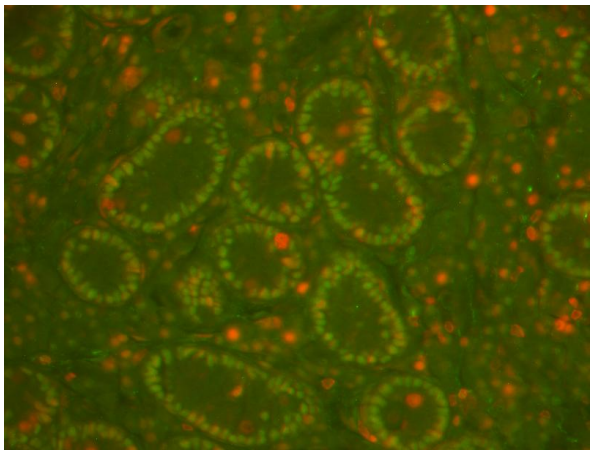
Appendix C.3. Using the 40x Objective, pictures were taken of the tissue samples stained for GATA-4 (A) of an area of the tissue that contained at least one round tubule. A subsequent picture was taken of the same area of the tissue, but stained for PCNA (B). A computer software package was then used to merge these two pictures together (C). These merged images were then used to determine the number of sertoli cells, germ cells and proliferating cells in the round tubule. These pictures are from a 21 day old boar.



A

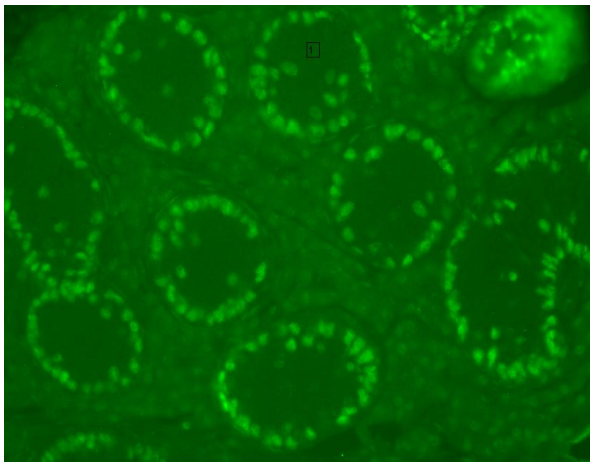


B

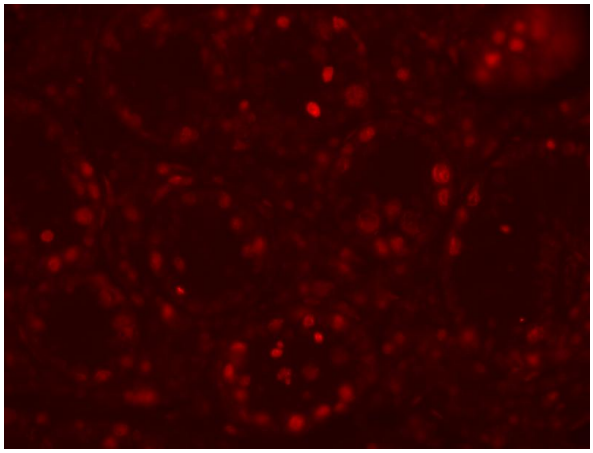


C

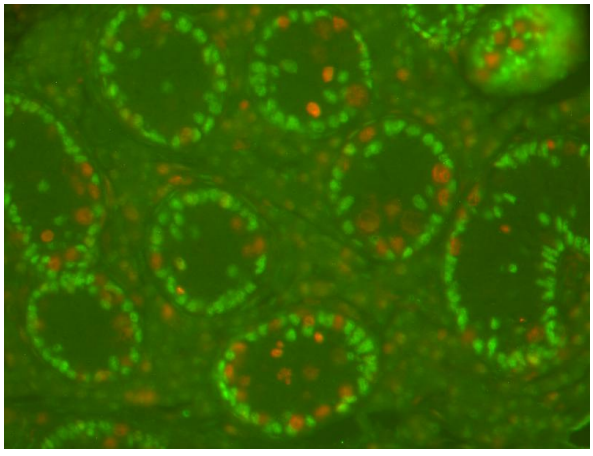
Appendix C.4. These pictures are the same as described in Appendix C.3. for GATA-4 (A), PCNA (B) and a merged image (C). However, these pictures are taken from a boar that was 42 days of age.



A

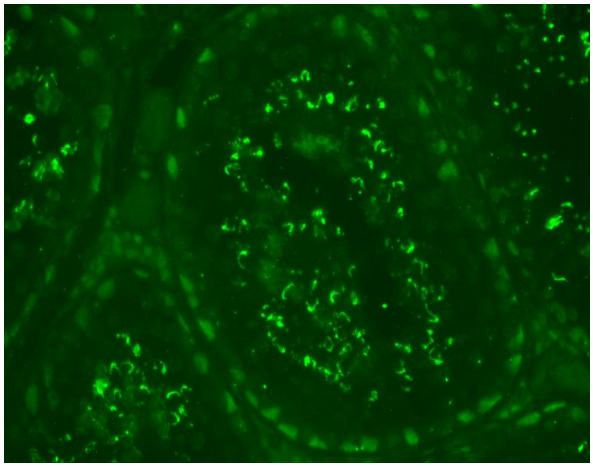


B

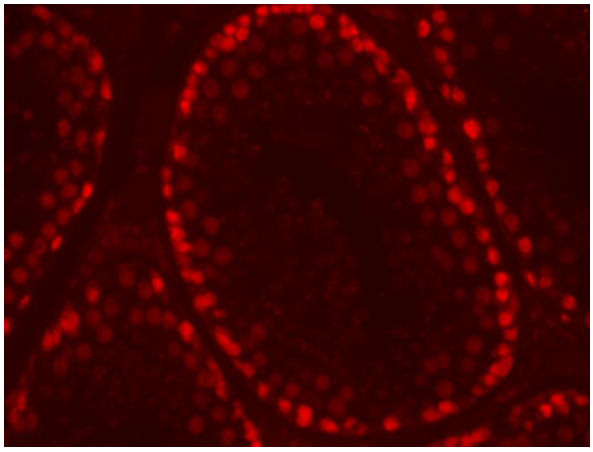


C

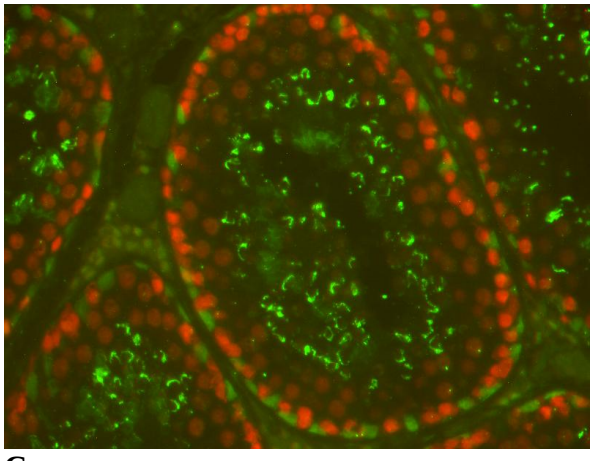
Appendix C.5. These pictures are the same as described in Appendix C.3. for GATA-4 (A), PCNA (B) and a merged image (C). However, these pictures are taken from a boar that was 98 days of age.



A

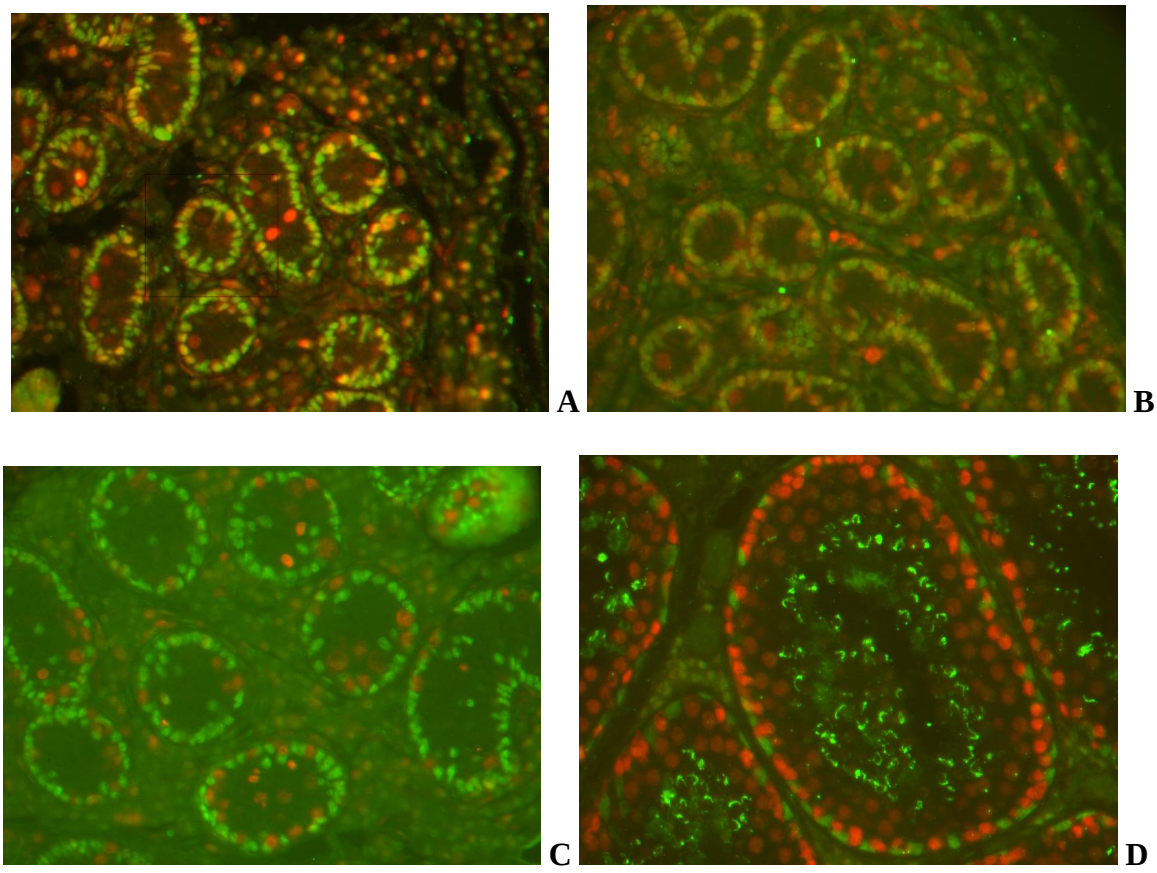


B

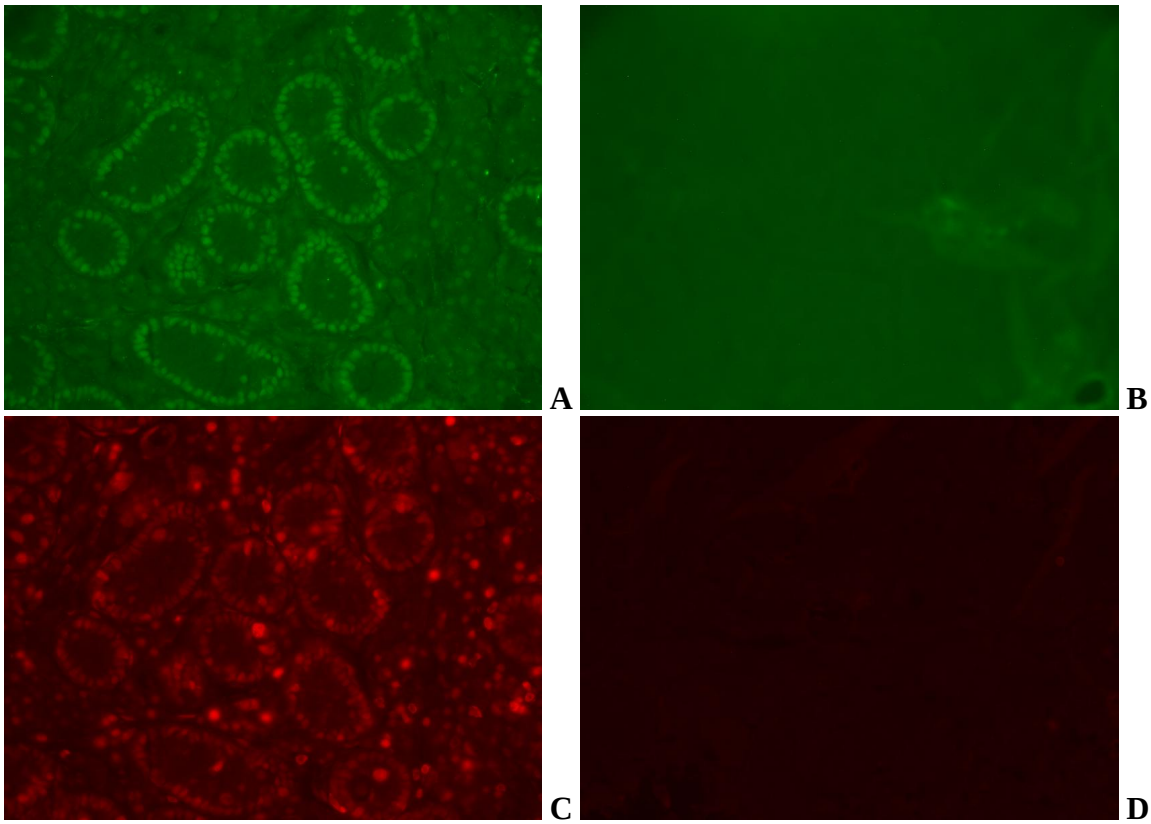


C

Appendix C.6. These pictures are the same as described in Appendix C.3. for GATA-4 (A), PCNA (B) and a merged image (C). However, these pictures are taken from a boar that was 330 days of age.

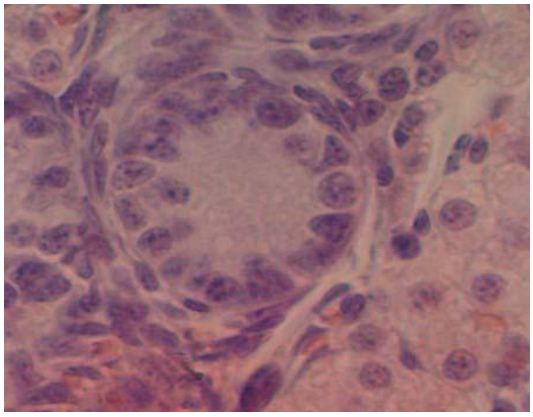


Appendix C.7. These merged pictures are taken from testicular tissue from 21 (A), 42 (B), 98 (C) and 330 (D) day old boars stained for GATA-4 (green) and PCNA (red). Within the seminiferous tubule, cells that are green are sertoli cells not undergoing proliferation. Cells that are red are either germ or sertoli cells that are proliferating. Sertoli cells that are proliferating appear yellow. From these pictures, it is easy to see that there are sertoli cells undergoing proliferation at days 21 and 42 and not at days 98 and 330.

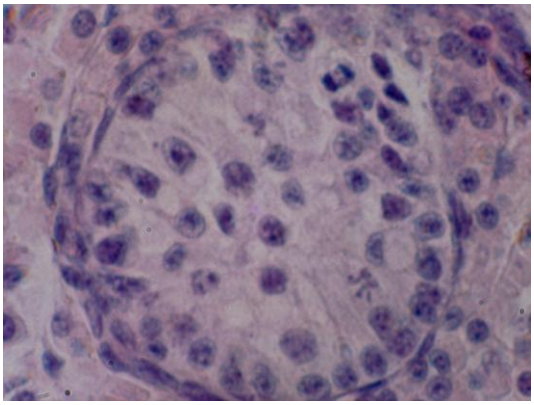


Appendix C.8. One piece of tissue on each slide did not have the primary antibodies for GATA-4 (B) or PCNA (D) added to the tissue. These tissue samples were used as a control for background staining. The tissue samples were then photographed at the same exposure time as the tissue samples where the primary antibodies GATA-4 and PCNA (A and C, respectively) were added to the tissue. From these example pictures, it can be determined that background staining was negligible. It should be noted that the background for GATA-4 was always slightly higher than for PCNA.

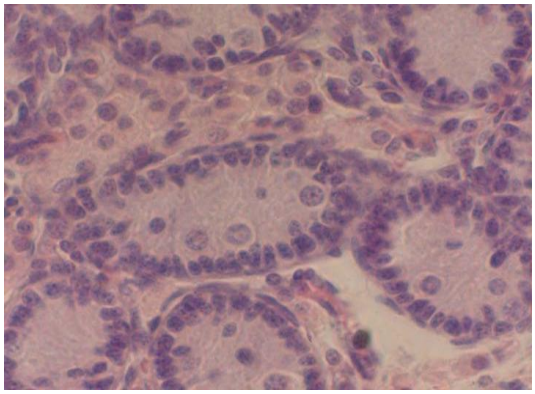
Haematoxylin and Eosin Pictures



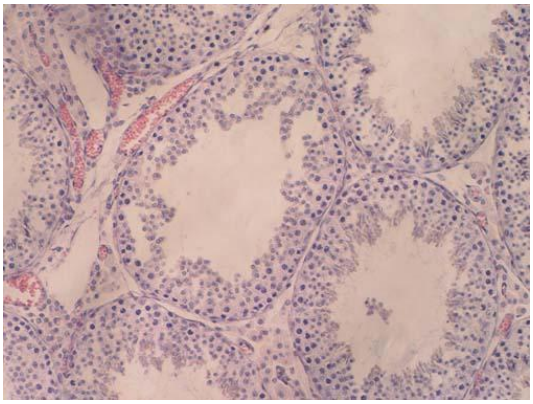
A



C

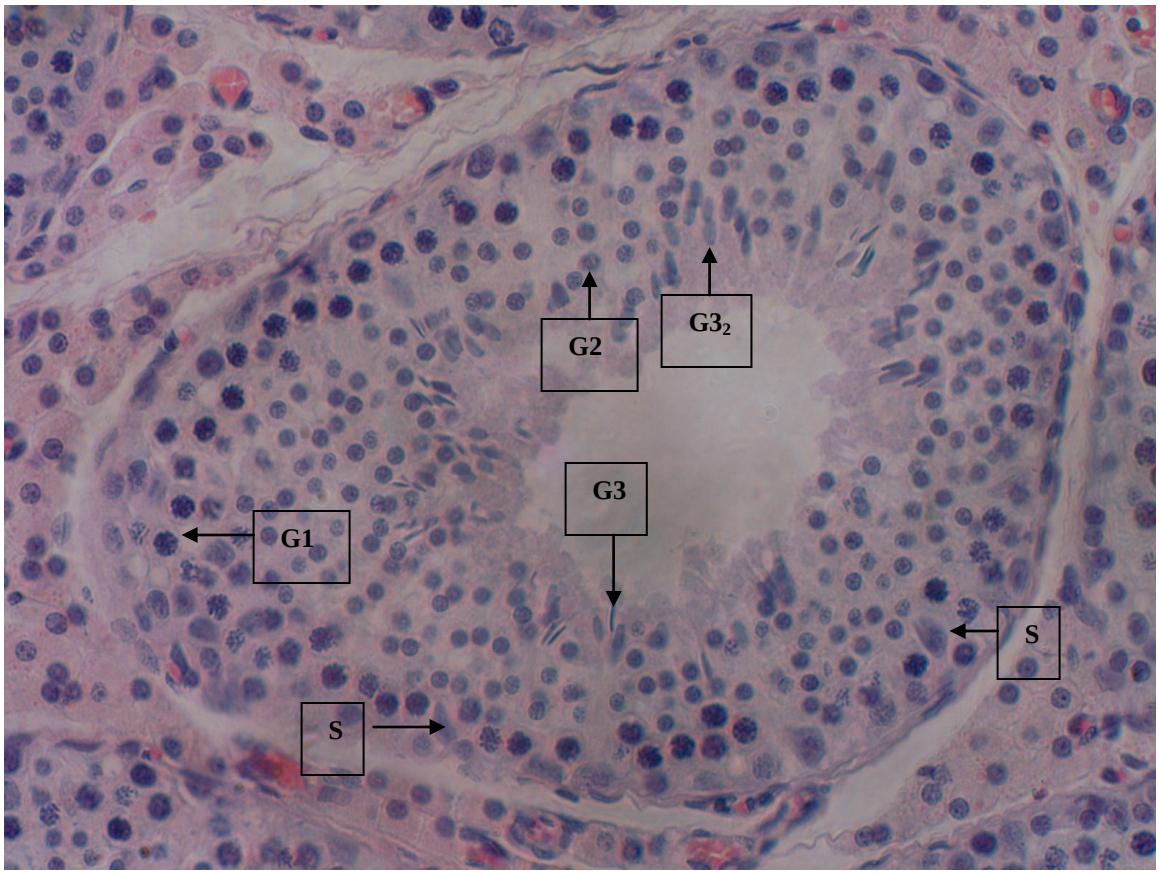


B



D

Appendix C.9. Haematoxylin and eosin stained testicular tissue samples from boars aged 21 days (A), 42 days (B), 98 days (C) and 330 days (D). These pictures were used to generally examine the tissue samples for any signs of poor tissue structure that could effect the immunofluorescent staining. These pictures were further used to enumerate germ cells within the seminiferous tubule.



Appendix C.10. Testicular tissue sample from a 330 day old boar stained with haematoxylin and eosin. These samples were used to enumerate germ cells. All germ cells were placed into one of three categories based on the size and shape of the cell. Category 1 (G1) consisted of large, round primary spermatocytes lying close to the basement membrane. Category 2 (G2) consisted of smaller, round spermatids. Category 3 consisted of elongated spermatozoa (G3)and spermatozoa that were beginning to elongate ($G3_2$) located closest to the lumen of the tubule. Some sertoli cell nuclei could also be identified by their irregular shaped nucleus (S)

Appendix D

SAS Coding for all Statistical Analysis

Repeated Measures for Serum Hormone Concentrations

```
proc glm;
class TRT WEEK;
model AVGFSH AVGLH AVGTEST AVGEST AVGIGF= TRT WEEK PIGID(TRT)
TRT*WEEK ;
test h = TRT e = PIGID(TRT);
lsmeans TRT WEEK TRT*WEEK / pdiff;
run;
quit;
```

Repeated Measures for Body Weight and Testicular Size

```
proc glm;
class TRT WEEK ;
model WTKG AREA TSBWR = TRT WEEK  TRT*WEEK ID(TRT);
test h = TRT e = ID(TRT);
lsmeans TRT TRT*WEEK / pdiff;
run;
quit;
```

Analysis of Variance for Histological Evaluations

Seminiferous Tubule and Interstitial Space Percentage

```
SQVOLDENS = SQRT (VOLDENS + 1);
proc glm;
class AGE TRT;
model SQVOLDENS = AGE TRT AGE*TRT;
lsmeans AGE*TRT /  pdiff;
run;
quit;
```

Mass of Seminiferous Tubule and Interstitial Space

```
proc glm;
class AGE TRT;
model ISMASS STMASS = AGE TRT AGE*TRT;
lsmeans AGE TRT /  pdiff;
run;
quit;
```

Number of Sertoli and Germ Cells per Organ and Average Nuclear Volume of Each

```
proc glm;
class AGE TRT ;
model CELLORGS CELLORGG AVGNUCVOLS AVGNUCVOLG = AGE TRT
AGE*TRT ;
lsmeans AGE  / pdiff;
run;
quit;
```

Percent of Proliferating Cells

```
SQPROLIF = SQRT ((PCTPROLIF/100) + 1);
proc glm;
class AGE TRT;
model SQPROLIF = AGE TRT AGE*TRT;
lsmeans AGE TRT / pdiff;
run;
quit;
```

Repeated Measures for Semen Evaluation

Total Sperm in the Ejaculate

```
proc glm;
class WEEK TRT;
model TSPERM =      WEEK TRT WEEK*TRT BOAR(TRT);
test h=TRT e=BOAR(TRT);
lsmeans TRT WEEK / pdiff;
run;
quit;
```

Semen Quality Estimates

```
ARCASOME = ARSIN (ASOME/100);
ARCNORMAL = ARSIN(NORMAL/200);
ARCDDROP = ARSIN (DDROP/200);
ARCPDROP = ARSIN(PDROP/200);
ARCTTAIL = ARSIN(TTAIL/200);
ARCTAIL = ARSIN(TAIL/200);
ARCHEAD = ARSIN(HEAD/200);
ARCMOT = ARSIN(AVGMOT/100);
ARCPMOT = ARSIN(AVGPMOT/100);

proc glm;
class WEEK TRT;
model TSPERM
SQRASOME SQRNORMAL SQRDDROP SQRPDROP SQRTTAIL SQRTAIL
SQRHEAD SQRMOT SQRPMOT
AVGDSL AVGVCL AVGVSL = WEEK TRT WEEK*TRT ID(TRT);
test h=TRT e=ID(TRT);
lsmeans TRT WEEK/ pdiff;
run;
quit;
```

Daily Sperm Production

```
proc glm;
class TRT ;
model DSP = TRT;
run;
quit;
```

Analysis of Variance for Seminal Plasma Protein Concentrations

Effects of FSH Treatment

```
proc glm;
class TRT ;
model A1 A2 A3 A4 A5 A6 A7 A8 A9 A10 A11 B1 B2 B3 C1 C2 C3 C4 C5 C6 C7 C8 C9
D1 D2 D3 D4 E1 F1 F2 F3 F4 F5 F6 F7 F8 F9 F10 G1 G2 G3
H1 H2 H3 H4 H5 H6 H7 I1 I2 I3 I4 I5 I6 TOTPROT HSP HSPPERMG TGFB TGFPERMG
TNFA TNFPERMG = TRT;
Lsmeans TRT / pdiff;
run;
quit;
```

Effects of Grouping Based on Percent Motility and Percent of Normal Sperm in Ejaculate

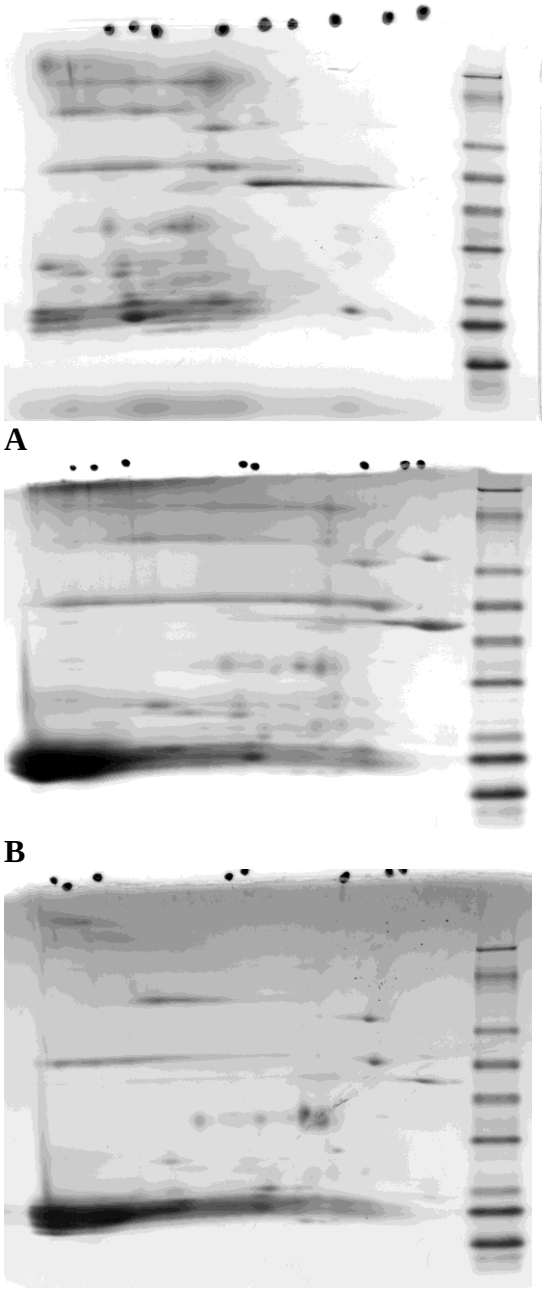
```
proc glm;
class GRPMOT GRPPMOT GRPNORM ;
model A1 A2 A3 A4 A5 A6 A7 A8 A9 A10 A11 B1 B2 B3 C1 C2 C3 C4 C5 C6 C7 C8 C9
D1 D2 D3 D4 E1 F1 F2 F3 F4 F5 F6 F7 F8 F9 F10 G1 G2 G3
H1 H2 H3 H4 H5 H6 H7 I1 I2 I3 I4 I5 I6 TOTPROT HSP HSPPERMG TGFB TGFPERMG
TNFA TNFPERMG = GRPMOT GRPPMOT GRPNORM ;
Lsmeans GRPMOT GROPMOT GRPNORM / pdiff;
run;
quit;
```

Analysis of Variance for Training to Mount and Collect

```
LENGTH = TRAINDT-STARTDT;
AGE = STARTDT - BDT;
proc glm;
class TRT FSH;
model LENGTH AGE= FSH TRT FSH*TRT;
lsmeans TRT FSH / pdiff;
run;
quit;
```

Appendix E

Pictures of 2-D Gels

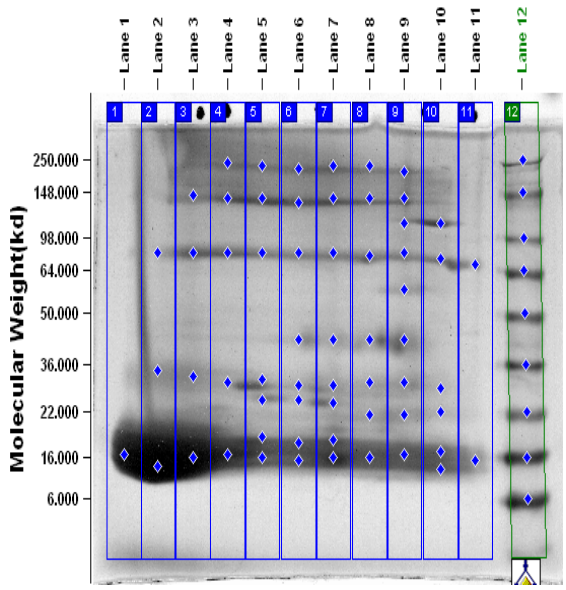


A

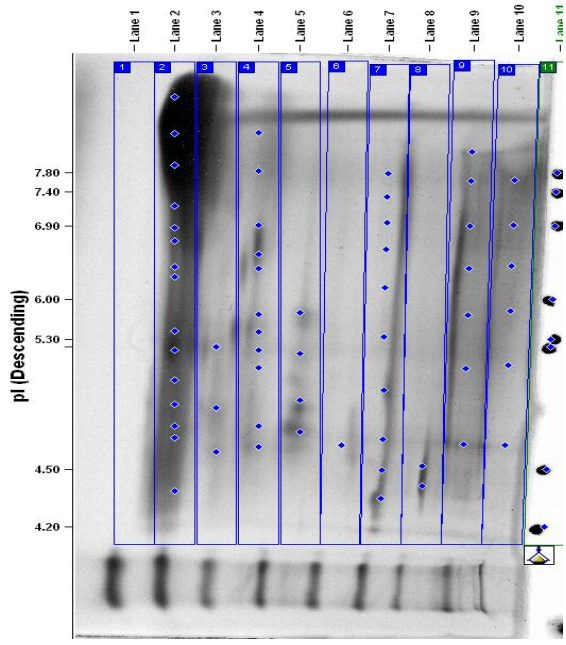
B

C

Appendix E.1. Scanned images of 2-D gel electrophoresis of seminal plasma proteins for 3 different boars. Boar 2901 (A) was used as an internal control and his seminal plasma was ran with each gel run. This boar also had the greatest number of different proteins in his seminal plasma. Boar 12808 (B) is representative of a boar that had a large amount of different proteins in his seminal plasma. Boar 14108 (C) is representative of a boar that had the lowest number of different proteins in his seminal plasma.



A

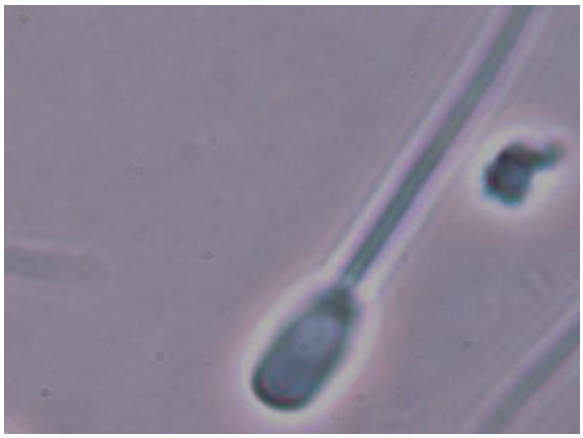


B

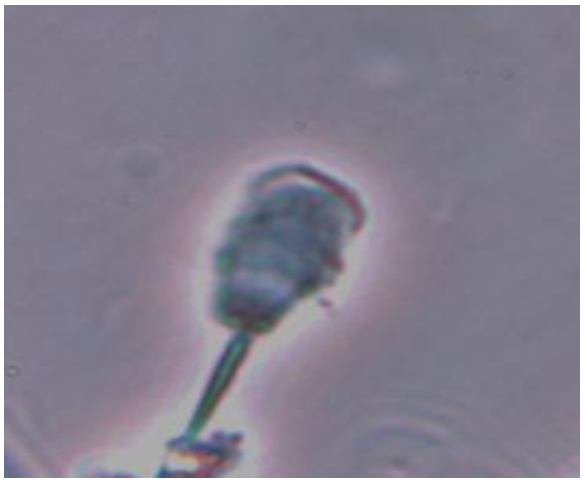
Appendix E.2. The 2-D gels were scanned into the computer in two different orientations for analysis of seminal plasma proteins. The gels were analyzed for molecular weights of proteins (A) and p.I.s of the proteins (B). Data obtained from these images was used to identify differences between boars in the composition of their seminal plasma protein profiles.

Appendix F

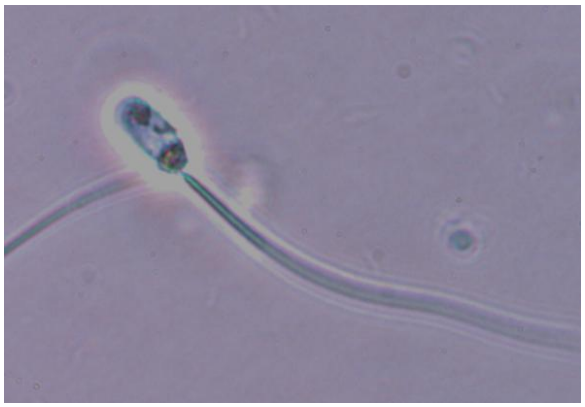
Semen Evaluation



A

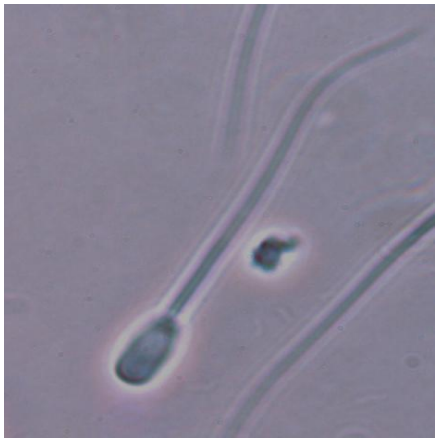


B

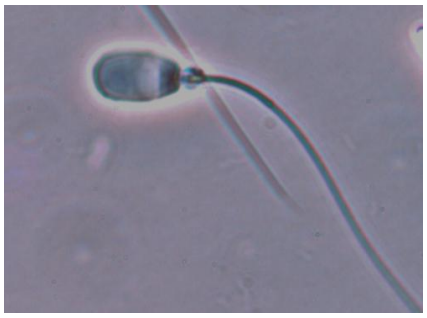


C

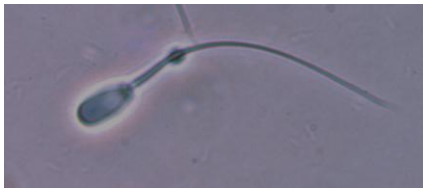
Appendix F.1. Spermatozoa were analyzed for the presence of a morphologically normal acrosome. An example of a normal acrosome is shown in picture A. Examples of abnormal acrosomes are pictured in B and C.



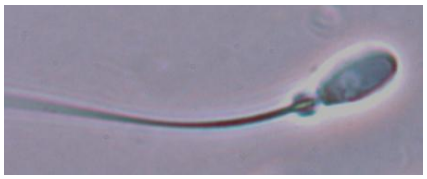
A



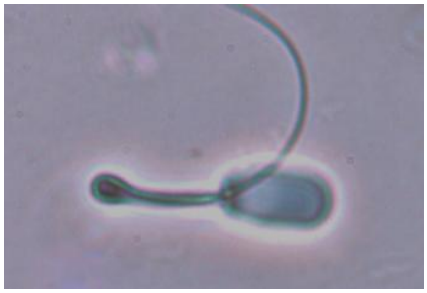
B



C



D



E

Appendix F.2. Spermatozoa were evaluated for morphological abnormalities. A spermatozoa with normal morphology is pictured in A. Some of the abnormalities identified were enlarged heads (B), distal (C) and proximal (D) cytoplasmic droplets and translocated tails (E).

Appendix G

Validation of Enzyme Linked Immunosorbent Assays

Before a particular ELISA can be used, a few tests for accuracy need to be performed. The first is a spike and recovery. This test is designed to test whether the sample matrix is interfering with the results of the assay. The second test is a test for the linearity of dilution. This test is designed to test for the precision of the assay at different dilutions. The final test is to calculate the inter and intra-assay coefficients of variation to determine the repeatability of the results.

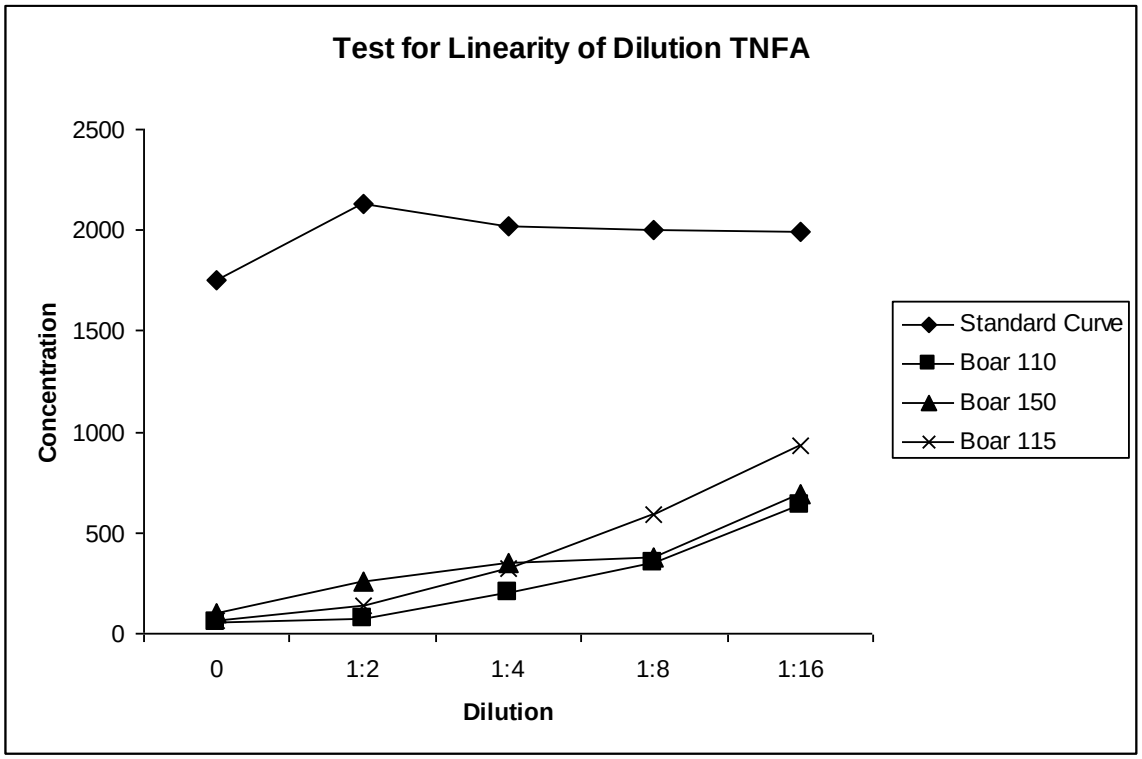
Tumor Necrosis Factor Alpha

The standard included in the kit was used to spike seminal plasma samples of 2 boars chosen at random. The standard is 4000pg/678µl of dilutent (4pg/µl).

Appendix G.1. Recovery data for TNFα

Boar	No Spike (0 pg/ml)	Low Spike (62.5 pg/ml) (0.8µl / 99.2 sample)	Medium Spike (125 pg/ml) (3.1µl / 96.9 sample)	High Spike (500 pg/ml) (12.5µl / 87.5 sample)
110	55.99	31.3	71.62	111.8
115	60.1	31.3	58.39	148.3
150	62.5	52.9	90.79	188.3
Mean Recovery		41.88%	38.63%	26.66%

Appendix G.2. Test for Linearity of Dilution for TNFα



From this table, it can be decided that precision of the assay is maintained to a dilution of 1:4. However, parallelism between the standard curve and the samples from the boars is lost after a 1:4 dilution. Samples were analyzed without any dilution.

The inter-assay coefficients of variation was 9.84% and the intra-assay coefficient of variation was 10.45%.

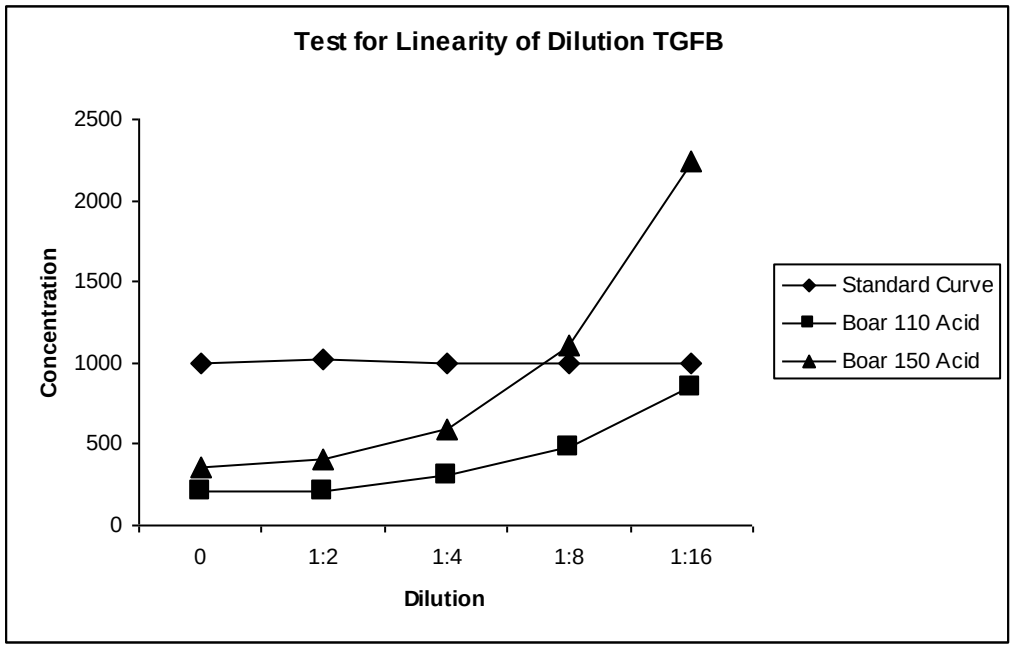
Transforming Growth Factor Beta

The standard included in the kit was used to spike seminal plasma samples of 2 boars chosen at random. The standard is 1µg/ml.

Appendix G.3. Recovery data for TGFβ

Boar	No Spike (0 pg/ml)	Low Spike (500 pg/ml) (0.7µl / 140 sample)	Medium Spike (250 pg/ml) (0.7 µl / 280 sample)	High Spike (125 pg/ml) 0.7 µl / 560 sample)
110	173.7	127.7	159.9	206.8
150	358	371.9	367.9	400.6
Mean Recovery		59.87%	48.18%	38.7%

Appendix G.4. Test for Linearity of Dilution for TGFβ



From this table, it can be decided that precision of the assay is maintained at a 1:2 and 1:4 dilution. Parallelism is not maintained past a 1:4 dilution. Samples were analyzed without any dilution.

The inter-assay coefficient of variation was 8.9% and the intra-assay coefficient of variation was 11.6%.

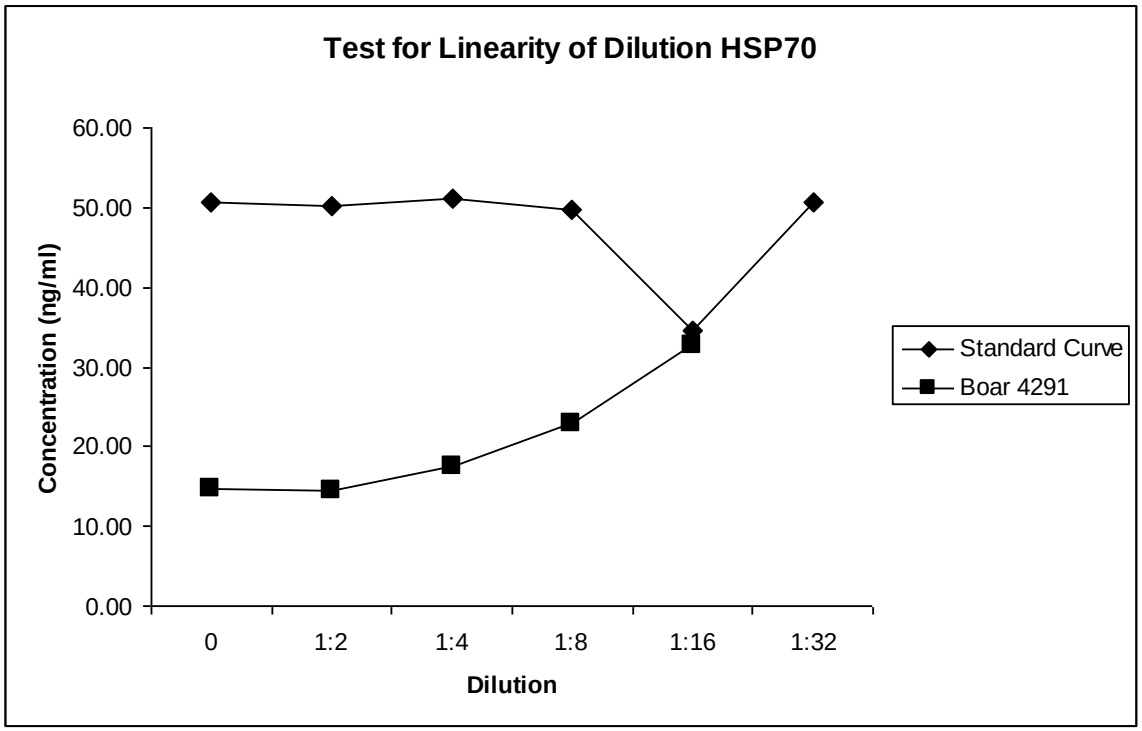
Heat Shock Protein 70

The standard for the kit was used to spike seminal plasma samples from one boar chosen at random. The standard is 10µg/ml.

Appendix G.5. Recovery Data for HSP70

Boar	No Spike (0 ng/ml)	Low Spike (5ng/ml)	Medium Spike (10ng/ml)	High Spike (20ng/ml)
158	0.78	3.527	6.573	11.351
Mean Recovery		54.62%	60.97%	61.02%

Appendix G.6. Test for Linearity of Dilution for HSP70



From this graph, it can be decided that precision of the assay is maintained through a 1:4 dilution. Parallelism is lost after a 1:4 dilution. Samples were ran without any dilution.

The inter-assay coefficient of variation was 0.339% and the intra-assay coefficient of variation was 5.73%.

Appendix H

Haematoxylin and Eosin Staining Procedures

1. Place slides containing the paraffin sections in a glass slide cradle
2. Deparaffinize and rehydrate sections
    a. 3 x 3min Xylene
    b. 3 x 3 min 100 % ETOH
    c. 1 x 3 min 95% ETOH
    d. 1 x 3 min 80% ETOH
    e. 1 x 5 min DI H2O
3. While sections are in water, skim the surface of the haematoxylin with a Kimwipe to remove oxidized particles. Blot excess H2O before continuing.
4. Haematoxylin staining
    a. 1 x 3 min Haematoxylin
    b. Rinse DI H2O
    c. 1 x 5 min TAP water (allows stain to develop)
    d. Dip 8-12x, fast in Acid ETOH to de-stain
    e. 2 x 1 min tap water
    f. 1 x 2 min DI H2O (can leave overnight if need be)
5. Blot excess water from slide before continuing
6. Eosin Staining
    a. 1 x 30 sec Eosin (up to 45 sec for older batch of eosin)

b. 3 x 5 min 95% ETOH

c. 3 x 5 min 100% ETOH

d. 3 x 15 min Xylene

7. You can leave slides in xylene overnight to get good clearing of any water.
8. Coversliping slides with Permount (xylene based)
    a. Place a small drop of Permount on the end of the slide using a glass rod. Avoid bubbles
    b. Angle the coverslip at the end of the slide and allow to fall gently onto the slide. Allow the Permount to spread evenly under the coverslip, covering all tissue.
    c. Dry overnight under hood.

Immunflourescence Staining

1. 5 min Xylene
2. 5 min Xylene
3. 5 min Xylene
4. 3 min 100% ETOH
5. 3 min 100% ETOH
6. 3 min 95% ETOH
7. 3 min 80 % ETOH
8. 3 min 70% ETOH

9. 3 min 50% ETOH
10. 5 min de-ionized water
11. Microwave samples (High power) covered in EDTA-NaOH buffer (pH 8.0) in a pyrex dish, 2 x 5 min
12. 45 min allow to sit and cool in the EDTA-NaOH buffer at room temperature
13. Rinse in deionized water
14. Wash in PBS-Tween for 5 minutes
15. Remove slides and tap on side to remove excess water. Allow to air dry for 1-2 minutes. Circle the tissue samples with a Super Pap Pen
16. 5 min Phosphate Buffered Saline (PBS) buffer with 1% Tween 20
17. 20 min, Block in 10% normal rabbit serum diluted in PBS-1%BSA, enough to cover samples, incubate in a humidified chamber (slide box with damp towel and aluminum foil), blot excess
18. Wash in PBS-Tween for 5 minutes
19. Add GATA-4 primary antibody (1:100 in PBS-1% BSA) enough to cover samples and PCNA (1:200 in PBS-1% BSA) enough to cover samples (about 50-100 microliters). Incubate for 48 hours at 4ºC in a humidified chamber.
20. 5 min PBS-Tween buffer
21. 5 min PBS-Tween buffer
22. Add fluorescently labeled Gata-4 rabbit anti-goat secondary antibody, enough to cover samples. Incubate at room temperature for 1 hour in a humidified chamber.
23. The slides are protected from light for the remainder of the procedure.

24. 5 min PBS-Tween buffer
25. Add 10% Normal goat serum diluted in PBS-1% BSA, enough to cover samples. Allow to incubate at room temperature for 20 minutes in a humidified chamber.
26. 5 min Tris buffer
27. Add fluorescently labeled PCNA goat anti-mouse secondary antibody enough to cover samples. Incubate at room temperature for 1 hour in a humidified chamber.
28. 5 min PBS-Tween buffer
29. 5 min PBS-Tween buffer
30. 3 min 50% ETOH
31. 3 min 70% ETOH
32. 3 min 80% ETOH
33. 3 min 95% ETOH
34. 3 min 100% ETOH
35. 15 min Xylene
36. 15 min Xylene
37. 15 min Xylene
38. Allow slides to air dry under a fume hood for 3-5 minutes.
39. Place 1 small drop of Floursave on each slide. Place a 24x60mm coverglass on each slide. Seal the edges of the coverglass to the slide with clear nail polish. Allow to dry.
40. Place slides in a slide box and store at 4ºC until histological evaluation.